Efectos de los pesticidas organoclorados sobre la neurotransmisión glutamatérgica en cultivos primarios neuronales. Interacciones con el sistema neuroendocrino.

Víctor Briz Herrezuelo

Efectos de los pesticidas organoclorados sobre la neurotransmisión glutamatérgica en cultivos primarios neuronales. Interacciones con el sistema neuroendocrino.

Tesis Doctoral presentada por

Víctor Briz Herrezuelo

Barcelona, 2011

Departamento de Neuroquímica y Neurofarmacología
Instituto de Investigaciones Biomédicas de Barcelona (IIBB)
Consejo Superior de Investigaciones Científicas (CSIC)
Institut d'Investigacions Biomèdiques August Pi i Sunyer (IDIBAPS)
Consorcio de Investigación Biomédica en Red especializado en Epidemiología y Salud Pública (CIBERESP)

Memoria presentada por

Víctor Briz Herrezuelo

Para optar al grado de

Doctor por la Universidad de Barcelona

Programa de Doctorado de Biomedicina

La directora: Fdo.

Dra. Cristina Suñol Esquirol
Investigadora científica
IIBB (CSIC-IDIBAPS)

Víctor Briz Herrezuelo

Este trabajo ha sido realizado gracias a una beca predoctoral IDIBAPS, desde febrero de 2007 hasta diciembre de 2010.

Este trabajo ha sido financiado por los siguientes proyectos:

"Identificación de dianas moleculares y funcionales alteradas en procesos de neurotoxicidad inducida por contaminantes ambientales y biocidas en cultivos neuronales" (PI 06/1212).

Grupo de Investigación consolidado "Farmacología y Toxicología" (2005/SGR/00826) y (2009/SGR/214)

"Neurotoxicidad de agentes ambientales, exposición de la población y salud pública" (CB06/02/0024)

"Neuroestrogenicidad de plaguicidas organoclorados" (CIBERESP, AA08-001).

"Estudio de mecanismos de neurotoxicidad crónica de contaminantes tóxicos: papel de los receptores GABA-A, de glutamato y de estrógenos. Posibles intervenciones terapéuticas" (PI10/00453)

A Elena

A mis padres

ÍNDICE

ABREVIATURAS

[^{35}S]-TBPS: ^{35}S- (t-butylbicyclophosphorothionate)

5HT$_3$: 5-hydroxytryptamine (serotonina) tipo 3

ADP: Adenosine Diphosphate

AF: Región del receptor de estrógenos con función activadora (del inglés "Activation Function"

AIF: Apoptosis-Inducing Factor

Akt: PKB (Proteína Kinasa B)

AMP: Adenosine Monophosphate

AMPA: 2-amino-3-(5-methyl-3-oxo-1,2- oxazol-4-yl)propanoic acid

AP: Activating Protein

ATP: Adenosine Triphosphate

BDNF: Brain-Derived Neurotrophic Factor

BGT1:Betaine-GABA Transporter 1

CaMKII: Calcio Calmodulina Kinasa II

CaMKIV : Calcio Calmodulina Kinasa IV

CREB: cAMP Response Element-Binding protein

DDT: Diclorodifeniltricloroetano

DIV: Días *In Vitro*

EAAT: Excitatory Amino-Acid Transporter

EGFR: Epidermal Growth Factor Receptor

ERK: Extracellular-signal-Regulated Kinase

ER-X: Estrogen Receptor X

ERα: Estrogen Receptor α

ERβ: Estrogen Receptor β

GABA: *gamma*-Aminobutyric acid

GAD: Glutamic Acid Decarboxylase

GAT: GABA Transporter

GluR: Glutamate Receptor

GPR: G-Protein coupled Receptor

GPR30: G-Protein-coupled Receptor 30

GRIP1: Glutamate Receptor Interacting Protein 1

GSK3β: Glucógeno Sintasa Kinasa 3β

HCH: Hexaclorociclohexano

Hsp: Heat shock protein

HT22: Línea celular "Human Teratocarcinoma 22"

ICI182780: Faslodex

IGF-I: Insulin-like Growth Factor-I

IP_3: Inositol trisphosphate

JNK: c-Jun N-terminal Kinase

KA: Subunidad del receptor de kainato (del inglés "Kainate Affinity")

KCC2: K^+/Cl^- Cotransporter 2

LD50: Lethal Dose 50

LTD: Long-Term Depression

LTP: Long-Term Potentiation

MAGUK: Membrane-Associated Guanylate Kinase

MAP: Mitogen-Activated Protein

MAPK: MAP Kinasa

MCF-7: Línea celular "Michigan Cancer Foundation" – 7

mGLUR: metabotropic Glutamate Receptor

MK801: Dizocilpine

MKP: MAP Kinase Phospatase

MPEP: 2-methyl-6-phenylethynyl-pyridine

NMDA: *N*-metil *D*-aspartato

nNOS: neuronal Nitric Oxide Synthase

NR: NMDA Receptor

PC12: Línea celular "PheoChromocytoma 12"

PCP: 1-(1-phenylcyclohexyl)piperidine

PDZ: PSD95, DlgA (Drosophila Disc Large Tumor Suppressor) and ZO1 (Zonula Occludens-1 Protein)

PHTPP: 4-[2-phenyl-5,7-bis(trifluoromethyl)pyrazolo[1,5-a] pyrimidin-3-yl] phenol

PI3K: Phosphatidylinositol 3-Kinase

PICK: Protein Interacting with C Kinase

PKA: Proteína Kinasa A

PKC: Proteína Kinasa C

PLC: Phospholipase C

PP1: Protein Phosphatase 1

PP2A: Protein Phosphatase 2A

PP2B: Phosphoprotein Phosphatase 2B

PSD: Post Synaptic Density

PSD95: Post Synaptic Density 95 (kDa)

PXR: Pregnane X Receptor

SAP102: Synapse-Associated **P**rotein 102 (kDa)

SAP97: Synapse-Associated Protein 97 (kDa)

SERM: Selective Estrogen Receptor Modulator

SNARE: SNAP [Soluble NSF (N-ethylmaleimide-Sensitive-Factor) Attachment Protein] Receptor

STEP: Striatal Enriched protein tyrosine Phosphatase

TGFβ: Transforming Growth Factor β

TM1-4: Transmembrane Domain 1-4

TNFα: Tumor Necrosis Factor α

vGAT: vesicular GABA Transporter

vGLUT: vesicular Glutamate Transporter

1. LA NEUROTRANSMISIÓN GLUTAMATÉRGICA

El ácido glutámico es un aminoácido no esencial y por tanto todas las células del organismo son capaces de sintetizarlo. Comúnmente se le denomina glutamato dado que a pH fisiológico siempre se encuentra en forma aniónica. Es un componente estructural de las proteínas y, además, es necesario para la síntesis de ácidos nucleicos y de moléculas antioxidantes como el glutatión, péptido que juega un papel importante en la supervivencia de las neuronas. El glutamato es también el principal neurotransmisor excitatorio en el sistema nervioso central. Puede ser sintetizado por dos vías distintas: a partir de la glutamina a través de la acción de la enzima glutaminasa (mecanismo predominante en las neuronas); o bien mediante la reacción de transaminación del α-cetoglutarato (mecanismo habitual en células gliales), que es un producto intermedio del ciclo de Krebs. Este neurotransmisor, una vez liberado por las vesículas presinápticas de las neuronas glutamatérgicas, actúa sobre los receptores de glutamato postsinápticos dando lugar a una respuesta fisiológica. Además, puede activar los receptores de glutamato presentes en la membrana presináptica y regular de esa manera su propia exocitosis, en un mecanismo de retroalimentación positiva o negativa en función del tipo de receptor de glutamato que se active. La concentración extracelular de glutamato está regulada por los transportadores de glutamato, que se dividen en dos grupos: dependientes de Na^+/K^+ o de alta afinidad (también llamados EAAT) e independientes de Na^+/K^+ o de baja afinidad. Por norma general, los EAAT captan el glutamato extracelular mientras que los independientes de Na^+/K^+ (como el antiportador cistina-glutamato) liberan glutamato mediante transporte activo; es decir, consumiendo energía. Los EAAT se expresan tanto en neuronas (EAAT4-5) como en las células gliales y endoteliales (EAAT1-3). Una vez captado, el glutamato es transformado en glutamina en las células gliales, que a su vez es transportada de nuevo a las neuronas para producir glutamato. Finalmente el glutamato es degradado a α-cetoglutarato por la glutamato deshidrogenasa o bien es transportado dentro de las vesículas exocitóticas a través de los transportadores vesiculares de glutamato (vGLUT1-3, independientes de Na^+/K^+), para ser secretado de nuevo. Este intercambio de aminoácidos entre neuronas y células gliales se conoce con el nombre de ciclo glutamato-glutamina (ver figura 1). Se cree que el glutamato liberado como neurotransmisor procede tanto del que es captado por transportadores del medio extracelular como del sintetizado a través de la glutaminasa presente en terminales nerviosos (Palmada & Centelles, 1998).

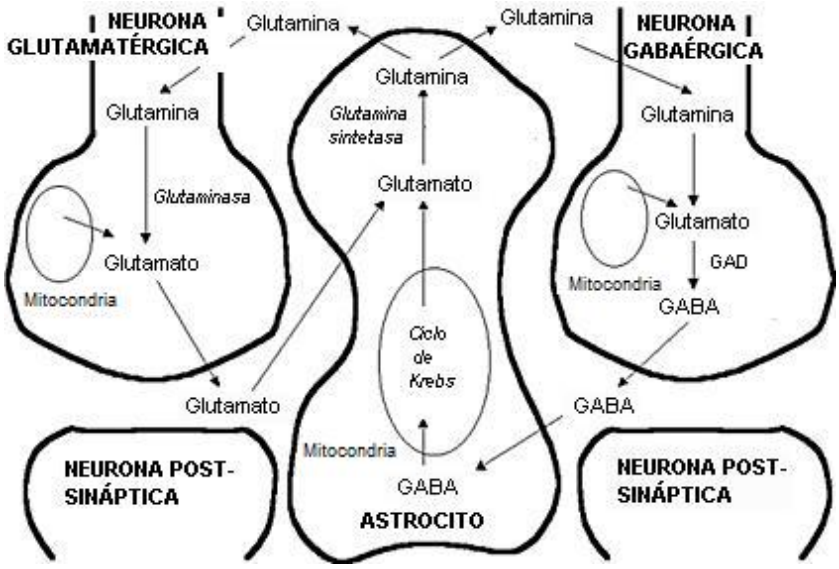


Figura 1. Ciclo glutamato-glutamina entre neuronas y astrocitos. Constituye la base bioquímica del metabolismo de los neurotransmisores glutamato y GABA (modificada de Palmada & Centelles, 1998).

A pesar de que el glutamato está presente en todas las células debido a su importancia en el metabolismo celular, siempre se ha pensado que solo las neuronas glutamatérgicas lo utilizaban como neurotransmisor. Es por ello que las neuronas glutamatérgicas a menudo son identificadas mediante el uso de anticuerpos contra el transportador vesicular vGLUT1, al ser el más común de todos ellos en el sistema nervioso central. Sin embargo, en los últimos años varios estudios han demostrado que el glutamato también puede ser secretado por otros tipos neuronales e incluso por células no neuronales. Por ejemplo, las motoneuronas liberan glutamato junto con la acetilcolina (Nishimaru *et al.*, 2005) y las neuronas monoaminérgicas expresan vGLUT2 y vGLUT3 (Trudeau, 2004). Por otro lado, los astrocitos también son capaces de liberar glutamato de manera dependiente de calcio (Parpura *et al.*, 1994) y expresan receptores de glutamato funcionales (Gallo & Russell, 1995), razón por la cual se piensa que participan de manera activa en la neurotransmisión glutamatérgica (Araque *et al.*, 1999).

1.1. CLASIFICACIÓN, ESTRUCTURA Y DISTRIBUCIÓN CEREBRAL DE LOS RECEPTORES DE GLUTAMATO

Existen dos grandes grupos de receptores de glutamato: los receptores ionotrópicos y los metabotrópicos. Los primeros regulan el paso de iones a través de la membrana plasmática y su permeabilidad a éstos se modifica cuando el glutamato se une al

receptor. Los receptores metabotrópicos, en cambio, tras la unión del glutamato, modifican su interacción con otras proteínas citosólicas y dan lugar a la activación de determinadas cascadas de señalización intracelulares.

1.1.1. Los receptores ionotrópicos de glutamato

Son canales iónicos activados por ligando de 4 dominios transmembrana con un extremo N-terminal extracelular que alberga el sitio de unión al ligando y otro C-terminal intracelular con diferentes sitios de fosforilación que regulan su actividad y la interacción con otras proteínas en la membrana (ver figura 2). Cuando el glutamato se une a ellos produce cambios conformacionales en el receptor dando lugar a la apertura del canal y permitiendo el flujo de iones Ca^{2+} y/o Na^{+} hacia el interior celular y de K^{+} hacia el espacio extracelular. Se dividen en tres tipos que se denominan respectivamente como los agonistas específicos que los activan: NMDA, AMPA y kainato.

1.1.1.1. Los receptores NMDA: Se caracterizan por poseer una alta permeabilidad al Ca^{2+} y una cinética de activación lenta. En condiciones de potencial de membrana en reposo, los receptores NMDA se encuentran inactivos debido al bloqueo del canal iónico por parte del Mg^{2+}. Cuando se produce una despolarización de la membrana, el Mg^{2+} desbloquea el canal permitiendo la entrada de Ca^{2+}, que actuará como segundo mensajero activando diversas cascadas de señalización.

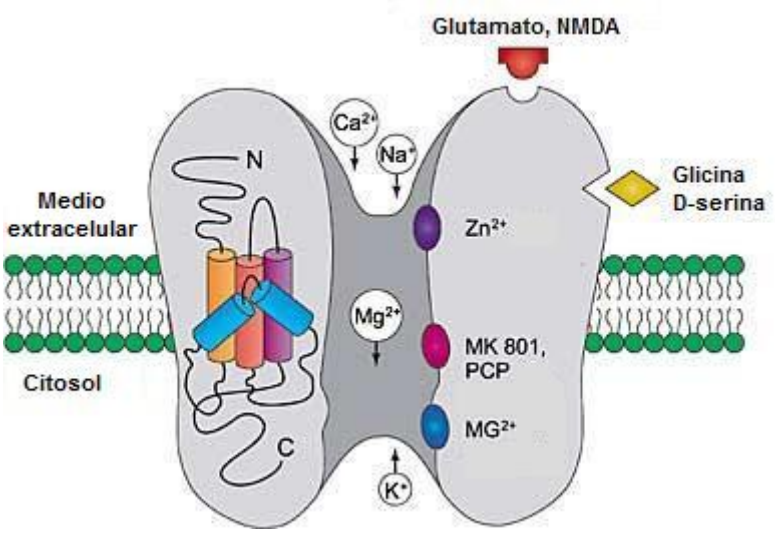


Figura 2. Estructura y farmacología del receptor NMDA. La unión de los agonistas glutamato o NMDA y glicina o D-serina estimula la apertura del canal, liberando el catión Mg^{2+} y permitiendo la entrada de los iones Ca^{2+} y Na^{+} al citosol y la salida de K^{+} al medio extracelular (modificada de Smith, 2002).

Los receptores NMDA son heterotetrámeros generalmente formados por dos subunidades NR1 y dos subunidades NR2. La subunidad NR1 es necesaria para la formación de receptores funcionales y contiene el sitio de unión de los aminoácidos glicina y D-serina (ver figura 2), que son co-agonistas endógenos del receptor junto al glutamato y necesarios para su activación (Kleckner & Dingledine, 1988). Existen 8 isoformas o variantes de *splicing* alternativo de NR1, derivadas del procesamiento post-transcripcional de su ARNm en 3 sitios diferentes: uno en el extremo N-terminal, denominado N1 (exón 5), que regula sus propiedades farmacológicas y dos en el extremo C-terminal, denominados C1 (exón 21) y C2 (exón 22), median la interacción con proteínas implicadas en el tráfico del receptor NMDA (ver apartado 1.3.2). Por su parte las subunidades NR2 son responsables de la unión del glutamato y del agonista NMDA (ver figura 2). Existen 4 subtipos diferentes (NR2A-D), procedentes de 4 genes distintos, que confieren al receptor NMDA propiedades farmacológicas y cinéticas exclusivas; más concretamente, difieren en el tiempo de desactivación del receptor; es decir, en el tiempo en que el glutamato permanece unido a ellas manteniendo activo el receptor, siguiendo este orden: NR2A<NR2B=NR2C<NR2D (Monyer *et al.*, 1994). Las subunidades NR2 contienen también el sitio de unión de ciertos moduladores alostéricos endógenos como el Zn^{2+} (inhibidor) y las poliaminas (potenciadores), las cuales son específicas de las subunidades NR2B. Además los receptores NMDA son sensibles al pH siendo inhibidos por los protones, en especial aquellos que contienen las subunidades NR2A y NR2B. También la sensibilidad a los antagonistas no competitivos como Mg^{2+}, MK801, fenciclidina (PCP) y ketamina varia en función de la subunidad NR2 presente, siendo mayor para NR2A y NR2B que para NR2C y NR2D. Sin embargo, la presencia del exón 5 de la subunidad NR1 convierte al receptor NMDA en insensible tanto al pH como a la modulación por parte del Zn^{2+} y las poliaminas (Cull-Candy *et al.*, 2001).

Los receptores NMDA están normalmente formados por diheterómeros siendo los más habituales los formados por NR1/NR2A y NR1/NR2B en la corteza e hipocampo, y por NR1/NR2C en cerebelo. Sin embargo, también existen triheterómeros entre los que destaca por su presencia en sinapsis neuronales el NR1/NR2A/NR2B (Tovar & Westbrook, 1999). Además, en los últimos años se han identificado dos nuevas subunidades, denominadas NR3A y NR3B, cuya presencia confieren al receptor NMDA una reducida permeabilidad al Ca^{2+} y características neuroprotectoras frente a la excitotoxicidad (Das *et al.*, 1998; Nishi *et al.*, 2001; Nakanishi *et al.*, 2009). La

subunidad NR1 está presente de manera abundante en todo el sistema nervioso central tanto en etapas tempranas del desarrollo como en el cerebro adulto. Por el contrario, las subunidades NR2 difieren bastante en cuanto a su distribución cerebral y su patrón de expresión durante el desarrollo. Las subunidades NR2B y NR2D son las más expresadas durante el desarrollo embrionario y postnatal temprano del cerebro, predominando en el telencéfalo (córtex e hipocampo) y en el diencéfalo y mesencéfalo respectivamente. Las subunidades NR2A y NR2C aumentan gradualmente a lo largo del desarrollo llegando a ser predominantes en algunas zonas del cerebro adulto, tales como el cerebelo. Además, en el hipocampo adulto, las subunidades NR2A y NR2B se encuentran mayoritariamente en las neuronas piramidales (glutamatérgicas) mientras que las subunidades NR2C y NR2D se expresan en las interneuronas (Monyer *et al.*, 1994). En cuanto a la localización celular, durante el desarrollo la subunidad NR2A desplaza gradualmente a la subunidad NR2B en las sinapsis neuronales, la cual pasa a ser mayoritaria en las zonas extrasinápticas. En el caso del cerebelo, este reemplazamiento de subunidades en las sinapsis implica no sólo la formación de receptores NR1/NR2A sino también NR1/NR2C e incluso receptores triheteroméricos NR1/NR2A/NR2C. Por el contrario los receptores NMDA que contienen la subunidad NR2D se localizan siempre en zonas extrasinápticas, ya sea en forma diheteromérica o triheteromérica NR1/NR2B/NR2D (Cull-Candy *et al.*, 2001).

1.1.1.2. Los receptores AMPA/Kainato: A menudo se les denomina también receptores no-NMDA por no ser sensibles a los antagonistas selectivos del receptor NMDA. Presentan una menor afinidad por el glutamato que los receptores NMDA pero por el contrario tienen una cinética de activación mucho más rápida que éstos. Los receptores AMPA están formados por 5 subunidades iguales (homómeros) o diferentes (heterómeros) denominadas GluR1-4, mientras que los de kainato normalmente se componen de las subunidades KA1-2 (de alta afinidad por kainato) junto a una subunidad GluR5-7 (de baja afinidad). A diferencia de los de kainato, los receptores AMPA son altamente permeables a Na^+ y K^+ pero poco al Ca^{2+}. Sin embargo, la ausencia de la subunidad GluR2 o el cambio en ésta de un aminoácido situado en el poro del canal les confiere una mayor permeabilidad al Ca^{2+} (Hollmann *et al.*, 1991; Mishina *et al.*, 1991). En lo que respecta a su distribución cerebral, los receptores AMPA están ampliamente expresados en todo el cerebro, siendo especialmente numerosos en el hipocampo y en el córtex. Concretamente las interneuronas GABAérgicas expresan la subunidad GluR2 en menor cantidad que las neuronas

piramidales glutamatérgicas y por ello presentan receptores AMPA permeables al Ca^{2+} (Kondo *et al.*, 1997). Por su parte las células granulares de cerebelo expresan solamente las subunidades GluR2 y GluR4. En cuanto a los receptores de kainato, destacan la alta expresión de KA1 en el hipocampo y la de GluR6 en células granulares de cerebelo (Ozawa *et al.*, 1998).

Los receptores AMPA/Kainato se localizan mayormente a nivel postsináptico en neuronas maduras. Su activación produce una despolarización de la membrana plasmática que conlleva la apertura de canales de Na^+ y de Ca^{2+} regulados por voltaje así como la activación de receptores NMDA, razón por la cual los receptores AMPA/Kainato son capaces de incrementar la concentración Ca^{2+} intracelular ($[Ca^{2+}]_i$) de manera notable. Por este motivo, los receptores AMPA/kainato están implicados en procesos de plasticidad sináptica (ver apartado 1.2.2) pero también pueden contribuir a la muerte excitotóxica (ver apartado 1.4).

1.1.2. Los receptores metabotrópicos de glutamato

Los receptores metabotrópicos de glutamato son receptores acoplados a proteínas G (GPRs) de 7 dominios transmembrana. Forman una superfamilia de GPRs junto a los receptores $GABA_B$, entre otros, que difiere de la de los receptores adrenérgicos en que son de mayor tamaño y tienen el sitio de unión del ligando en el extremo N-terminal. Además esta clase de GPRs se caracterizan por encontrarse normalmente en forma de dímeros con los extremos N-teminal unidos mediante puentes de disulfuro (Romano *et al.*, 1996), teniendo incluso la capacidad de que la unión de un sólo ligando active los dos receptores que forman el dímero (Brock *et al.*, 2007). En conjunto son un total de 8 subtipos (mGLUR1-8) que se dividen en 3 clases en función de su homología genética, sus características farmacológicas y su señalización intracelular (ver figura 3).

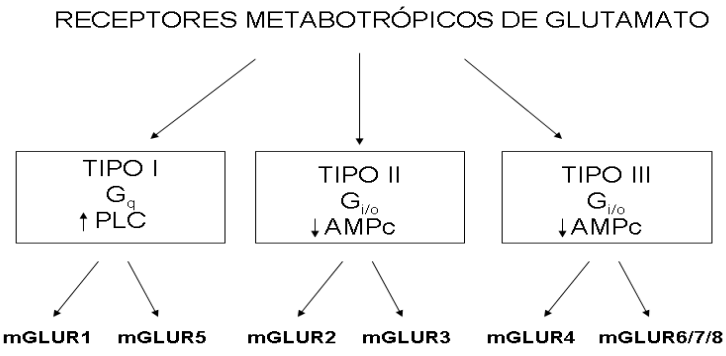


Figura 3. Clasificación de los receptores metabotrópicos de glutamato

1.1.2.1. Receptores metabotrópicos de glutamato de tipo I: Formado por los subtipos mGLUR1 y mGLUR5, están generalmente acoplados a proteínas G_q aunque en ciertos casos también median sus efectos a través de proteínas $G_{i/o}$. Tras la estimulación del receptor, la subunidad α de la proteína G_q activa la fosfolipasa-C (PLC) produciendo inositol trifosfato (IP_3) y diacilglicerol, los cuales inducirán respectivamente la liberación de Ca^{2+} del retículo endoplasmático y la activación de la proteína kinasa C (PKC). A pesar de tener el mismo mecanismo de señalización, los mGLUR de tipo-I presentan funciones diferentes y en ocasiones opuestas (ver apartado 1.4.3) y su expresión cerebral es también desigual. Mientras que mGLUR1 se encuentra expresado principalmente en el cerebelo, el tálamo y el hipocampo, el patrón de expresión de mGLUR5 en el cerebro es más difuso aunque destaca, entre otras zonas, en las capas superficiales del córtex y en el hipocampo (Ferraguti & Shigemoto, 2006). Por otro lado, la expresión de mGLUR1 y mGLUR5 a lo largo del desarrollo cambia de modo inverso; incrementando en el caso del primero y disminuyendo de manera general (excepto en el córtex) para el segundo (Catania *et al.*, 1994), razón por la que se le ha asignado a mGLUR5 un papel importante en la sinaptogénesis (Wijetunge *et al.*, 2008). En cuanto a su localización celular, es mayoritariamente postsináptica (concretamente en la zona perisináptica o adyacente a la densidad postsináptica-PSD-) (Lujan *et al.*, 1997) aunque también se han detectado en la membrana presináptica modulando la liberación de glutamato (Musante *et al.*, 2008). Cabe destacar que también han sido localizados en la membrana nuclear de neuronas corticales modulando los niveles de Ca^{2+} dentro del núcleo (O'Malley *et al.*, 2003; Jong *et al.*, 2007). Además de estar altamente expresado en neuronas, mGLUR5 también está presente en astrocitos (Miller *et al.*, 1995).

1.1.2.2. Receptores metabotrópicos de glutamato de tipo II y III: Los mGLURs de tipo II están compuestos por mGLUR2 y mGLUR3, mientras que los de tipo-III los forman mGLUR4 y mGLUR6-8. En ambos casos su señalización es a través de proteínas $G_{i/o}$, cuya activación inhibe la adenilato ciclasa y disminuye por tanto la formación de AMP cíclico (ver figura 3). Los de tipo II se expresan principalmente en las capas corticales profundas, en el hipocampo y en el cerebelo (Berthele *et al.*, 1999; Ferraguti & Shigemoto, 2006). En cuanto a los de tipo III es de destacar la marcada expresión de mGLUR4 en células granulares de cerebelo. Su patrón de expresión durante el desarrollo se asemeja al de los mGLUR de tipo-I; el de mGLUR3 se corresponde con el de mGLUR5 (temprano), mientras que el de mGLUR2 es tardío como el de mGLUR1

(Catania *et al.*, 1994). Se localizan principalmente a nivel presináptico modulando la liberación de glutamato y GABA, así como de diferentes hormonas (Bonci *et al.*, 1997; van den Pol *et al.*, 1998; Durand *et al.*, 2008), aunque también se encuentran altamente expresados en los astrocitos (Yao *et al.*, 2005).

1.2. FUNCIONES Y SEÑALIZACIÓN DE LOS RECEPTORES DE GLUTAMATO

Entre las funciones fisiológicas reguladas por el glutamato, ya sea durante el desarrollo como también en el cerebro adulto, destacan la neurogénesis, el crecimiento de neuritas, la sinaptogénesis, la plasticidad sináptica y la supervivencia neuronal. Como hemos descrito anteriormente, tanto la activación de los receptores ionotrópicos de glutamato como de los metabotrópicos de tipo I produce un incremento en la concentración de Ca^{2+} intracelular ($[Ca^{2+}]_i$), que será más o menos intenso y/o duradero según la composición estructural del receptor que se trate. Por ello, el Ca^{2+} constituye el principal segundo mensajero de la señalización mediada a través de receptores de glutamato.

Cuando los receptores de glutamato activados son autoreceptores o receptores presinápticos, el Ca^{2+} movilizado generalmente se unirá a la sinaptotagmina activando el complejo de proteínas SNARE que conforma la maquinaria de la exocitosis. Una vez activado éste, se inducirá la fusión de las vesículas secretoras con la membrana plasmática llevando a término la liberación de neurotransmisores. En cambio, la activación de los receptores de glutamato postsinápticos y el consecuente incremento de $[Ca^{2+}]_i$ puede estimular la activación de numerosas rutas de señalización (ver figura 4). Algunas de las proteínas activadas por Ca^{2+} en respuesta al glutamato son kinasas, como PKC, calmodulina kinasa II (CaMKII) y PKA; o fosfatasas, como la calcineurina (PP2B); las cuales actúan modulando la función de los propios receptores AMPA y NMDA mediante fosforilación y desfosforilación, respectivamente. También existen sensores de Ca^{2+} como la hipocalcina que recluta a la proteína AP-2 para regular el tráfico de receptores de glutamato (ver apartado 1.3.3); o proteasas como la calpaina, que controlan la actividad de éstos mediante degradación proteolítica (ver apartado 1.4.2.2). Otras, como la proteína *Ras*, activan cascadas de señalización intracelulares tales como las rutas MAPK y PI3K/Akt que a su vez regulan la expresión génica. De esta manera, el incremento de $[Ca^{2+}]_i$ producido por la estimulación de los receptores de

glutamato dará lugar a la activación de determinadas cascadas de señalización intracelular en función del tipo y localización del receptor que se trate así como de sus proteínas asociadas, provocando por tanto una gran variedad de respuestas fisiológicas (Sheng & Kim, 2002). Sin embargo, los receptores metabotrópicos de tipo I también tienen efectos independientes del Ca^{2+}, como en el caso de la activación de ERK1/2 o la modulación de los canales de Ca^{2+} (Herrero *et al.*, 1998; Yang *et al.*, 2004).

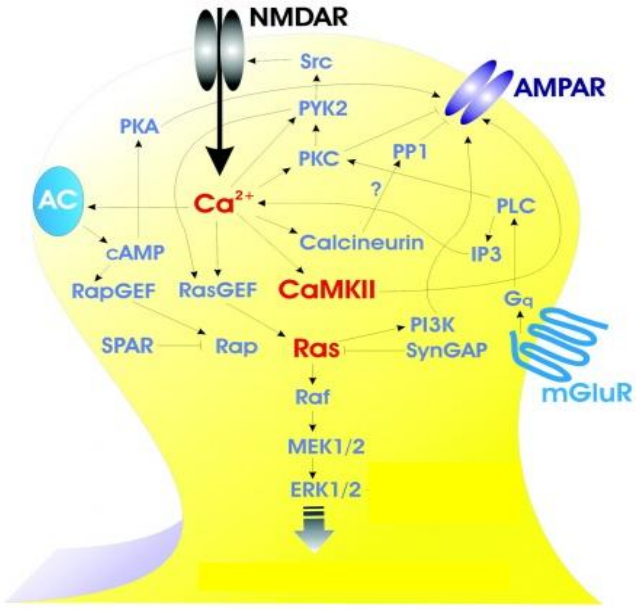


Figura 4. Vías de señalización asociadas a los receptores de glutamato. La entrada de Ca^{2+} desencadena la activación de las rutas MAPK y PI3K/Akt, entre otras (modificada de Sheng & Kim, 2002).

1.2.1. Diferenciación neuronal

Las neuronas y células gliales se originan a partir de la diferenciación de las células madre neurales. En este sentido, el glutamato estimula la neurogénesis ya que regula la expresión de factores neurotróficos, como el BDNF o IGF-I; de citokinas y de otras moléculas señalizadoras, como el óxido nítrico (NO); que detienen la proliferación de los progenitores neurales e inducen su diferenciación a neuronas. Por otro lado, el glutamato inhibe el crecimiento dendrítico (pero no axonal) y estimula la formación de nuevas sinapsis a través de la regulación de las proteínas del citoesqueleto. Así, el aumento de la $[Ca^{2+}]_i$ inducido por la activación de receptores de glutamato inhibe, por un lado, la polimerización de la tubulina deteniendo la elongación de la neurita, y por otro lado, activa la polimerización de la actina a nivel local estimulando la formación de

extensiones filopódicas en el cono de crecimiento, las cuales establecerán los futuros contactos sinápticos. Por otro lado, la producción de factores neurotróficos inducida por el glutamato también contribuirá a los procesos de sinaptogénesis (Mattson, 2008). Se cree que, en particular, los receptores NR1/NR2B tienen un papel determinante en la formación de sinapsis, puesto que se han encontrado formando *clusters* o agrupamientos en dendritas, antes incluso de que éstas sean inervadas por los terminales presinápticos (Li *et al.*, 1998). De acuerdo con esta idea, la actividad a través de los receptores NR1/NR2B y de los canales de canales de Ca^{2+} de tipo L es determinante para la expresión de la subunidad NR2A a lo largo del desarrollo (Hoffmann *et al.*, 2000), fenómeno clave en la maduración de las sinapsis (van Zundert *et al.*, 2004). Además, se ha postulado que el cambio de predominancia de las subunidades NR2 en las sinapsis, mencionado anteriormente, no se debe a alteraciones en la expresión o localización de NR2B sino a la creación de nuevas sinapsis formadas por receptores NMDA que sólo contienen la subunidad NR2A (Liu *et al.*, 2004).

1.2.2. Plasticidad sináptica

Los receptores de glutamato son los principales responsables de los procesos de plasticidad sináptica, tales como la potenciación a largo plazo (LTP, del inglés "Long-Term Potentiation") y la depresión a largo plazo (LTD, del inglés "Long-Term Depression"). Estos cambios en la actividad sináptica constituyen las bases fisiológicas subyacentes a los procesos de aprendizaje y memoria, ya que refuerzan o debilitan las conexiones entre las neuronas en respuesta a un estímulo externo. A nivel molecular, estas alteraciones se producen por cambios en la actividad y localización en la membrana de los receptores de glutamato, como consecuencia de la acción de las kinasas y fosfatasas mencionadas anteriormente. Existen diferentes mecanismos responsables de la plasticidad sináptica, cuya participación varía según la zona del cerebro que se trate. De este modo, encontramos que tanto la LTP como la LTD pueden ser dependientes o independientes del receptor NMDA, y además los receptores involucrados pueden ser presinápticos y/o postsinápticos. Para añadir mayor complejidad, algunos receptores participan en la inducción de LTP (o LTD) y otros en su expresión o mantenimiento. La forma más habitual de plasticidad sináptica es la dependiente del receptor NMDA, en donde la activación de éstos produce un aumento en la PSD de los receptores AMPA permeables al Ca^{2+}, manteniendo así la expresión de la LTP. Por el contrario, la posterior internalización de los receptores AMPA dará lugar

a la depresión prolongada de la actividad sináptica o LTD. Ambos procesos están regulados de manera opuesta por las kinasas y fosfatasas activadas por Ca^{2+}. Por un lado, las kinasas CaMKII y PKA estimulan rápidamente los receptores AMPA; y por otro las fosfatasas, de cinética más lenta, los desactivarán al cabo de un tiempo determinado. Además, la intensidad de la señal de Ca^{2+} también determinará que se activen unas u otras (Sheng & Kim, 2002). En este sentido, se ha observado que la actividad de GSK3β (regulada negativamente por PI3K/Akt y positivamente por la fosfatasa PP1) juega un papel importante en la transición de LTP a LTD (Peineau *et al.*, 2008).

En cuanto a las subtipos de receptores involucrados, los receptores NR1/NR2A sinápticos parecen responsables de la formación de LTP en la corteza cerebral, mientras que la inducción de LTD se debe a la activación de receptores NR1/NR2B extrasinápticos (Massey *et al.*, 2004). Sin embargo, los receptores NMDA presinápticos también contribuyen a ambas formas de plasticidad en ésta y en otras zonas cerebrales (Duguid & Sjostrom, 2006). Por su parte, los receptores metabotrópicos de tipo I han sido implicados en generación de LTD en hipocampo y cerebelo (Zhuo & Hawkins, 1995), y, más recientemente, en la inducción y expresión de LTP dependiente del receptor NMDA (Lu *et al.*, 1997; Cheyne & Montgomery, 2008). Respecto al LTP independiente del receptor NMDA, se sabe que, además de los canales de Ca^{2+} dependientes de voltaje, los receptores de kainato también están implicados en su formación (Bortolotto *et al.*, 1999).

1.2.3. Supervivencia neuronal

Durante el desarrollo se producen más neuronas de las que finalmente formarán parte del sistema nervioso. De modo que aquellas neuronas que no sean lo suficientemente estimuladas (por el glutamato o por otros neurotransmisores excitatorios), no se integrarán en los circuitos neuronales y, como consecuencia, desencadenarán la muerte celular programada o apoptosis. Por el contrario, la activación de los receptores de glutamato inducirá la expresión de factores neurotróficos que, una vez liberados, actuarán sobre sus receptores situados en la membrana presináptica estimulando la producción de proteínas antiapoptóticas, como *Bcl*-2, y de enzimas antioxidantes (Mattson, 2008). En concreto, la actividad mediada por los receptores NMDA juega un papel clave en el mantenimiento de la supervivencia neuronal mediada por el glutamato. Un caso paradigmático es el de las células granulares de cerebelo; puesto que, para su

adecuada diferenciación y supervivencia, requieren de una constante activación de los receptores NMDA. Por esta razón, han de ser cultivadas en presencia de una alta concentración de K^+ (25 mM) para facilitar la neurotransmisión, reproduciendo así, *in vitro*, la inervación procedente de las fibras musgosas que ocurre en el cerebro (Gault & Siegel, 1998). De hecho, un modelo frecuente para inducir apoptosis en células granulares de cerebelo es el de reducir la concentración de K^+ (a 5 mM) en el medio de cultivo.

Del mismo modo que durante el desarrollo, la supervivencia neuronal también depende de la actividad sináptica a través de los receptores NMDA en el cerebro adulto. Además, se ha demostrado que la activación selectiva de los receptores NMDA sinápticos tiene efectos neuroprotectores frente a diferentes modelos de muerte neuronal *in vitro* (Soriano *et al.*, 2006; Leveille *et al.*, 2010). Sin embargo, la neuroprotección mediada por el receptor NMDA en modelos de isquemia, tanto *in vitro* como *in vivo*, ha sido atribuida a la activación específica de los receptores NR1/NR2A, independientemente de su localización sináptica (Liu *et al.*, 2007). A pesar de ello, la activación de los receptores NR1/NR2B también tiene efectos neuroprotectores frente a la apoptosis inducida por la sobreactivación de GSK3β (Habas *et al.*, 2006). Por tanto, no está totalmente clara todavía la función de los receptores NMDA en la supervivencia neuronal, aunque varios estudios apuntan a que la activación de vías de señalización intracelular, MAPK y PI3K/Akt, y su posterior regulación de la expresión de BDNF y de las proteínas mitocondriales de la familia *Bcl*-2, son determinantes al respecto (Zhu *et al.*, 2005; Xu *et al.*, 2007a; Hardingham, 2009).

1.3. TRÁFICO DE LOS RECEPTORES DE GLUTAMATO

El tráfico de receptores es un proceso complejo controlado por muchos factores que regula la expresión funcional de éstos en la membrana plasmática. Se compone de dos fases diferenciadas: la secreción o inserción de receptores en la membrana, que abarca desde la síntesis del receptor hasta su localización en la superficie celular; y la internalización o endocitosis, que finaliza con la degradación del receptor o bien el reciclaje para su nuevo transporte hacia la membrana. Además, la unión de los receptores a las proteínas *scaffold* o de anclaje a la membrana determinará su estabilidad en la membrana así como su localización sináptica, funcionalidad y señalización intracelular.

1.3.1. Secreción de los receptores de glutamato

Una vez sintetizadas en los ribosomas, las subunidades que conforman los receptores de glutamato son transportadas al retículo endoplasmático. Allí serán ensambladas entre sí a través de puentes de disulfuro dando origen a la estructura cuaternaria definitiva de los receptores. Éstos son entonces transportados al aparato de Golgi, desde donde partirán en vesículas secretoras hacia la membrana plasmática. En cambio, aquellos receptores que no han sido ensamblados correctamente serán degradados en el retículo endoplasmático. En el caso de los receptores NMDA, la presencia de la subunidad NR1 es totalmente necesaria para que los receptores sean exportados hacia la membrana celular. Como hemos mencionado anteriormente, el extremo C-terminal de la subunidad NR1 está compuesto por 3 dominios o *cassettes* denominados C0, C1 y C2 (exones 20,21 y 22, respectivamente). El procesamiento alternativo del ARNm de C1 y C2 dará lugar a 4 subunidades NR1 diferentes (ver figura 5). El dominio C1 tiene un motivo formado por 3 aminoácidos (arginina-X-arginina, siendo X un aminoácido cualquiera) que determina la retención del receptor en el retículo endoplasmático. La unión con las subunidades NR2 oculta este motivo permitiendo la secreción del receptor, mientras que las subunidades NR1 que carecen de la región C1 son capaces de alcanzar la membrana por sí solas. Además, la presencia del dominio C2' (procedente del truncamiento de C2) acelera el tráfico de los receptores NMDA hacia la membrana (Perez-Otano & Ehlers, 2004).

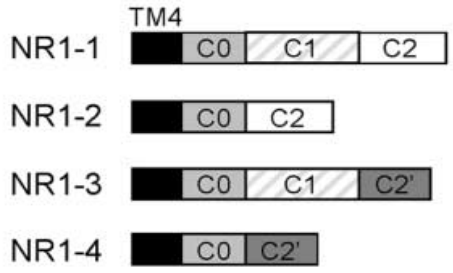


Figura 5. Variantes de *splicing* derivadas del procesamiento los cassettes C1 y C2 situados en el extremo C-terminal de la subunidad NR1 del receptor NMDA (obtenida de Mu *et al.*, 2003).

El tráfico de los receptores NMDA también puede estar regulado mediante modificaciones post-traduccionales que se producen a lo largo del proceso de secreción. De esta manera, la fosforilación coordinada por parte de PKC y PKA en el dominio C1

favorece el transporte de receptores NMDA a la membrana (Scott *et al.*, 2003). En cambio, la palmitoilación de las subunidades NR2 en su extremo C-terminal retiene a los receptores en el aparato de Golgi (Hayashi *et al.*, 2009). Por otro lado, la interacción con las proteínas PSD95 y SAP102 es también otro mecanismo de regulación del tráfico de los receptores NMDA hacia la membrana, de tal manera que facilitan la unión a las proteínas del complejo exocisto, quienes a su vez median la fusión de las vesículas secretoras con la membrana plasmática (Sans *et al.*, 2003). Existen diferencias entre las subunidades NR2 en lo que respecta a su secreción: mientras que para los receptores NR1/NR2B se trata de un proceso constitutivo, en el caso de los receptores NR1/NR2A, ésta depende de la actividad sináptica (Perez-Otano & Ehlers, 2004). El transporte de receptores a través de la maquinaria secretora es relativamente lento (2-3 horas); sin embargo, los receptores NMDA también pueden ser incorporados a la membrana de manera rápida, a través de la exocitosis mediada por el complejo SNARE. Este proceso está regulado por diferentes factores tales como PKC, insulina o la activación de los mGLURs de tipo I (Lan *et al.*, 2001; Skeberdis *et al.*, 2001).

1.3.2. Localización sináptica e interacción con las proteínas *scaffold*

Existe también un tipo de transporte dentro de la membrana plasmática denominado difusión lateral, mediante el cual los receptores se mueven de zonas sinápticas a zonas extrasinápticas y viceversa. Se cree que tiene una gran importancia en el reciclaje de los receptores, permitiendo bien su acceso a la zona de endocitosis para su posterior internalización o su posicionamiento en la sinapsis tras su inserción en la membrana, y está regulado por la interacción con las proteínas *scaffold*. En el caso de los receptores NMDA, este proceso es constitutivo siendo especialmente dinámico durante el desarrollo, y afecta tanto a los receptores NR1/NR2A como a los NR1/NR2B. En cambio, la difusión lateral de los receptores AMPA depende de la actividad sináptica y parece estar implicada en los procesos de plasticidad (Perez-Otano & Ehlers, 2004).

Los receptores de glutamato situados en la membrana plasmática se encuentran asociados a las llamadas proteínas *scaffold* formando un complejo que estabiliza a los receptores en determinadas zonas de la membrana plasmática (por ejemplo en la PSD), ya que funcionan como punto de anclaje entre el receptor, la membrana y el citoesqueleto de actina. Sirven además para reclutar otras proteínas, como CaMKII o nNOS, que se encargan de la transducción intracelular de la señalización mediada por los receptores de glutamato (Gardoni *et al.*, 2006; d'Anglemont, X *et al.*, 2007;

d'Anglemont, X *et al.*, 2009; Gardoni *et al.*, 2009). En la tabla 1 se detallan las proteínas *scaffold* a las que se encuentran asociados los receptores de glutamato más habitualmente. Entre ellas, las proteínas de la familia MAGUK (guanilato kinasas asociadas a la membrana), como PSD95 y SAP102, son las que generalmente se unen a los receptores NMDA. Esta interacción se produce a través del dominio de unión a PDZ presente en la región C-terminal de las subunidades NR2 y de algunas variantes de *splicing* de NR1 (Kornau *et al.*, 1995; Muller *et al.*, 1996). La palmitoilación, tanto de PSD95 como de las subunidades NR2, favorece esta unión y también estimula la fosforilación en residuos de tirosina de éstas últimas por parte de la familia de kinasas Src, estabilizando el complejo en la membrana e inhibiendo al mismo tiempo su degradación por las calpaínas (Rong *et al.*, 2001; Dong *et al.*, 2004; Hayashi *et al.*, 2009). SAP102, en cambio, no contiene sitios de palmitoilación y por ello se cree que no tiene la capacidad de formar *clusters* ni tampoco impide la proteolisis del receptor NMDA inducida por las calpaínas (Dong *et al.*, 2004). Por el contrario, la fosforilación en residuos de serina de NR2B en respuesta a la actividad sináptica inhibe su asociación con PSD95 y SAP102 y como consecuencia reduce su expresión en la membrana (Chung *et al.*, 2004). A pesar de que la expresión y la localización de la subunidad NR1 no se ven alteradas en ratones *knockout* para PSD95, la plasticidad sináptica y el aprendizaje sí que están afectados en estos ratones (Migaud *et al.*, 1998). Esto parece ser debido a que el incremento progresivo en la expresión de PSD95 a lo largo del desarrollo (paralelo al de NR2A) es decisivo en el cambio de predominancia de las subunidades NR2 en las sinapsis (Elias *et al.*, 2008), las cuales a su vez son determinantes en los procesos de plasticidad sináptica, como hemos explicado previamente. De este modo, en neuronas corticales maduras, los receptores NR1/NR2A se encuentran normalmente unidos a PSD95 en las sinapsis, mientras que los receptores NR1/NR2B se asocian preferentemente a SAP102 y se localizan en zonas perisinápticas o extrasinápticas (van Zundert *et al.*, 2004). A pesar de ello, la subunidad NR2A no requiere de la unión con PSD95 para su localización sináptica, mientras que en el caso de NR2B es requisito indispensable (Prybylowski *et al.*, 2005). Además, la interacción de PSD95 con las subunidades NR2 está también regulada por CaMKII, cuya activación o inhibición induce la disociación de PSD95 con NR2A o con NR2B, respectivamente, alterando por tanto la localización sináptica de esta última (Gardoni *et al.*, 2006; Gardoni *et al.*, 2009).

PSD95 y SAP102 también interaccionan con los receptores AMPA aunque no de manera directa sino a través de la proteína transmembrana *stargazin* (van Zundert *et al.*, 2004; Elias *et al.*, 2008). Además, se ha observado que PSD95 y SAP102 pueden interaccionar entre sí a través de la calmodulina, lo que se ha propuesto como un mecanismo de formación de *clusters* o agrupamientos de receptores de glutamato (Masuko *et al.*, 1999). Sin embargo, las proteínas normalmente unidas a los receptores AMPA en la PSD son SAP97, para GluR1, y PICK1 o GRIP1, para GluR2/3. En cuanto a los receptores metabotrópicos de glutamato, su principal proteína *scaffold* se llama *Homer*, la cual forma complejos multiméricos acoplando los mGLUR de tipo I con los receptores de IP3 o de rianodina, de modo que facilita la transducción de su señalización. Además, se ha visto que *Homer* también es capaz de unirse a PSD95 a través de otra proteína llamada *Shank* (Tu *et al.*, 1999), lo que parece contribuir al acoplamiento funcional de los mGLURs de tipo I con los receptores NMDA (Guo *et al.*, 2004).

Proteínas *scaffold*	**Receptores AMPA**	**Receptores de Kainato**	**Receptores NMDA**	**mGluR**
PSD95	✓		✓	
SAP97	✓			
SAP102			✓	✓
Stargazin	✓			
PICK1/GRIP1	✓	✓		✓
AP-2	✓		✓	
Homer				✓

Tabla 1. Proteínas *scaffold* asociadas normalmente a los receptores de glutamato en la membrana plasmática (modificada de http://www.bristol.ac.uk/synaptic/)

1.3.3. Endocitosis de los receptores de glutamato

Existen dos tipos de endocitosis para los receptores de glutamato: la constitutiva y la inducida por ligando. La primera es más habitual durante el desarrollo, cuando las

sinapsis no son todavía maduras, o en zonas extrasinápticas; puesto que en estos casos los receptores no se encuentran fuertemente anclados a la membrana. En el segundo caso, la activación del receptor por el ligando induce cambios en la asociación con las proteínas *scaffold*, directamente o a través de las vías de señalización intracelular, que darán lugar a su desestabilización en la membrana y consecuente difusión a la zona perisináptica, donde está situada la maquinaria endocitótica. En el caso de los receptores NMDA, es necesaria la unión de los dos ligandos (glutamato y glicina) para inducir la endocitosis. En cambio, la internalización de los receptores AMPA requiere la activación previa de los receptores NMDA. A pesar de interaccionar con distintas proteínas *scaffold* en la membrana plasmática, los receptores ionotrópicos de glutamato comparten el mismo mecanismo de internalización: la endocitosis mediada por clatrina. El primer paso después de la disociación con las proteínas de anclaje a la membrana es la unión de los receptores a otra proteína, AP-2, que sirve de adaptador para la interacción posterior de la clatrina con el complejo (por ello aparece en la tabla 1 como proteína *scaffold*). Varias moléculas de clatrina polimerizan produciendo una invaginación de la membrana que, tras la acción de la dinamina, dará lugar a la formación de la vesícula endocítica. A continuación, las vesículas endocíticas se fusionan entre sí originando los endosomas tempranos, los cuales podrán seguir dos caminos: devolver a los receptores a la membrana a través de los endosomas de reciclaje, o bien enviarlos a los endosomas tardíos para su posterior degradación en los lisosomas. De manera general, en la endocitosis constitutiva los receptores se suelen reciclar y en la inducida por ligando se degradan. A pesar de ello, los mecanismos que determinan que los receptores sigan una u otra vía vienen marcados por las proteínas *Rab* y ciertas cascadas de señalización, y varían con cada tipo de receptor. Por ejemplo, la subunidad GluR2 de los receptores AMPA será reciclada o degradada, respectivamente, en función de si su internalización es inducida por el propio agonista AMPA o por NMDA (Lee *et al.*, 2004), mientras lo contrario sucede con la subunidad GluR1 (Ehlers, 2000). En el caso de los receptores NMDA, las subunidades NR2A y NR2B tienen diferentes motivos endocíticos (formados por 4 aminoácidos) en el extremo C-terminal que determinan; primero, la afinidad por las proteínas AP; y después la ruta de internalización que seguirán, siendo preferente la de reciclaje para NR2B (Lavezzari *et al.*, 2004). Se ha observado que la inhibición de GSK3β reduce de manera rápida y específica los receptores NR1/NR2B mediante su disociación con PSD95 (Chen *et al.*, 2007). En cambio, la subunidad NR1 está regulada por

ubiquitinación y posterior degradación por el proteosoma (Kato *et al.*, 2005). Respecto a los receptores metabotrópicos de glutamato, éstos siguen un mecanismo de endocitosis diferente: la constitutiva se produce a través de la proteína caveolina-1 y las "balsas lipídicas" (Francesconi *et al.*, 2009), mientras que la dependiente de ligando está mediada por las arrestinas (Mundell *et al.*, 2001).

1.3.4. Plasticidad homeostática

Existen dos tipos de plasticidad homeostática: la metaplasticidad y la conocida en inglés como *synaptic scaling* ("balance sináptico"). La metaplasticidad consiste en cambios funcionales (generalmente a nivel de los receptores de NMDA) que alteran la capacidad de las neuronas de inducir los propios procesos de plasticidad sináptica (LTP y LTD). En cambio, el "balance sináptico" se trata de un proceso fisiológico de regulación del número o densidad de componentes que participan en la neurotransmisión (principalmente receptores) en respuesta a cambios a largo plazo en la actividad sináptica. Este proceso fisiológico es un mecanismo compensatorio que permite mantener en equilibrio el balance entre la excitación y la inhibición neuronal, con el fin de evitar las consecuencias fatales que supone, por ejemplo, el exceso de activación de receptores de glutamato (ver apartado 1.4). A diferencia de la gran movilidad en la membrana que caracteriza a los receptores AMPA, acorde con su descrita función en la plasticidad sináptica, los receptores NMDA siempre se han considerado bastante estables en la superficie celular (con una vida media de unas 20 horas). Sin embargo, en los últimos años se ha demostrado que el tráfico de receptores NMDA es muy dinámico y está controlado en gran medida por la actividad sináptica, considerándose incluso el principal mecanismo molecular responsable de los fenómenos de plasticidad homeostática, junto al de los receptores AMPA (Perez-Otano & Ehlers, 2005; Turrigiano, 2008). De esta manera, el bloqueo continuado de la actividad sináptica produce un incremento reversible del número de receptores NMDA en la membrana postsináptica (Rao & Craig, 1997), mientras que la actividad prolongada produce el efecto contrario (Crump *et al.*, 2001; Swann *et al.*, 2007). Sin embargo, este proceso no se limita a la actividad sináptica sino que el bloqueo crónico de los transportadores de glutamato tiene un efecto similar (Cebers *et al.*, 1999), a pesar de que en este caso se produce también la activación de receptores extrasinápticos. Como hemos mencionado anteriormente, la plasticidad homeostática es además un fenómeno bidireccional que no sólo afecta a la neurotransmisión excitatoria (glutamatérgica) sino

también a la neurotransmisión inhibitoria (GABAérgica). Así, la activación mantenida de los receptores NMDA por un lado disminuye la funcionalidad de los receptores de glutamato (Brandoli *et al.*, 1998; Shi *et al.*, 2001), y por otro incrementa la del receptor $GABA_A$ (Aamodt *et al.*, 2000). Por el contrario, el tratamiento crónico con antagonistas NMDA incrementa la neurotransmisión glutamatérgica (Rao & Craig, 1997) y disminuye la GABAérgica (Kinney *et al.*, 2006). Finalmente, en la plasticidad homeostática no intervienen exclusivamente los receptores de glutamato y de GABA sino también las proteínas *scaffold*, las kinasas de señalización intracelular, ciertos factores neurotróficos (como BDNF) o incluso algunas citoquinas (como TNFα) liberadas por las células gliales (Perez-Otano & Ehlers, 2005; Turrigiano, 2008).

1.4. EXCITOXICIDAD DEL GLUTAMATO

A pesar de las numerosas e importantes funciones que tiene el glutamato en el sistema nervioso central, también puede tener efectos letales para las neuronas cuando se encuentra en grandes concentraciones en el medio extracelular. Esta situación constituye la base etiofisiopatológica del daño neurológico producido por la isquemia, el trauma craneoencefálico o la epilepsia, y también contribuye al progreso de enfermedades neurodegenerativas como el Alzheimer o el Huntington. El término excitotoxicidad se refiere a la muerte celular producida por la excesiva activación de los receptores de glutamato y fue acuñado originariamente por (Olney, 1969). Inicialmente fue asociado a un incremento del volumen celular y a la depleción de las reservas energéticas de la célula. Más adelante, (Choi, 1987) estableció que existen dos componentes en la muerte neuronal causada por el glutamato: uno marcado por un hinchamiento celular a raíz de la entrada masiva de Na^+ y Cl^-, seguida de agua por ósmosis, que puede dar lugar a la rotura de la membrana celular; y otro más lento y predominante a menores concentraciones de glutamato, causado por la entrada de Ca^{2+} y la consecuente activación de proteasas y fosofolipasas. Posteriormente, se asoció el primer componente de la excitotoxicidad a la muerte por necrosis y el segundo a la apoptosis causada por la activación de caspasas (Meli *et al.*, 2004), aunque la contribución de cada componente a la muerte excitotóxica es todavía fuente de amplio debate.

1.4.1. Determinantes moleculares de la excitotoxicidad

(Choi, 1987) fue el primero en sugerir que la entrada de Ca^{2+} a través de los receptores NMDA (pero no a través de los canales de Ca^{2+} regulados por voltaje o de los receptores AMPA/Kainato) era la principal causante de la excitotoxicidad mediada por glutamato, hecho que se confirmó en trabajos posteriores (Michaels & Rothman, 1990; Koh & Choi, 1991; Tymianski *et al.*, 1993). Debido a estos hallazgos, comenzó a formarse el concepto de "compartimentalización del Ca^{2+} citosólico" y a debatirse su implicación en la señalización así como en la muerte neuronal. En este sentido, la activación de diferentes receptores de glutamato, a pesar de compartir el mismo mensajero (el Ca^{2+}), puede dar lugar a respuestas fisiológicas o patológicas que variarán en función de la intensidad y duración del estímulo por un lado, y de la localización del receptor así como de las proteínas asociadas a éste, por otro. Así, por ejemplo, la activación de un mismo subtipo de receptor metabotrópico de glutamato puede favorecer o prevenir la excitotoxicidad dependiendo de factores tan diversos como su asociación con los receptores NMDA o la presencia de astrocitos (ver apartado 1.4.3). De manera similar, se han asignado papeles opuestos a los receptores NMDA dependiendo de su composición así como de su localización en la membrana celular. Si bien está ampliamente aceptado que la activación de los receptores NMDA sinápticos estimula la supervivencia neuronal y la de los extrasinápticos desencadena la cascadas de muerte celular; en cambio, la implicación de las subunidades NR2A y NR2B en ambos procesos es todavía un aspecto controvertido (Hardingham, 2009). Así, tanto los receptores NR1/NR2B como los NR1/NR2A son capaces de mediar la muerte inducida por glutamato en neuronas corticales maduras (von Engelhardt *et al.*, 2007). Por ello, se ha sugerido que las rutas de señalización asociadas al receptor NMDA tienen un papel crítico en el desarrollo de la excitotoxicidad. En este sentido, la interacción con las proteínas PSD95 y nNOS parece ser determinante en la conexión de los receptores NMDA con los procesos excitotóxicos (Cui *et al.*, 2007). Asimismo, se ha propuesto que la exposición a dosis bajas de NMDA favorece la activación de los receptores sinápticos y con ello la de las rutas de supervivencia PI3K/Akt y MAPK. Por el contrario, dosis altas de NMDA favorecen la activación de los receptores extrasinápticos estimulando así la activación de calpaínas, la sobrecarga de Ca^{2+} en la mitocondria y la producción excesiva de óxido nítrico (Hardingham, 2009).

Por otro lado, la entrada de Cl^- a través de los receptores $GABA_A$ y de los canales de Cl^- contribuyen también a la excitotoxicidad. Por ejemplo, los antagonistas de los receptores $GABA_A$ y de los canales de Cl^- protegen contra a la toxicidad inducida por alto K^+ en células granulares de cerebelo al prevenir la liberación de glutamato (Babot *et al.*, 2005). En cambio, en neuronas corticales estos mismos antagonistas de los receptores $GABA_A$ no tienen efecto protector frente al tratamiento con NMDA, mientras que los de los canales de Cl^- sensibles a volumen sí que lo tienen, pero su mecanismo es a nivel postsináptico previniendo el hinchamiento celular y la posterior necrosis (Inoue & Okada, 2007). Otro estudio muestra cómo la distribución diferencial de los receptores NMDA y AMPA/Kainato en las distintas capas corticales determina su contribución a la excitotoxicidad (Young *et al.*, 2007). Por tanto, a pesar de que los receptores NMDA se consideran los mayores responsables del daño neuronal mediado por el glutamato, muchos otros receptores (no sólo de glutamato) intervienen en el proceso y su contribución específica dependerá de la región cerebral, de su localización celular y del tipo de estímulo excitotóxico.

Además del origen del Ca^{2+} citosólico, se ha demostrado que la duración del estímulo es un factor determinante en la muerte excitotóxica, pero no así la magnitud de $[Ca^{2+}]_i$ alcanzada (Tymianski *et al.*, 1993). En este sentido, se ha observado que la activación de los receptores AMPA/Kainato, pero no de los receptores NMDA, ha de ser prolongada para producir neurodegeneración (Koh & Choi, 1991), a pesar de que el efecto máximo sobre la $[Ca^{2+}]_i$ es similar para ambos receptores (Carriedo *et al.*, 1998). Sin embargo, la entrada rápida de Ca^{2+} a través de los receptores AMPA/Kainato permeables al mismo también es capaz de producir muerte, de manera similar a la producida por NMDA. Esto es debido a que, en estos casos, la $[Ca^{2+}]_i$ se mantiene alta incluso una vez retirado el agonista, mientras que el incremento de $[Ca^{2+}]_i$ causado por la activación de canales de Ca^{2+} o de receptores AMPA/Kainato impermeables a Ca^{2+} es transitorio (Carriedo *et al.*, 1998). La activación de receptores NMDA estimula además la liberación de glutamato produciendo un efecto de retroalimentación positiva que contribuye de manera importante a la excitotoxicidad (Fogal *et al.*, 2005).

Por último, se ha observado que la susceptibilidad a la excitotoxicidad crece a lo largo del desarrollo, hecho que se atribuyó inicialmente al aumento de expresión de receptores de glutamato a medida que las neuronas maduran, a pesar de que no se correspondía con una mayor funcionalidad de los receptores (Koroshetz *et al.*, 1990;

Frandsen & Schousboe, 1990; Mizuta *et al.*, 1998). Más recientemente, algunos autores han sugerido que la localización extrasináptica de los receptores NMDA y AMPA permeables a Ca^{2+} es determinante en este fenómeno (Sinor *et al.*, 2000; King *et al.*, 2006), mientras que otros lo han achacado a una mayor liberación de glutamato posterior al estímulo excitotóxico en neuronas maduras (Fogal *et al.*, 2005).

1.4.2. Rutas de señalización implicadas en la excitotoxicidad

Existen diferentes mecanismos que contribuyen en mayor o menor medida a la excitotoxicidad del glutamato dependiendo del tipo de estímulo y de la célula que se trate. En la mayoría de las ocasiones, al menos en aquellas en las que intervienen los receptores NMDA, es la combinación de todos ellos la que produce efectos letales para las neuronas. En la figura 6 se ilustran las rutas más habitualmente implicadas en la muerte excitotóxica derivada de la excesiva activación de los receptores NMDA.

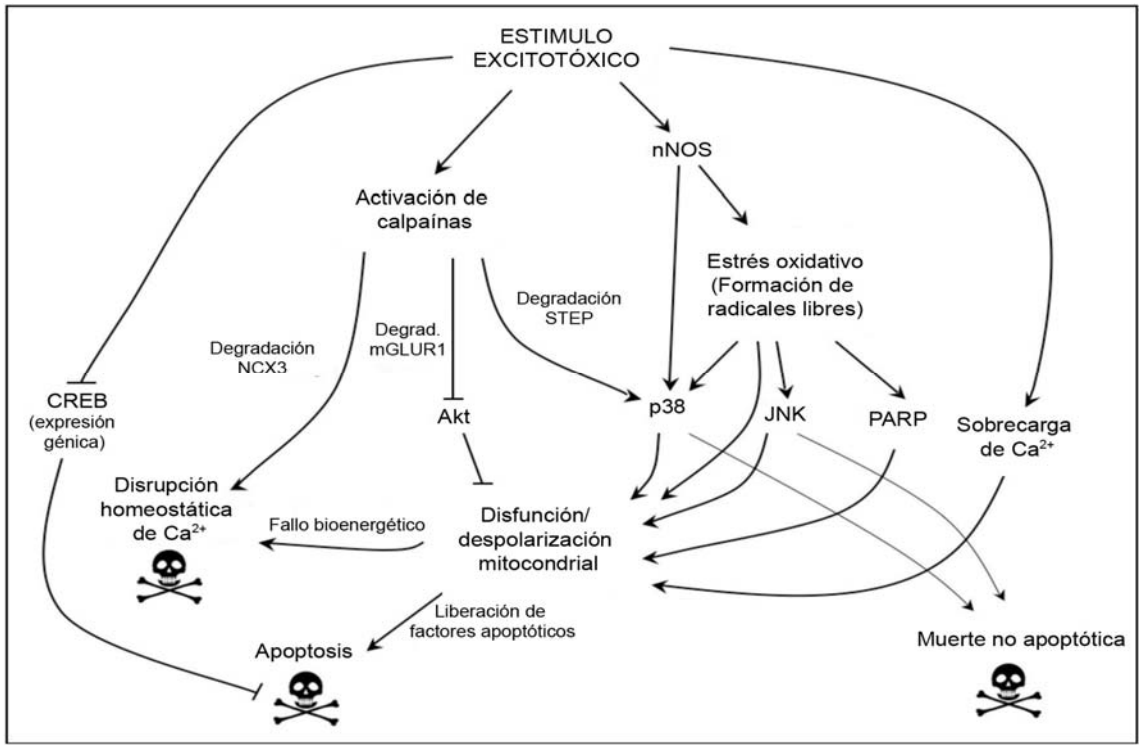


Figura 6. Rutas intracelulares implicadas en la excitotoxicidad (modificada de Hardingham, 2009))

1.4.2.1 Daño mitocondrial y estrés oxidativo: Es bien sabido que las mitocondrias captan el Ca^{2+} citosólico a través de un uniportador, lo que facilita la síntesis de ATP. Además, de modo parecido al retículo endoplasmático, las mitocondrias pueden

funcionar como depósitos intracelulares transitorios de Ca^{2+}, de manera tal que regulan la $[Ca^{2+}]_i$ a nivel local y, con ello, procesos fisiológicos tales como la liberación de neurotransmisores (Aldea *et al.*, 2002). Sin embargo, en situaciones patológicas en las que la $[Ca^{2+}]_i$ se mantiene alta puede llegar a producirse una sobrecarga de Ca^{2+} en la mitocondria, acompañada de la disrupción de su potencial de membrana. Esta situación tendrá múltiples consecuencias fatales para la célula, tales como: (i) la reversión de la actividad ATP sintasa que ahora consumirá energía en lugar de sintetizarla (ii) el desacoplamiento de la cadena de transporte electrónico a la fosforilación oxidativa lo que generará la producción masiva de radicales libres; (iii) la formación del poro de transición de permeabilidad mitocondrial que dará paso a la liberación de factores pro-apoptóticos al citosol, como el citocromo *c*, el AIF (factor inductor de la apoptosis) o precursores de las caspasas (pro-caspasas). La familia de proteínas *Bcl*-2 (como la propia *Bcl*-2 o *Bax*) regulan estos procesos impidiendo o permitiendo la formación de este poro. Finalmente, la apoptosis puede desencadenarse de manera dependiente o independiente de caspasas. En el primer caso, el citocromo *c* liberado al citosol se asocia con las proteínas Apaf-1 y la pro-caspasa 9 formando el apoptosoma. Éste complejo activa la caspasa-9 que a su vez activará por proteolisis a la caspasa-3, la cual se encarga de procesar enzimas digestivas nucleares como poli-(ADP-ribosa) polimerasa (PARP). Además, las caspasas pueden autoactivarse amplificando la llamada cascada de las caspasas. En cambio, la liberación de AIF desencadena la apoptosis independiente de caspasas, puesto que por sí solo es capaz de causar la degradación del ADN y la condensación de la cromatina (Le Bras *et al.*, 2006).

Por otro lado, la excesiva formación de óxido nítrico a causa de la sobreactivación de los receptores NMDA también puede contribuir al estrés oxidativo, ya sea por sí mismo o por la formación de derivados como los peroxinitritos, puesto que ambos inhiben la respiración mitocondrial amplificando así la producción de radicales libres y además pueden oxidar lípidos y proteínas e incluso fragmentar el ADN (Hardingham, 2009).

1.4.2.2 Calpaínas: Las calpaínas, al igual que las caspasas, pertenecen a la familia de las cisteín-proteasas ya que contienen un residuo de cisteína en el centro activo que media su acción proteolítica. A diferencia de éstas, las calpaínas son activadas directamente por Ca^{2+}. Dado que requieren una alta $[Ca^{2+}]_i$ para su activación, se han asociado mayoritariamente a la activación de los receptores NMDA. Su principal función fisiológica es la degradación de receptores de membrana (como los propios rceptores

NMDA), proteínas del citoesqueleto (como la α-espectrina) y proteínas *scaffold*, de manera tal que modulan su función o facilitan su degradación. Por esta razón, se les ha asignado un papel importante en la regulación de distintos aspectos de la neurotransmisión. Sin embargo, debido a la baja selectividad de su capacidad proteolítica, también se han relacionado con el desarrollo de ciertas enfermedades neurodegenerativas, como el Alzheimer o el Parkinson (Wu & Lynch, 2006). Asimismo, la activación descontrolada de las calpaínas está implicada en el daño neuronal provocado por la epilepsia, en donde éstas pueden originar la activación de AIF o de la endonucleasa G a través del procesamiento de proteínas de la familia *Bcl*-2 (Fujikawa, 2005). Otras dianas celulares de las calpaínas son mGLUR1 (ver apartado 1.4.3) o el intercambiador Na^+/Ca^{2+} (NCX3), cuya proteolisis conduce a la disrupción homeostática del Ca^{2+} y, con ello, contribuye a la excitotoxicidad (ver figura 6).

1.4.2.3 Rutas de señalización MAPK: La familia de proteínas MAPK se organizan en 3 cascadas de señalización que se conocen con el nombre de sus kinasas efectoras: ERK1/2, p38 y JNK. Éstas comparten el mismo mecanismo de activación, por fosforilación en dos residuos de treonina y de tirosina, y de acción, ya que se traslocan al núcleo, donde inducen la expresión de proteínas implicadas en la proliferación y la diferenciación celular, entre otras funciones, a través de su acción sobre determinados factores de transcripción. Las rutas MAPK pueden ser estimuladas por un gran número de estímulos extracelulares, como neurotransmisores, factores neurotróficos, citoquinas, hormonas, etcétera. Además, son activadas en respuesta al estrés oxidativo o al aumento de $[Ca^{2+}]_i$, por lo que han sido implicadas en la muerte neuronal asociada a numerosas patologías (Colucci-D'Amato *et al.*, 2003; Kim & Choi, 2010). Todas estas rutas se han relacionado también con la excitotoxicidad mediada por los receptores NMDA. Así, por ejemplo, p38 media la muerte excitotóxica en células granulares de cerebelo, mientras que tanto p38 como JNK contribuyen a ella en neuronas corticales. En este contexto, la activación de p38 depende del acoplamiento entre los receptores NMDA y nNOS a través de PSD95, y además puede ser estimulada a través de la degradación de la proteína STEP por las calpaínas (ver figura 6)

En cambio, el papel de la vía de señalización ERK1/2 en la excitotoxicidad parece ser más complejo, habiéndose mostrado tanto efectos protectores como perjudiciales dependiendo del modelo de muerte usado. Por ejemplo, la supervivencia inducida a través de los receptores NMDA sinápticos depende de la activación de ERK1/2 y CREB

y de la subsiguiente expresión de factores antiapoptóticos, mientras que la estimulación de los extrasinápticos inhibe esta misma ruta (Hardingham, 2009). Por el contrario, la activación crónica de ERK1/2 ha sido relacionada con el con la muerte neuronal producida por la isquemia o la epilepsia, así como con el desarrollo de cáncer o de algunas enfermedades de neurodegenerativas. Sin embargo, existen diferencias en cuanto a la localización celular de la forma activa de ERK1/2 entre las distintas patologías (Murray *et al.*, 1998; Colucci-D'Amato *et al.*, 2003). Por tanto, no sólo la cinética sino también la localización determinan el efecto que tendrá la activación de ERK1/2 sobre la viabilidad neuronal.

1.4.3. Implicación de los receptores metabotrópicos de glutamato en la excitotoxicidad

Si bien a los receptores metabotrópicos de glutamato de tipo II y III se les ha atribuido generalmente un efecto neuroprotector frente a la excitotoxicidad; ya sea por limitar la liberación de glutamato, por incrementar la captación de éste por los astrocitos o por inducir la liberación glial de TGFβ (Sabelhaus *et al.*, 2000; D'Onofrio *et al.*, 2001; Yao *et al.*, 2005), el papel de los mGLUR de tipo I al respecto es, cuanto menos, controvertido. Por un lado, se ha descrito que sus agonistas incrementan la toxicidad causada por NMDA tanto *in vitro* como *in vivo* (McDonald & Schoepp, 1992; Bruno *et al.*, 1995) y, por el contrario, estos mismos compuestos previenen la muerte inducida por NMDA en células granulares de cerebelo (Pizzi *et al.*, 1996), a pesar de que ambos efectos están mediados por PKC. Con el fin de aclarar estos resultados contradictorios, (Nicoletti *et al.*, 1999) estableció diferentes factores que podían influir en este comportamiento dual de los mGLUR de tipo I en la excitotoxicidad. El primero de ellos es la presencia de la subunidad NR2C en los receptores NMDA, la cual está altamente expresada en células granulares de cerebelo maduras y cuya actividad está disminuida por la acción de PKC, lo que explicaría el efecto protector derivado de la activación de los mGLUR de tipo I sobre la toxicidad del NMDA en estas neuronas. En cambio, la fosforilación de las subunidades NR2A y NR2B por PKC aumenta su actividad y por tanto puede incrementar sus efectos neurotóxicos. Otro factor es el llamado cambio funcional ("functional switch", en inglés), que consiste en que una primera activación de los mGLUR de tipo I tiene un efecto facilitador de la transmisión sináptica, pero la repetida estimulación de éstos en un breve lapso de tiempo (< 45 minutos) o el aumento de la concentración extracelular de glutamato produce el efecto contrario (inhibitorio).

Este fenómeno fue observado inicialmente a nivel presináptico modulando la liberación de glutamato a través de la distinta regulación de los canales de K^+ y de Ca^{2+}, respectivamente (Herrero *et al.*, 1998). Más adelante, se comprobó que este cambio funcional de los mGLUR de tipo I también sucedía a nivel postsináptico y frente a un estímulo excitotóxico y además parece implicar la desensibilización del receptor mediada por PKC (Bruno *et al.*, 2001). En cambio, la activación de la calcineurina por estimulación de los receptores NMDA induce la desfosforilación de mGLUR5, lo que potencia su actividad causando la "resensibilización" del receptor (Alagarsamy *et al.*, 2005). Finalmente, (Nicoletti *et al.*, 1999) también postularon que la presencia de mGLUR5 en astrocitos también podría contribuir a la excitotoxicidad, hecho que se confirmó posteriormente (Ding *et al.*, 2007).

En cuanto a la contribución específica de los subtipos mGLUR1 y mGLUR5 a la excitotoxicidad, se han descrito mecanismos diferentes para cada uno de ellos. Por ejemplo los antagonistas de mGLUR1 son neuroprotectores frente a la excitotoxicidad a causa de que incrementan la liberación de GABA (Battaglia *et al.*, 2001), mientras que el de mGLUR5, MPEP, lo es porque inhibe la secreción de glutamato pero también debido a su acción sobre los receptores postsinápticos (Popoli *et al.*, 2004). En células granulares de cerebelo, los efectos protectores derivados de la activación de mGLUR5 se deben a la inhibición de la actividad de nNOS inducida por NMDA (Llansola & Felipo, 2010). En cambio, el papel de mGLUR1 en la excitotoxicidad en estas células es ambiguo puesto que, por un lado, su sobreexpresión en condiciones de privación trófica causa muerte apoptótica de manera independiente de ligando y, al mismo tiempo, es responsable de la neuroprotección mediada por glutamato bajo este modelo (Pshenichkin *et al.*, 2008). Además, recientemente se ha descubierto que la activación de los receptores NMDA induce el truncamiento de la región intracelular de mGLUR1 por las calpaínas, lo que le permite conservar la capacidad de incrementar la $[Ca^{2+}]_i$ pero impide la activación de la ruta PI3K/Akt por éste, y por ello contribuye a la excitotoxicidad (Xu *et al.*, 2007b) (ver figura 6).

En los últimos años ha habido varios estudios que demuestran la existencia de un acoplamiento funcional entre los receptores NMDA y los metabotrópicos de tipo I en aspectos fisiológicos tales como la regulación de la expresión génica y la transmisión del impulso nervioso (Stoop *et al.*, 2003; Yang *et al.*, 2004; Guo *et al.*, 2004). Es más, la estimulación simultánea de ambos receptores tiene un efecto sinérgico sobre la

fosforilación de ERK1/2 a través de un mecanismo independiente de Ca^{2+} (Yang *et al.*, 2004). En cambio, debido a esta interacción también pueden darse efectos inhibitorios recíprocos sobre el incremento de $[Ca^{2+}]_i$ entre mGLUR5 y el receptor NMDA (Perroy *et al.*, 2008). Estos efectos, en apariencia contradictorios, parecen explicarse por el tipo de interacción entre los receptores; en el primer caso el efecto sinérgico está mediado a través de la formación de un complejo entre las proteínas Homer y PSD95, mientras que en el segundo se trata de una interacción directa entre los receptores y requiere el extremo C-terminal de mGLUR5 libre. Por todo ello, la interacción con los receptores NMDA así como la asociación con las proteínas *scaffold* y las vías de señalización intracelulares determina la diferente modulación de la excitotoxicidad por los receptores metabotrópicos de glutamato.

1.4.4. Toxicidad oxidativa del glutamato

Murphy *et al.* (1989) descubrieron un mecanismo alternativo por el cual el glutamato causaba muerte de manera independiente de sus receptores en una línea celular derivada de neuronas. Se le dio el nombre de toxicidad oxidativa porqué implica la producción de radicales libres y se previene con antioxidantes. El mecanismo a nivel molecular está basado en la inhibición del transporte de cistina (dímero de cisteína) por el glutamato, de modo tal que reduce los niveles intracelulares de cisteína, precursor y factor limitante en la biosíntesis del péptido antioxidante glutatión. La disminución de glutatión puede conducir a la muerte neuronal puesto que ésta molécula no sólo actúa como el mayor antioxidante endógeno en las células, sino que también participa en la síntesis y reparación del ADN. Posteriormente, la toxicidad oxidativa del glutamato se observó también en otras líneas celulares (PC12 y HT22) y en neuronas corticales inmaduras, en las que no se expresan receptores NMDA funcionales (Murphy *et al.*, 1990; Schubert *et al.*, 1992; Maher & Davis, 1996). Este tipo de muerte requiere la presencia de monoaminas como fuente de radicales libres (Maher & Davis, 1996) y está modulada por factores neurotróficos y por la activación de los receptores metabotrópicos de glutamato de tipo I (Schubert *et al.*, 1992; Sagara & Schubert, 1998). Es más, la ruta ERK1/2 juega un papel determinante al respecto; primero, mediando los efectos protectores derivados de la activación de los mGLURs de tipo I durante la fase inicial, pero contribuyendo a la muerte en la fase tardía de la exposición a glutamato (Luo & DeFranco, 2006).

2. LA NEUROTRANSMISIÓN GABAÉRGICA

El GABA es el mayor neurotransmisor inhibitorio del sistema nervioso central. Es un derivado del glutamato sintetizado por la enzima glutamato descarboxilasa (GAD), presente exclusivamente en las neuronas GABAérgicas. Existen dos isoformas, GAD65 y GAD67, que difieren en su distribución cerebral y también a nivel celular. La primera está localizada principalmente en los terminales nerviosos en su forma inactiva (apoGAD65) y se activa en respuesta a una demanda rápida de GABA. En cambio, GAD67 está distribuida de manera uniforme por la célula, razón por la cual suele usarse como marcador de neuronas GABAérgicas. El GABA es introducido en las vesículas secretoras a través del transportador vesicular vGAT y, una vez liberado, actúa sobre los receptores postsinápticos. Este neurotransmisor sigue un ciclo similar al glutamato (ver figura 1), siendo captado por los astrocitos o por las propias neuronas GABAérgicas mediante los transportadores de GABA (GAT1-3 y BGT1, todos ellos dependientes de Na^+/Cl^-). En los astrocitos es degradado por la enzima GABA transaminasa transformándose en un intermediario del ciclo de Krebs, mientras que en las neuronas GABAérgicas es reutilizado como neurotransmisor (Martin & Rimvall, 1993; Madsen *et al.*, 2008). El GABA puede ser liberado junto a glicina o ATP, neurotransmisores inhibitorio (cuando activa los receptores de glicina pero no los de NMDA) y excitatorio, lo que potenciará o atenuará la respuesta fisiológica de GABA, respectivamente (Alger & Le Beau, 2001). Además, durante el desarrollo el glutamato es secretado junto a GABA y glicina lo que parece contribuir al refinamiento de las sinapsis inhibitorias (Gillespie *et al.*, 2005).

Hasta el momento se han identificado dos tipos de receptores de GABA: uno es ionotrópico, el receptor $GABA_A$, y el otro es metabotrópico, el receptor $GABA_B$. Los receptores $GABA_A$ se caracterizan por ser permeables al Cl^- de manera que producen generalmente una hiperpolarización de la membrana plasmática y la consecuente inhibición de la neurotransmisión (ver apartado 2.3). En cuanto a los receptores $GABA_B$, pertenecen a la misma superfamilia de GPRs que los receptores metabotrópicos de glutamato, como ya mencionamos anteriormente, y están acoplados a proteínas G_i, con lo que inhiben la formación de AMPc y contribuyen así al efecto inhibitorio del GABA. Destaca su alta expresión en las células granulares de cerebelo (Möhler *et al.*, 2001).

2.1. ESTRUCTURA Y COMPOSICIÓN DEL RECEPTOR $GABA_A$

Los receptores $GABA_A$ son heteropentámeros formados por la combinación de hasta 18 subunidades diferentes (ver tabla 2), 20 incluyendo las variantes de *splicing* de β2 y de γ2. Pertenecen a la llamada superfamilia de receptores ionotrópicos "Cys-loop" (por tener todos ellos un puente de disulfuro entre dos cisteínas en el extremo N-terminal), que incluye, además de los receptores $GABA_A$, los receptores nicotínicos de acetilcolina, los de serotonina $5HT_3$ y los de glicina. Todas las subunidades de los receptores $GABA_A$ están formadas por 4 dominios transmembrana (llamados TM1-4), un dominio extracelular en el extremo N-terminal y un dominio intracelular entre TM3 y TM4, susceptible de ser regulado por fosforilación. Los dominios TM2 de cada una de las 5 subunidades que componen el receptor forman el poro del canal de Cl^-.

Tabla 2. Subunidades del receptor $GABA_A$

α1-6	β1-3	γ1-3	δ	ε	π	ρ1-3

Los receptores $GABA_A$ están normalmente formados por dos subunidades *α*, dos subunidades *β* y una subunidad *γ* ó δ (ver figura 7), siendo el más expresado en el cerebro adulto el compuesto por las subunidades *α*1/*β*2/*γ*2. La presencia de unas u otras subunidades confiere al receptor propiedades farmacológicas muy diferentes (ver apartado 2.2). Un caso particular es el de la subunidad *α*6, la cual se expresa exclusivamente en las células granulares del cerebelo en el cerebro adulto. Esta subunidad forma habitualmente receptores de tipo *α*6/*β*/*δ* que, al igual que los *α*4/*β*/*δ* en otras células, suelen localizarse en sitios extrasinápticos y son responsables de la inhibición tónica o constitutiva, dado que son más sensibles a GABA. Existe también un subtipo de receptores $GABA_A$, a menudo llamados receptores $GABA_C$, cuya composición estructural es diferente al resto de receptores $GABA_A$, aunque conserva las principales propiedades funcionales de éstos, como la permeabilidad al Cl^-. Se localizan casi exclusivamente en la retina y están formados por homopentámeros de la subunidad *ρ*. Se han descrito como receptores de GABA insensibles a bicuculina y baclofen, antagonistas respectivos de los receptores $GABA_A$ y $GABA_B$, aunque ligeramente sensibles al antagonista no competitivo del receptor $GABA_A$ picrotoxinina (Möhler *et al.*, 2001).

Los niveles de expresión de las distintas subunidades del receptor $GABA_A$ varían a lo largo del desarrollo cerebral, especialmente en el caso de las subunidades α y β. Este fenómeno sigue el mismo patrón *in vitro*, de modo que en cultivos de neuronas corticales las subunidades α3, α5 y β3 disminuyen, mientras que α2 aumenta progresivamente. Se ha comprobado que estos cambios de expresión están regulados por la actividad a través del propio receptor $GABA_A$; así, el neuroesteroide alopregnanolona los acelera y, en cambio, el antagonista TBPS no sólo los inhibe sino que los invierte (Poulter *et al.*, 1997). Por el contrario, en las células granulares de cerebelo, el incremento es general para todas las subunidades allí expresadas excepto α1 y α6, cuya expresión se mantiene constante (Behringer *et al.*, 1996), aunque está estimulada por factores neurotróficos, como el BDNF (Bulleit & Hsieh, 2000), así como por la activación del receptor $GABA_A$ (Elster *et al.*, 1995; Carlson *et al.*, 1997). Además, la concentración de KCl en el medio de cultivo tiene efectos muy diferentes sobre unas y otras; de manera que, cuando se mantiene alta (25mM), disminuye la expresión de α1, α6 y β2 y aumenta la de α4, β3 y δ, como consecuencia de la activación de los de los receptores NMDA y AMPA/Kainato (Gault & Siegel, 1998; Payne *et al.*, 2008).

2.2. FARMACOLOGÍA DEL RECEPTOR $GABA_A$

Existe un elevado número de sustancias químicas cuyos efectos son mediados por su acción sobre los receptores $GABA_A$. Algunas de ellas son compuestos endógenos como el propio neurotransmisor GABA, el catión Zn^{2+} o los neuroesteroides; otras son drogas de abuso o anestésicos, como las benzodiacepinas, los barbitúricos, ciertos éteres o el etanol; y también encontramos ciertos productos naturales, como los flavonoides y los terpenos; e industriales como los pesticidas organoclorados o el tolueno (Johnston, 2005). En la figura 7 se ilustran los diferentes sitios de unión al receptor $GABA_A$ de algunos de estos compuestos.

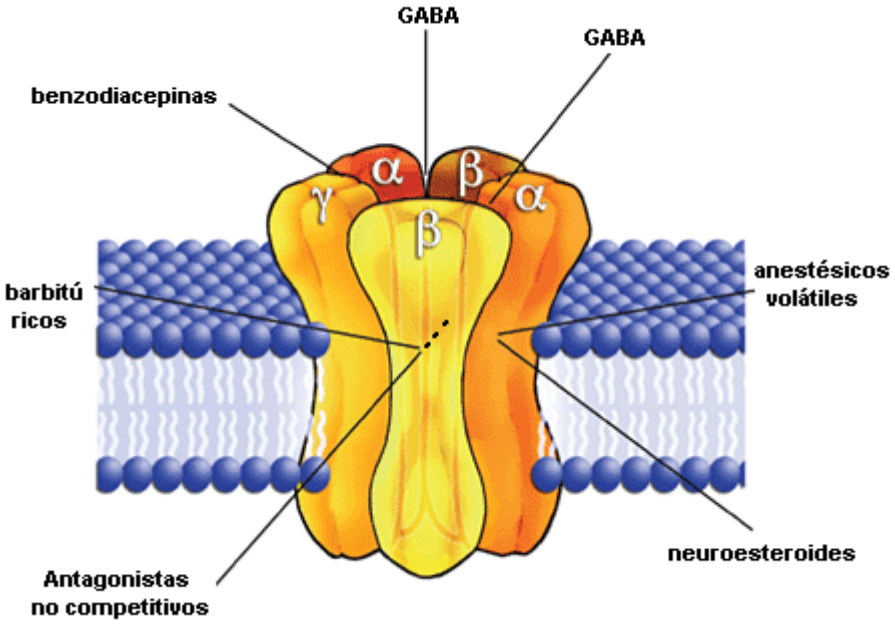


Figura 7. Estructura y farmacología del receptor $GABA_A$ (modificada de http://www.niaaa.nih.gov/Resources/GraphicsGallery/Neuroscience/PublishingImages/)

2.2.1. Benzodiacepinas y barbitúricos

Las benzodiacepinas son moduladores alostéricos positivos que potencian la acción fisiológica de GABA, al aumentar la frecuencia de apertura del canal iónico asociado al receptor $GABA_A$. La presencia de las subunidades α y γ es necesaria para la formación del sitio de unión a benzodiacepinas. Los receptores $GABA_A$ que contienen las subunidades α1-3 o α5 tienen una alta afinidad por éstas. En cambio, los que están formados por α4 o α6 presentan una baja afinidad por las mismas. Mientras que la subunidad α1 es responsable de los efectos sedativos y sobre la memoria de estos compuestos, α2-3 y α5 median sus efectos ansiolíticos y de relajación motora. Hasta el momento no se han descubierto moléculas endógenas que activen este lugar de acción (Möhler *et al.*, 2001; Johnston, 2005). La afinidad de un compuesto determinado por el sitio de acción de las benzodiacepinas suele evaluarse mediante el ensayo de unión de [^{3}H]-flunitrazepam, el cual puede usarse también para evaluar la funcionalidad del receptor $GABA_A$ (Suñol *et al.*, 2006).

Los barbitúricos y otros anestésicos (como el cloroformo o el halotano) también actúan como moduladores alostéricos positivos del receptor $GABA_A$ aunque, a diferencia de las benzodiacepinas, son capaces de activarlo a altas concentraciones en ausencia de

GABA. Las subunidades α y β se han mostrado determinantes en la acción de estos compuestos. Sin embargo, debido a su alta inespecificidad, no sólo dentro de los receptores $GABA_A$ sino porqué además inhiben los receptores AMPA/Kainato y los receptores nicotínicos de acetilcolina, han sido progresivamente substituidos por otros anestésicos intravenosos más específicos, como el etomidato o el propofol (Möhler *et al.*, 2001; Johnston, 2005).

2.2.2. Antagonistas no competitivos

Los antagonistas no competitivos del receptor $GABA_A$ actúan sobre el sitio de unión de la picrotoxinina, bloqueando el paso de iones Cl^- a través del canal. Esta acción inhibitoria sobre el receptor $GABA_A$ les confiere propiedades convulsivas. La afinidad de un compuesto determinado por el sitio de acción de la picrotoxinina suele evaluarse mediante el ensayo de unión de [^{35}S]-TBPS. De esta manera se ha evaluado la acción sobre el receptor $GABA_A$ de un gran número de compuestos, entre ellos los pesticidas organoclorados (Lawrence & Casida, 1984; Pomes *et al.*, 1993; Pomes *et al.*, 1994b). Los antagonistas no competitivos del receptor $GABA_A$ generalmente constan de una región hidrofóbica y, en algunos casos, también contienen una zona polar dentro de su estructura química. La presencia de esta última, como en el caso de los pesticidas dieldrin y endosulfan así como en el de la picrotoxinina, determina su mayor afinidad por el receptor $GABA_A$ que por el de glicina (Vale *et al.*, 2003). Es más, la mutación de un residuo de alanina (en la posición 302) por otro de serina situado en el dominio MT2 que forma el canal iónico es responsable de la formación de receptores $GABA_A$ resistentes a los ciclodienos (como el dieldrin) y, a su vez, de un 60% de los casos de resistencia a estos pesticidas en la especie *Drosophila* (ffrench-Constant *et al.*, 1993). Sin embargo, la acción inhibitoria del diedrín no parece alterada por la distinta composición del receptor $GABA_A$ pero sí su acción potenciadora, que requiere la presencia de la subunidad γ (Nagata *et al.*, 1994). Se ha demostrado que el propio GABA, a concentraciones altas, puede actuar sobre este sitio de unión, aunque en este caso la subunidad γ sí que es necesaria (Korpi & Luddens, 1993).

A pesar de que tradicionalmente se ha considerado a estos compuestos como bloqueantes del poro del canal; sin embargo, un estudio reciente sugiere que su acción es de tipo alostérica negativa de modo que impide la apertura del canal más que producir directamente su bloqueo por impedimento estérico (Law & Lightstone, 2008). Esto puede explicar, tal vez, los efectos de modulación alostérica sobre los sitios de

unión de GABA y de las benzociacepinas que se les han asignado a alguno de estos compuestos (Solà *et al.*, 1993; Vale *et al.*, 1997). De manera inversa, las benzodizepinas y los barbitúricos son capaces de revertir el efecto inhibitorio de la picrotoxinina pero no, en cambio, del pesticida endosulfán (Pomes *et al.*, 1994b), por lo que algunas diferencias parecen darse entre estos compuestos en lo respectivo a su perfil farmacológico.

2.2.3. Esteroides neuroactivos

A lo largo de los años 80 varios estudios descubrieron que algunos esteroides endógenos, como la alopregnanolona, así como ciertos derivados sintéticos, potenciaban los efectos no sólo de GABA sino también de las benzodiacepinas y de los barbitúricos sobre el receptor $GABA_A$. También se observó que estos efectos alostéricos se producían a niveles fisiológicos (en el rango nanomolar) pero que, además, a concentraciones mayores eran capaces de activar el receptor por sí solos. Respecto a su mecanismo de acción, los esteroides aumentan el tiempo en que el canal iónico está abierto al reducir la cinética de desensibilización, de modo similar a los barbitúricos, pero también incrementan la frecuencia de apertura del canal actuando a nivel presináptico (Lambert *et al.*, 2001; Mellon, 2007). Se ha postulado que la presencia de la subunidad δ confiere a los receptores $GABA_A$ insensibilidad frente a los efectos alostéricos de estos compuestos, aunque no afecta a sus efectos miméticos de GABA, como se ha comprobado que sucede en las células granulares de cerebelo a medida que maduran (Zhu *et al.*, 1996). Sin embargo, más recientemente, se han establecido dos sitios de unión para los esteroides; uno responsable de sus efectos moduladores, localizado en la subunidad α; y otro que media su acción directa sobre el receptor y se sitúa en la interfaz entre las subunidades α y β (Hosie *et al.*, 2006). Además, otros autores han verificado que es precisamente la subunidad δ la que confiere una mayor sensibilidad a estas hormonas (Mihalek *et al.*, 1999; Wohlfarth *et al.*, 2002). Por tanto, no está totalmente claro cómo la distinta composición de los receptores $GABA_A$ altera los efectos de los esteroides sobre el mismo.

2.3. FUNCIONES Y SEÑALIZACIÓN DEL RECEPTOR $GABA_A$

La expresión temprana de los receptores $GABA_A$ en el sistema nervioso central (presente a partir del 14º día embrionario) ha hecho pensar que este receptor estaba involucrado en el desarrollo del cerebro. Varios estudios confirmaron que,

efectivamente, GABA estimulaba la migración y la diferenciación neuronales, siendo estos efectos mediados, en parte, por el aumento de $[Ca^{2+}]_i$, lo que sugería que este neurotransmisor tenía, además, una naturaleza excitatoria durante este periodo (ver apartado 2.3.2). Así, por ejemplo, la presencia del ARNm de algunas de las subunidades del receptor $GABA_A$ en los conos de crecimiento durante los primeros días *in vitro*, así como la inhibición de la elongación de neuritas o de la diferenciación de neuronas monoaminérgicas por antagonistas del receptor, indican que el GABA juega un papel importante en los procesos de sinaptogénesis y diferenciación neuronal (Liu *et al.*, 1997a; Poulter & Brown, 1999). Paralelamente, estudios con animales K.O. para algunas de las subunidades del receptor $GABA_A$ han revelado numerosas funciones en las que participa este receptor, como la memoria y el aprendizaje o la coordinación motora. Por último, se ha demostrado que las alteraciones en la funcionalidad de este receptor, ya sean de naturaleza genética o por factores ambientales, están fuertemente implicadas en determinados trastornos neurológicos, tales como la epilepsia, la ansiedad o el alcoholismo (Möhler *et al.*, 2001).

2.3.1. Inhibición de la neurotransmisión

Entre las funciones reguladas por GABA, ya sea a través del receptor $GABA_A$ o del $GABA_B$, la principal de ellas es el control de la excitabilidad neuronal. Como hemos mencionado anteriormente, existen dos formas por las cuales el GABA puede ejercer esta acción: regulando la liberación de los neurotransmisores a través de los receptores presinápticos o inhibiendo a nivel postsináptico la transmisión del impulso nervioso. El receptor $GABA_B$ se encarga principalmente de inhibir la secreción de neurotransmisores, tales como GABA y glutamato, y de algunas hormonas, a través del bloqueo de los canales de Ca^{2+} dependiente de voltaje, aunque también contribuyen a la hiperpolarización de la membrana postsináptica mediada por GABA al favorecer la salida de K^+ de la célula. En cambio, la modulación de la actividad neuronal mediada por el receptor $GABA_A$, independientemente de su localización, se basa en la inhibición de la generación del potencial de acción. Esta acción inhibitoria se produce a través de dos mecanismos diferenciados derivados de la entrada de Cl^-: por un lado, hiperpolariza la membrana situando el potencial de membrana más alejado (más negativo) del potencial de acción y, por otro, disminuye la resistencia de la membrana con lo que reduce el efecto de otras corrientes (como las de Na^+ y Ca^{2+}) sobre el propio potencial de membrana. Estos efectos tienen como consecuencia la inhibición de los canales

iónicos dependientes de voltaje así como del receptor NMDA, cuya inhibición por Mg^{2+}, como ya hemos comentado, es también dependiente del voltaje. De esta manera, los receptores $GABA_A$ pueden también influir en los procesos de plasticidad sináptica mediados por el receptor NMDA, como LTP y LTD, retardando o favoreciendo su aparición, respectivamente (Alger & Le Beau, 2001).

2.3.2. Efectos excitatorios del GABA

A pesar ser el neurotransmisor inhibitorio más importante en el cerebro adulto, GABA también produce efectos de naturaleza excitatoria, principalmente durante las primeras etapas del desarrollo. Esto es debido a que en neuronas inmaduras el gradiente de iones Cl^- está invertido con respecto a las maduras; es decir, que la activación del receptor $GABA_A$ produce una salida de Cl^- de la célula en lugar de una entrada, y por lo tanto causa una despolarización de la membrana y la consecuente entrada de Ca^{2+}. Al contrario de lo sucedido en el cerebro adulto, el glutamato a través de los mGLURs inhibe la actividad excitatoria de GABA, tanto a nivel presináptico como postsináptico (van den Pol *et al.*, 1998). Finalmente, la expresión del co-transportador de K^+/Cl^-, KCC2, induce la inversión gradiente de iones Cl^- y, con ello, el cambio funcional de los receptores $GABA_A$ (Rivera *et al.*, 1999).

Sin embargo, los efectos excitatorios de GABA no se reducen al cerebro en desarrollo, puesto que también pueden suceder en neuronas maduras e incluso simultáneamente a los efectos inhibitorios. En este caso, el mecanismo fisiológico es diferente al de las inmaduras y no implica a los aniones Cl^-, sino que se debe a la salida de iones HCO_3^- por el propio receptor $GABA_A$ (Alger & Le Beau, 2001). Por otro lado, se ha observado que GABA es capaz de estimular la liberación de glutamato, de manera independiente de Ca^{2+}, a través de los canales de Cl^- y de los transportadores EAAT, gracias a su acción sobre GAT-1 (Raiteri *et al.*, 2005).

2.4. TIPOS DE NEURONAS GABAÉRGICAS

A diferencia de las neuronas glutamatérgicas, que fundamentalmente de dividen en piramidales o de proyección y granulares, existen numerosos tipos de interneuronas GABAérgicas cuya clasificación tradicionalmente se ha hecho en base a su morfología: células en cesto, células en candelabro, células estrelladas, etcétera. Una organización más reciente de las neuronas GABAérgicas se ha basado en las diferentes proteínas de unión a Ca^{2+} (como las positivas para parvalbumina, calbindina o calretinina) y

neuropéptidos (como las positivas para colecistoquinina o somatostatina) que expresan. En la corteza cerebral se encuentran muchas de ellas, siendo las más abundantes las parvalbumina positivas (Kinney *et al.*, 2006). En el cerebelo, además de las neuronas de Golgi o en cesto, encontramos también las neuronas de Purkinje que son exclusivas de esta zona del cerebro. A pesar de que el córtex y el cerebelo son dos de las regiones cerebrales con mayor número de interneuronas GABAérgicas, en cultivos primarios de neuronas corticales generalmente hay una mayor proporción de éstas (15-20%) que en los de células granulares de cerebelo (5%) (Sonnewald *et al.*, 2004; Kinney *et al.*, 2006).

3. EL SISTEMA NEUROENDOCRINO

El hipotálamo y la glándula pituitaria constituyen las zonas cerebrales responsables de la función neuroendocrina, puesto que producen una gran cantidad de hormonas que, a su vez, regulan la síntesis y liberación de muchas otras en los demás órganos endocrinos. De esta manera, controlan procesos tan importantes como la función reproductiva, la toma de alimentos, la presión sanguínea, la temperatura corporal, el crecimiento y el metabolismo. De modo inverso, las hormonas producidas en sus respectivas glándulas endocrinas, una vez liberadas al torrente sanguíneo, son capaces de atravesar la barrera hematoencefálica y llegar al sistema nervioso central, donde ejercerán algunas de sus funciones. Entre ellas, encontramos acciones sobre el hipotálamo regulando la propia función neuroendocrina (mediante un mecanismo de retroalimentación positiva o negativa) o el comportamiento sexual, y de manera más general en otras áreas del cerebro modulando las funciones cognitivas (Sonntag, 2002).

Existen 3 grandes grupos de hormonas en base a su naturaleza química: las derivadas de aminoácidos, como las catecolaminas o la tiroxina; las peptídicas, como la insulina; y las lipídicas, como los esteroides y las prostaglandinas. Las hormonas esteroideas se dividen, a su vez, en corticoesteroides; producidos en la glándula adrenal y formados por los glucocorticoides (como el cortisol) y los mineralocorticoides (como la aldosterona), y en hormonas sexuales; producidas principalmente en las gónadas y entre las que encontramos: los andrógenos u hormonas sexuales masculinas (como la testosterona), los estrógenos u hormonas sexuales femeninas (como el estradiol) y los progestágenos (como la progesterona).

3.1. ESTEROIDOGÉNESIS EN EL CEREBRO

Todas las hormonas esteroideas son sintetizadas por diferentes miembros de la familia de enzimas *citocromo P450*, entre otras, a partir del colesterol (ver figura 8). Por ejemplo, la enzima *citocromo P450arom* (más conocido como aromatasa), que transforma la testosterona en estradiol, se encuentra muy expresado en los ovarios pero también en el cerebro, en especial en las neuronas del hipotálamo y del córtex. Su actividad está regulada por la propia testosterona y también por la actividad a través de los receptores NMDA y $GABA_A$ (Roselli *et al.*, 1984; Amateau *et al.*, 2004; Hojo *et al.*, 2004; Zhou *et al.*, 2007). Además, los niveles de la aromatasa aumentan en los astrocitos en respuesta a diferentes modelos de neurodegeneración y, en este sentido, su

actividad es determinante para la supervivencia neuronal. Esto, unido a su presencia en las membranas pre- y postsinápticas, indican que el estradiol sintetizado a nivel local en el cerebro tiene un papel importante en las neuronas, tanto en situaciones fisiológicas como patológicas (Garcia-Segura *et al.*, 2003; Hojo *et al.*, 2004). A pesar de ello, el estradiol no se considera un neuroesteroide, ya que se cree que se sintetiza exclusivamente a partir de la testosterona circulante (Robel *et al.*, 1999). No obstante, dado que la mayoría de las enzimas esteroidogénicas se han detectado en el cerebro, esta parece todavía una cuestión abierta (Mellon & Compagnone, 1999).

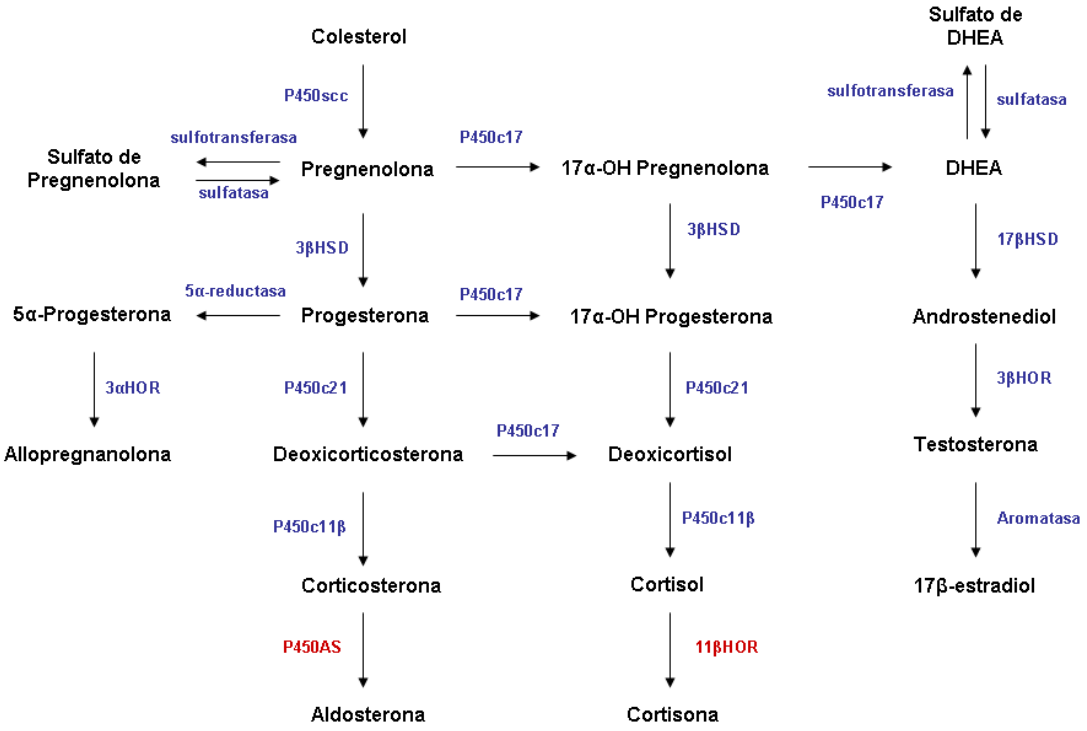


Figura 8. Síntesis y metabolismo de las hormonas esteroideas. Las enzimas destacadas en azul han sido detectadas en el cerebro (modificado de (Mellon & Compagnone, 1999).

Algunos precursores de las hormonas, como la pregnenolona y la dehidroepiandrosterona (DHEA), así como sus respectivos derivados sulfatados, son también sintetizados en el cerebro y, además, actúan como esteroides neuroactivos puesto que modulan la actividad de los receptores NMDA, AMPA y $GABA_A$. Por ello, favorecen la consolidación de la memoria e intervienen en procesos como la diferenciación y la supervivencia neuronales. Otros neuroesteroides, se originan a partir de la metabolización de las hormonas esteroideas. Tal es el caso de la alopregnanolona,

que se obtiene a partir de la progesterona por la acción sucesiva de las enzimas 5α-reductasa y 3α-hidroxi oxidoreductasa, las cuales colocalizan en las neuronas piramidales del córtex y en las de Purkinje en el cerebelo, entre otras (Mellon, 2007). La alopregnanolona estimula la neurogénesis y la supervivencia neuronal, principalmente a través de la modulación del receptor $GABA_A$, aunque su acción sobre el receptor pregnano X (PXR) o sobre el poro de transición de permeabilidad mitocondrial también parecen contribuir a sus efectos neuroprotectores (Charalampopoulos *et al.*, 2004; Xilouri & Papazafiri, 2006; Mellon, 2007; Kelley *et al.*, 2008; Sayeed *et al.*, 2009).

3.2. LOS RECEPTORES DE ESTRÓGENOS

Durante muchos años se ha pensado que el estradiol mediaba sus efectos a través de un único receptor, más tarde denominado ERα. Sin embargo, (Kuiper *et al.*, 1996) descubrieron la existencia de un nuevo receptor de estrógenos que poseía una afinidad por el estradiol similar a la del primero (0,1-0,5nM), y al que bautizaron como ERβ. Ambos pertenecen a la familia de receptores nucleares junto con el receptor de progesterona o el receptor de andrógenos, entre otros. Todos ellos presentan una estructura similar (Figura 9), dividida en 5 dominios: el dominio A/B, situado en el extremo N-terminal y con una ligera actividad transcripcional independiente de ligando (función activadora 1 o AF1, del inglés "Activation Function 1"); el dominio C, de unión al ADN; el dominio D o región bisagra, implicado en el tráfico y localización del receptor; el dominio E, que contiene el sitio de unión al ligando y a los co-reguladores y la función activadora 2 (AF2); y el dominio F, situado en el extremo C-terminal.

La distribución en el sistema nervioso central de las dos isoformas del receptor de estrógenos es similar, aunque ERα predomina en el hipocampo y en el hipotálamo, mientras que la expresión de ERβ es mayoritaria en la corteza cerebral y en el cerebelo (Mitra *et al.*, 2003). Los receptores de estrógenos tienen un patrón de expresión diferente a lo largo del desarrollo postnatal en el córtex del ratón, tanto *in vivo* como *in vitro*, observándose una disminución progresiva de los niveles de ERα acompañada de un incremento en los de ERβ (Prewitt & Wilson, 2007). En cambio, en las células granulares del cerebelo el patrón de expresión de ambas isoformas es prácticamente el inverso, predominando inicialmente ERβ y después ERα (Belcher, 1999).

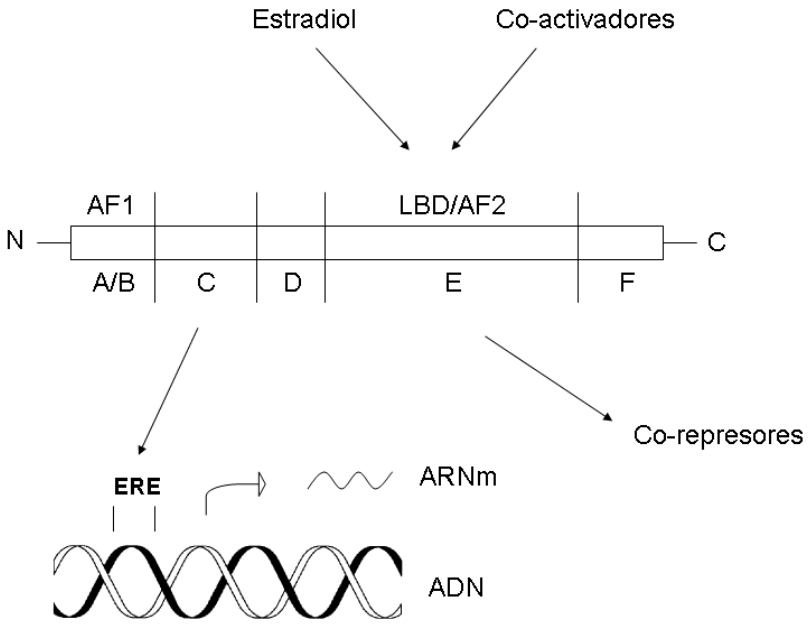


Figura 9. Esquema de la estructura primaria del receptor de estrógenos, dividida en 5 dominios (A-F). Las regiones AF1 y AF2 regulan la actividad transcripcional independiente y dependiente de ligando, respectivamente. La unión del estradiol al LBD (dominio de unión al ligando) estimula el reclutamiento de los co-activadores y la disociación de los co-represores, permitiendo al receptor unirse a la secuencia ERE (elemento de respuesta a estrógenos) del ADN y activar la transcripción del gen diana, lo que dará lugar al correspondiente ARN mensajero (ARNm).

En los últimos años, varios estudios han mostrado que el estradiol media algunos de sus efectos a través de un receptor acoplado a proteínas G, denominado GPR30, en líneas celulares y neuronas (Filardo *et al.*, 2000; Terasawa *et al.*, 2009). Este receptor tiene una farmacología diferente a los receptores de estrógenos clásicos, ya que es activado por tamoxifen e ICI182780 (Filardo *et al.*, 2000). GPR30 se encuentra ampliamente distribuido en el cerebro (Hazell *et al.*, 2009), aunque dada su localización intracelular predominante, su relevancia fisiológica no está todavía clara (Matsuda *et al.*, 2008). Además, se ha probado la existencia de otro receptor de estrógenos asociado a la membrana neuronal, bautizado como ER-X, que tiene igual afinidad por 17α- que por 17β-estradiol (Toran-Allerand, 2005).

3.2.1. Efectos genómicos de los receptores de estrógenos

En ausencia de ligando, los receptores de estrógenos, ERα y ERβ, se encuentran habitualmente en el citosol unidos a las llamadas *heat shock proteins* (*Hsp*). Cuando el ligando se une al receptor, éste se disocia de las *Hsp*, se dimeriza consigo mismo y se

transloca al núcleo, donde se unirá a determinadas secuencias del ADN, llamadas elementos de respuesta a estrógenos (ERE), y regulará la transcripción de sus genes diana (ver figura 9). Así, los receptores nucleares son capaces de estimular o inhibir la transcripción de determinados genes, en función de a qué tipo de co-reguladores se unan (co-activadores o co-represores, respectivamente), ya que estos últimos actúan como enzimas facilitando o dificultando el acceso de la maquinaria de la transcripción al ADN (Shibata *et al.*, 1997). De manera general, los agonistas del receptor de estrógenos, como el estradiol, estimulan la actividad transcripcional mientras que los antagonistas, como tamoxifen o ICI182780, la inhiben (Andersen *et al.*, 1999). Asimismo, se ha descrito que ERα es un factor de transcripción más potente que ERβ, el cual funciona, además, como un regulador o inhibidor de la actividad transcripcional del primero (Hall & McDonnell, 1999). Sin embargo, existen numerosas excepciones y los efectos genómicos de cada modulador selectivo del receptor de estrógenos (SERM, del inglés "Selective Estrogen Receptor Modulator") dependen del tipo de receptor de estrógenos y de los correguladores presentes en cada tipo celular (Shibata *et al.*, 1997; Andersen *et al.*, 1999). Además, el estradiol es capaz de estimular la expresión de genes que no contienen ERE en su promotor a través de la unión de ERα a otros factores de transcripción como c-fos, c-jun y CREB. En este mismo contexto, los SERMs producen efectos genómicos independientes de ERE tanto a través de ERα como de ERβ (Paech *et al.*, 1997; Wang *et al.*, 2004). Se ha observado también que la actividad transcripcional del receptor de estrógenos está regulada por ciertos factores de crecimiento, como IGF-I, ya sea en presencia o ausencia de ligando (Kato *et al.*, 1995; Mendez & Garcia-Segura, 2006). Un modelo muy utilizado para estudiar los efectos genómicos producidos por el estradiol y otros SERMs es la línea celular MCF-7 (Michigan Cancer Foundation-7), derivada de cáncer de mama humano. Estas células proliferan exclusivamente en respuesta a la actividad transcripcional mediada por el receptor de estrógenos. Por esta razón, se han usado también para evaluar la estrogenicidad de numerosos compuestos ambientales y, de esta manera, su potencial carcinogénico, mediante el llamado E-screen (Soto *et al.*, 1995).

Existe un elevado número de genes regulados por el receptor de estrógenos en el cerebro (tanto en neuronas como en células gliales), algunos de los cuales expresan proteínas implicadas en la neurogénesis, la plasticidad sináptica, la sinaptogénesis y la supervivencia neuronal, tales como hormonas peptídicas, factores neurotróficos, receptores de membrana, componentes del citoesqueleto, etc (McEwen, 1999; Garcia-

Segura *et al.*, 1999). Por otro lado, se ha observado que los estrógenos regulan la expresión de diferentes genes en tejidos reproductivos, dependiendo de si activan ERα o ERβ (Waters *et al.*, 2001). Además, el estradiol regula la expresión del propio receptor de estrógenos, de manera que reduce la expresión de ERα y aumenta ligeramente la de ERβ, en un mecanismo de retroalimentación negativa y positiva, respectivamente (Waters *et al.*, 2001; Grunfeld & Bonefeld-Jorgensen, 2004). Este regulación autóloga del receptor de estrógenos se ha descrito también en neuronas, observándose efectos diversos tras la exposición a estradiol; incremento de ERα y disminución o aumento de ERβ (Prange-Kiel *et al.*, 2003; Aguirre *et al.*, 2010).

3.2.2. Efectos no genómicos de los receptores de estrógenos

A lo largo de los últimos años, se han reportado numerosas acciones mediadas por el receptor de estrógenos que transcurren en un período de tiempo demasiado corto (segundos o unos pocos minutos) como para poder involucrar cambios en la expresión génica, y que, por tanto, no requieren la síntesis *de novo* de proteínas (proceso que implica varias horas). Estas incluyen la modulación de algunas vías de señalización intracelular, como MAPK y PI3K/Akt (ver más abajo), de receptores de neurotransmisores (ver apartado 3.3) y de canales iónicos de membrana, como los canales de Ca^{2+} de tipo L o los de K^{+}. De esta manera, el estradiol es capaz de regular procesos como la excitabilidad neuronal, la plasticidad sináptica o la secreción hormonal a través de su acción rápida sobre receptores de estrógenos asociados a la membrana plasmática (Watson *et al.*, 2007; Ogiue-Ikeda *et al.*, 2008; Kelly & Ronnekleiv, 2009). A pesar de que tanto ERα como ERβ se sitúan principalmente en el núcleo, también se han detectado en las membranas pre- y postsinápticas y asociados a las mitocondrias (Jelks *et al.*, 2007; Ogiue-Ikeda *et al.*, 2008; Milner *et al.*, 2008; Sheldahl *et al.*, 2008). Toran-Allerand (1999) propuso un modelo en donde el receptor de estrógenos se sitúa en el lado citoplasmático de la membrana neuronal formando un complejo con las proteínas Hsp90 y *src*, en modo tal que se asocia con los receptores de la familia tirosin-kinasa, como el receptor de IGF-I, y, al mismo tiempo, con las kinasas de la ruta MAPK, siendo capaz de activar ambos tras la unión del estradiol. De manera similar, la activación del receptor de IGF-I puede activar el ERα en ausencia de ligando. En otra serie de estudios, se ha observado que el receptor de estrógenos forma diversos complejos con PI3K (mediante interacción con su subunidad reguladora, p85) y con GSK3β, junto a otras proteínas, de modo que la unión de estradiol a su receptor causa la

activación de Akt (por fosforilación en serina 473 mediada por la subunidad catalítica, p110) y la consecuente inhibición de GSK3β (por fosforilación en serina 9 a cargo de ésta última), lo que dará lugar a múltiples efectos celulares (ver figura 10). En este contexto, se ha observado que la activación conjunta de ERα y el receptor de IGF-I tiene efectos sinérgicos sobre esta ruta de señalización (Mendez *et al.*, 2006). Otras proteínas *scaffold* que asocian el receptor de estrógenos con receptores tirosin-kinasa, proteínas G y sus vías de señalización en la membrana son las caveolinas y Shc (Evinger, III & Levin, 2005; Mermelstein, 2009).

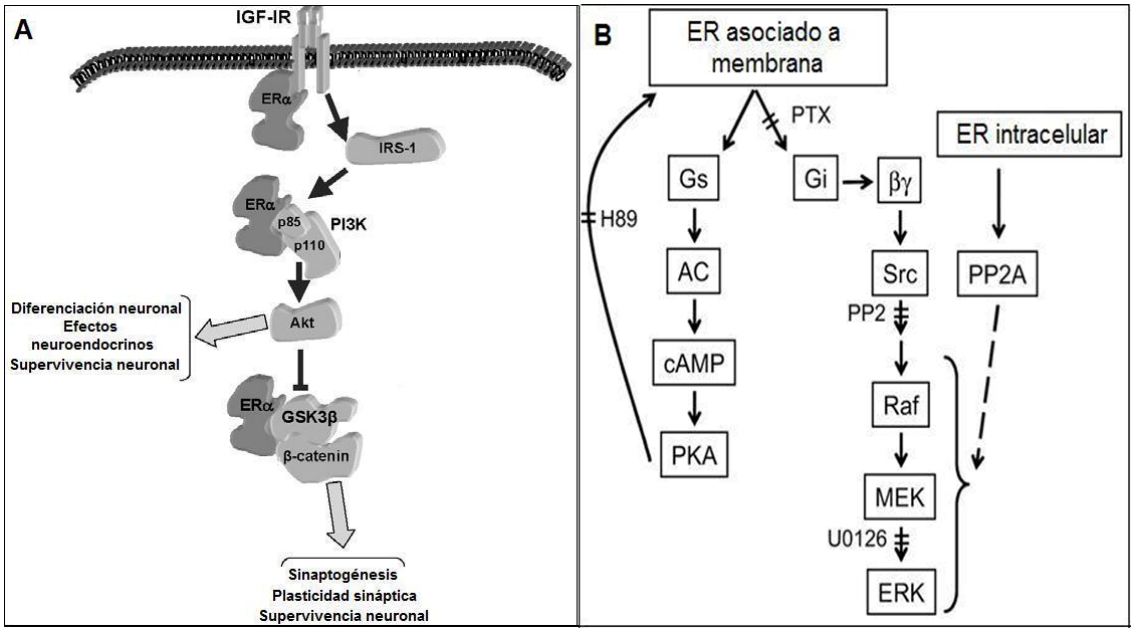


Figura 10. Rutas de señalización intracelular activadas por estradiol. A) Ruta PI3K/Akt (modificada de Mendez *et al.*, 2006). B) Ruta MAPK (modificada de Belcher *et al.*, 2005).

Las cascadas de fosforilación MAPK y PI3K/Akt son dos de las vías de señalización más estudiadas, dada su gran implicación en algunos de los fenómenos regulados por el estradiol en la membrana, como los mencionados anteriormente, pero también porque sus kinasas efectoras (Akt y ERK1/2), desencadenan la activación de ciertos factores de transcripción (incluido el propio receptor de estrógenos, por fosforilación en residuos de tirosina) mediando, a más largo plazo, otras funciones como la diferenciación y supervivencia neuronal. Por ello, estas vías de señalización suponen un nexo de unión entre los efectos no genómicos y genómicos de los estrógenos (Toran-Allerand, 1999; Mendez *et al.*, 2006). Sin embargo, los mecanismos por los cuales los estrógenos

activan estas kinasas son diferentes según el tipo celular. Así, la activación de ERK1/2 por estradiol en líneas celulares derivadas de cáncer de mama está mediada por GPR30 y ERα con la participación del receptor del factor de crecimiento epitelial (EGFR) (Filardo *et al.*, 2000; Evinger, III & Levin, 2005), mientras que, en células granulares de cerebelo, no implica la activación de EGFR pero sí la de PKA y la de un receptor acoplado a proteínas G sin identificar (Belcher *et al.*, 2005) (ver figura 10). En cambio, en neuronas corticales los efectos del estradiol sobre ERK1/2 y Akt dependen de la interacción de ERα con p85, aunque otros mecanismos, como la activación de PKC y de canales de Ca^{2+}, también contribuyen a la activación de MAPK por estradiol, en estas y otras células (Cordey *et al.*, 2003; Mannella & Brinton, 2006; Zhao & Brinton, 2007). A pesar de ello, se ha propuesto que el receptor de estrógenos implicado en estas rutas en el córtex podría el recién descubierto ER-X (Toran-Allerand, 2005). Por el contrario, el estradiol es incapaz de activar Akt en células granulares de cerebelo, hecho que se ha relacionado con su incapacidad de proteger frente a la excitotoxicidad en estas neuronas (Miñano *et al.*, 2007). Además de las cascadas explicadas anteriormente, la modulación rápida de las rutas MAPK, p38 y JNK, por estradiol se ha visto también implicada en sus efectos protectores frente al daño celular (Razandi *et al.*, 2000a; Razandi *et al.*, 2000b; Cordey *et al.*, 2003; Mendez *et al.*, 2005; Zhao & Brinton, 2007; Bourque *et al.*, 2009).

3.3. EFECTOS DEL ESTRADIOL SOBRE LA NEUROTRANSMISIÓN

La disminución de los niveles circulantes de estrógenos en las mujeres tras la menopausia, como consecuencia de una pérdida de la función neuroendocrina, se ve acompañada de trastornos cognitivos y de una mayor incidencia de enfermedades neurodegenerativas, síntomas que son mejorados gracias a la terapia hormonal sustitutiva con estrógenos y/o progestágenos (Sonntag, 2002). Los efectos positivos del estradiol al respecto, se han explicado a través de dos mecanismos diferenciados como son, por un lado, la regulación del metabolismo de la acetilcolina en las neuronas colinérgicas del estriado y, por otro, la inducción de la sinaptogénesis de manera dependiente del receptor NMDA en la región CA1 del hipocampo, la cual tiene un papel clave en los procesos de aprendizaje y memoria (McEwen *et al.*, 1995). Más recientemente, se observado que ambos efectos pueden estar relacionados (Daniel & Dohanich, 2001). Por otro lado, el estradiol ha demostrado ser potencialmente

beneficioso en el tratamiento de algunas enfermedades neurológicas, como el Parkinson o la depresión, debido a su modulación positiva de las neurotransmisiones dopaminérgica y serotoninégica, respectivamente (Ostlund *et al.*, 2003; Bourque *et al.*, 2009).

3.3.1. Efectos del estradiol sobre los receptores NMDA

Con el fin de esclarecer los mecanismos moleculares responsables de la potenciación de la memoria por los estrógenos, Smith *et al.* (2009) han propuesto un modelo fisiológico en donde el aumento de los niveles de estradiol en el cerebro (durante la fase *proestrus* del ciclo reproductivo femenino) estimula la sinaptogénesis y la plasticidad sináptica (LTP) en esta región hipocampal durante un periodo de dos días, a través de la incorporación de receptores NR1/NR2B a estas nuevas sinapsis. Este efecto es reversible ya que al cabo de 3-4 días, cuando los niveles de estrógenos disminuyen y los de progesterona aumentan, se recupera el estado basal. Además, el estradiol promueve la expresión de otras proteínas sinápticas clave en estos procesos como sinaptofisina, vGLUT1, PSD95 y la subunidad GluR1 (Jelks *et al.*, 2007; Liu *et al.*, 2008).

Varios estudios coinciden en que la regulación estrogénica de los receptores NMDA está mediada por ERα y es de naturaleza genómica (Brann *et al.*, 1993; Jelks *et al.*, 2007; Morissette *et al.*, 2008b), aunque también parece implicar cambios post-transcripcionales (Gazzaley *et al.*, 1996). A pesar de ello, estudios con ratones *K.O.* para ERβ demuestran su importancia en la plasticidad sináptica hipocampal y en la formación de la memoria inducidas por estradiol (Liu *et al.*, 2008). Más controvertidos son los efectos del estradiol en la corteza cerebral, en donde se han observado tanto incrementos como disminuciones de los receptores NMDA en la membrana y, al parecer, ambas isoformas (ERα y ERβ) contribuyen (Brann *et al.*, 1993; Morissette *et al.*, 2008b). En cambio, en el hipotálamo, el mecanismo por el cual el estradiol estimula la sináptogénesis implica la liberación de glutamato pero no afecta a la densidad de receptores NMDA (Schwarz *et al.*, 2008).

Se ha comprobado que el estradiol también modula los receptores NMDA, por fosforilación en residuos de tirosina, a través de la activación de *src* y de la ruta MAPK (Bi *et al.*, 2000). De esta manera, los estrógenos son capaces de regular, de manera rápida, procesos tales como la plasticidad sináptica, la supervivencia neuronal y la sinaptogénesis (Bi *et al.*, 2000; Ogiue-Ikeda *et al.*, 2008; Xu *et al.*, 2010). Por último,

también se ha descrito que, a dosis altas (>10μM), el estradiol inhibe directamente el receptor NMDA (Weaver, Jr. *et al.*, 1997).

3.3.2 Efectos del estradiol sobre los receptores metabotrópicos de glutamato

Recientemente, se ha descubierto que los receptores de estrógenos median algunos de sus efectos a través de su interacción con los receptores metabotrópicos de glutamato en la membrana celular. Esta interacción se produce a través de las caveolinas y es independiente del glutamato. Por un lado, el estradiol estimula los mGLUR de tipo I (mGLUR1 en hipocampo y mGLUR5 en el estriado) a través de ERα, causando la activación de PKC y la liberación de Ca^{2+} de depósitos intracelulares, y la posterior fosforilación de CREB por la ruta MAPK. Por el contrario, en condiciones despolarizantes (alto K^{+}), el estradiol previene la activación de CREB inducida por la entrada de Ca^{2+} a través de los canales de Ca^{2+} de tipo L, y este efecto está mediado por la asociación de ERα y ERβ con los mGLUR de tipo II (mGLUR2 en hipocampo y mGLUR3 en el estriado). Esta asociación funcional entre los receptores de estrógenos y los mGLURs se ha observado también en el hipotálamo, tanto en neuronas como en células gliales, e incluso en el sistema nervioso periférico, habiéndose relacionado con la modulación estrogénica del comportamiento sexual y de la transmisión del dolor, respectivamente (Mermelstein, 2009).

3.3.3 Efectos del estradiol sobre la neurotransmisión GABAérgica

Dos estudios diferentes han mostrado, tanto *in vivo* como *in vitro*, que el estradiol disminuye los niveles de la enzima GAD65 (pero no de GAD67) y, consecuentemente, la neurotransmisión GABAérgica, mecanismo que han señalado como responsable de la inducción de la sináptogénesis por los estrógenos en el hipocampo (Murphy *et al.*, 1998; Rudick & Woolley, 2001). Sin embargo, una serie de experimentos posteriores desmintieron esta hipótesis (Smith *et al.*, 2009). Otros trabajos, en cambio, han mostrado que los SERMs, tamoxifen e ICI182780, y los inhibidores de la aromatasa también reducen la expresión de GAD65 (Ikeda *et al.*, 2006; Zhou *et al.*, 2007). Además, el estradiol reduce los niveles de GABA en la corteza cerebral, presumiblemente a través de ERβ, lo que, a su vez, estimula la producción de BDNF en las neuronas piramidales (Blurton-Jones & Tuszynski, 2006). Por otro lado, el estradiol regula la expresión de las subunidades del receptor $GABA_A$, de manera que aumenta los receptores α4/β/δ y disminuye los α1/β/γ2 en el hipocampo, mientras que en el córtex incrementa las subunidades α1, α2 y γ2 y con ello la sensibilidad de los receptores

$GABA_A$ a las benzodiacepinas pero no a los neuroesteroides (Shen *et al.*, 2005; Calza *et al.*, 2010). Además, el estradiol potencia el efecto excitatorio del GABA durante el desarrollo a través de la estimulación de la actividad los canales de Cl^- y de Ca^{2+} de tipo L (Nuñez *et al.*, 2005). Finalmente, los efectos neuroprotectores del estradiol frente a la excitotoxicidad en neuronas hipocampales se han atribuido a la disminución de la expresión de los mGLUR de tipo I (Hilton *et al.*, 2006).

4. LOS PESTICIDAS ORGANOCLORADOS

Los pesticidas organoclorados son compuestos sintéticos utilizados principalmente como insecticidas. Se clasifican en 5 grupos en base a su naturaleza química: a) derivados del clorobenceno, como el DDT o el metoxicloro; b) hexaclorociclohexanos (HCH), formados por los isómeros α-HCH, β-HCH, γ-HCH (lindano) y δ-HCH; c) ciclodienos, como el dieldrín, el α-endosulfan (a partir de ahora endosulfan) o el Aldrin; d) terpenos clorados, como el toxafeno; e) otros derivados del ciclopentadieno, como la clordecona o el mirex. En la figura 11 se detallan las estructuras químicas de algunos de ellos.

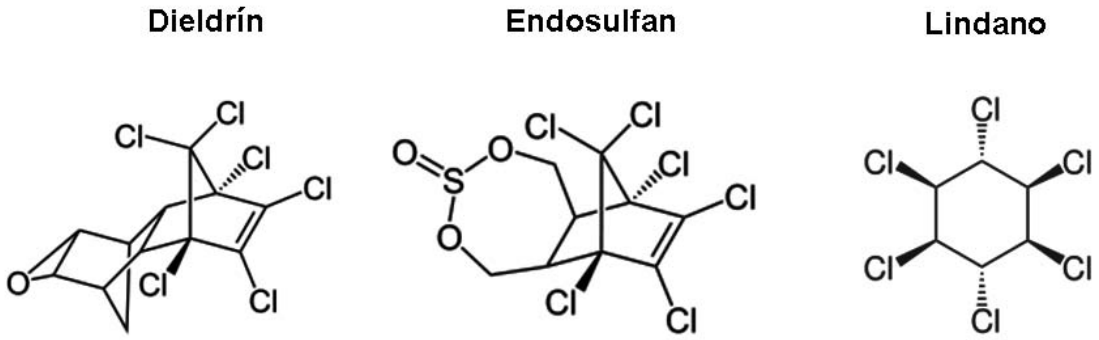


Fig.11. Estructura química de los pesticidas organoclorados estudiados

4.1. EXPOSICIÓN A LOS PESTICIDAS ORGANOCLORADOS

Los pesticidas organoclorados han sido usados de manera masiva para el control de plagas en la agricultura desde los años 40 hasta los 70. En las décadas posteriores, fueron prohibiéndose sucesivamente en la mayor parte del mundo debido al impacto medioambiental y al riego para la salud que suponen. A pesar de ello, todavía siguen utilizándose de manera discontinua en algunos países contra las termitas, como es el caso del dieldrín, o contra la malaria, con sucede con el DTT, e incluso con fines farmacéuticos en champúes o lociones para combatir los piojos, como sucede con el lindano en Estados Unidos. Actualmente, muchos de ellos han sido incluidos (el dieldrín en 2004, el lindano en 2009 y el endosulfán en abril de este año) en el Convenio de Estocolmo para contaminantes orgánicos persistentes (POPs, 2011) http://www.pops.int) con el fin de restringir o eliminar su uso y producción a nivel mundial.

De manera general, son compuestos muy estables frente a la luz, el aire o el calor y tienen una polaridad baja, características físico-químicas que convierte a los pesticidas organoclorados en sustancias con una alta persistencia ambiental y con una gran capacidad de biomagnificación, ya que tienden a acumularse en los tejidos grasos de los animales concentrándose, de este modo, a lo largo de la cadena trófica. Por ejemplo, su vida media en el aire es 40-60 horas, mientras que en agua y suelos puede ser desde unos pocos días, como en los casos de endosulfán y lindano, hasta varios años, como sucede con el dieldrín (Hazardous Substances Data Bank (HSDB, 2011). Debido a su capacidad de bioconcentración, se han detectado muchos de estos pesticidas (en ocasiones a niveles superiores a los permitidos por la legislación vigente) en todo tipo de alimentos, animales y vegetales, (Kalantzi *et al.*, 2001; Schafer & Kegley, 2002; Gonzalez *et al.*, 2003; Hites *et al.*, 2004; Hilber *et al.*, 2008; Chopra *et al.*, 2011) así como en diferentes tejidos humanos (Botella *et al.*, 2004; Cerrillo *et al.*, 2005). Por esta razón, su principal vía de exposición para la población general es a través de la dieta, aunque en casos de exposición ocupacional se da por absorción respiratoria y dérmica (HSDB, 2011). Dado que son capaces de atravesar la placenta, los fetos también están expuestos a estos contaminantes a través de la dieta materna. De hecho, se ha llegado a correlacionar los niveles de pesticidas en cordón umbilical de madres españolas con el consumo de determinados alimentos (Mariscal-Arcas *et al.*, 2010). En un estudio longitudinal, llevado a cabo entre los años 1969-1976, se calculó el consumo diario medio de varios pesticidas, observándose que, en el caso del dieldrín, éste era de hasta 0,7 μg/Kg en recién nacidos (Duggan *et al.*, 1983). Posteriormente, la Organización Mundial de la Salud estableció el consumo diario aceptable en 0.1, 6 y 8 μg/Kg de peso para el dieldrín, endosulfan y lindano, respectivamente (WHO, 2004).

La biotransformación de los pesticidas organoclorados realizada por las enzimas citocromo P450 hepáticos es lenta, a pesar de ser inductores de estas mismas enzimas (Coumoul *et al.*, 2002; Johri *et al.*, 2008). La metabolización de estos pesticidas da lugar a derivados conjugados glucurónicos (dieldrín), sulfúricos (endosulfan) y fenólicos (lindano) que se eliminan a través de la orina, la bilis, las heces y la leche materna, siendo esta última una fuente importante de exposición en humanos tras el nacimiento. Además, el aldrín es transformado en dieldrín por animales, insectos, bacterias e incluso por acción de la luz solar (HSDB, 2011), y este último puede, además, originar el aldrín-transdiol dentro del organismo, que también es un compuesto tóxico(van den & Narahashi, 1974).

Finalmente, gracias a su carácter lipofílico, los pesticidas organoclorados son capaces de atravesar la barrera hematoencefálica y alcanzar el cerebro, habiéndose encontrado en tejido cerebral de individuos *post mortem* a concentraciones de 0,05-1 μg/g de lípido (Fleming *et al.*, 1994; Corrigan *et al.*, 2000).

4.2. TOXICIDAD DE LOS PESTICIDAS ORGANOCLORADOS

La toxicología es la ciencia que estudia los efectos adversos que determinadas sustancias químicas causan en el organismo. En primer lugar, sería conveniente definir algunos conceptos básicos de la toxicología. Tal vez el más importante es el de la dosis; ya en el año 1538, Paracelso introdujo la idea de que sólo la dosis determina que algo sea venenoso. Por ello, tanto en farmacología como en toxicología, es necesario establecer la relación dosis-respuesta a la hora de estudiar el efecto de un compuesto dado sobre cualquier parámetro fisiológico. Por otro lado, el tiempo es también un factor importante a tener en cuenta, debiéndose distinguir entre toxicidad aguda y toxicidad crónica. En estudios con animales *in vivo*, la primera se define como la resultante de una sola dosis en un periodo de 24 horas, mientras que en el segundo caso se trata de dosis repetidas (diarias normalmente) o una dosis única pero un tiempo de exposición más largo (varios días o meses). En los estudios *in vitro* los tiempos de exposición se reducen, siendo de unas pocas horas para la toxicidad aguda y de al menos 1-2 días *in vitro* (DIV) para la crónica.

3.2.1. Toxicidad aguda de los pesticidas organoclorados

La toxicidad aguda de un determinado compuesto químico se calcula mediante el parámetro LD50 (del inglés "Lethal Dose 50"), que indica la dosis necesaria para matar al 50% de los animales tratados. En la tabla 3 se recogen los valores de LD50 en rata para algunos pesticidas organoclorados, tras exposición oral y dérmica. En humanos, la dosis sintomáticas están en torno a 5-10 mg/Kg de peso corporal para dieldrín y endosulfan y 15-20 mg/Kg para el lindano (FAO/UNEP, 1991). La sintomatología clínica tras una intoxicación aguda con pesticidas organoclorados incluye excitabilidad, vértigo, cefalea, náusea, vómito, diarrea, ataxia, hiperestesias y parestesias en la cara, temblor, confusión mental, contracciones mioclónicas, y en casos severos se dan directamente convulsiones tónicas o tónico-clónicas generalizadas e incluso depresión respiratoria que puede llevar a la muerte. El tratamiento habitual en casos que presentan convulsiones es con diazepam, y en casos más severos con fenobarbital. Asimismo, el

hígado y los riñones son otros órganos afectados por la intoxicación aguda con altas concentraciones de estos pesticidas (ATSDR, 2002).

LC50 (µg/Kg peso)	Dieldrín	Endosulfan	Lindano
Vía oral	40	30-80	90-270
Vía dérmica	60-90	75	900-1000

Tabla 3. Letalidad en rata de los pesticidas organoclorados (fuente: (FAO/UNEP, 1991; WHO, 2004).

3.2.2. Toxicidad crónica de los pesticidas organoclorados

Entre los síntomas descritos a nivel clínico, encontramos cefalea, somnolencia, visión borrosa y movimientos musculares y oculares involuntarios así como persistencia de disturbios del sueño, la memoria y la conducta durante varios días o semanas (ATSDR, 2002). También se han documentado efectos inmunodepresores y la aparición de tumores en el hígado en animales de experimentación, en los casos de dieldrín y lindano. Por esta razón, la Agencia de Protección Medioambiental estadounidense (US EPA, 1987) incluyó al primero ellos en la categoría 2A (probable carcinógeno en humanos) y al segundo en la 2B (posible carcinógeno en humanos). A pesar de ello, según la Agencia Internacional para la Investigación del Cáncer (IARC, 1987) las evidencias en animales son insuficientes e inadecuadas en humanos, en el caso del dieldrín, por lo que permanece en la categoría 3 (no clasificable como carcinógeno). En cuanto al endosulfán, no se ha clasificado como carcinógeno aunque algunos estudios indican que tiene un riesgo potencial de inducir cáncer de mama, al igual que el diedrín, a concentraciones altas (Soto *et al.*, 1994; Soto *et al.*, 1995).

4.3. EFECTOS DE LOS PESTICIDAS ORGANOCLORADOS SOBRE EL SISTEMA ENDOCRINO

Los pesticidas organoclorados tienen efectos perjudiciales sobre la reproducción animal (incluida la humana), tales como disminución de la fertilidad, atrofia de los órganos sexuales y alteraciones en el comportamiento sexual y en la función tiroidea, y por ello son considerados disruptores endocrinos (Naqvi & Vaishnavi, 1993; Schantz &

Widholm, 2001; Maranghi *et al.*, 2007; Tiemann, 2008). (Johnson *et al.*, 1992) fueron los primeros en demostrar la estrogenicidad de los pesticidas organoclorados metoxicloro, DDT y sus derivados. Estudios posteriores confirmaron que algunos otros pesticidas, incluidos el dieldrín y endosulfan pero no el lindano, tenían actividad estrogénica en el ensayo E-Screen, comentado anteriormente, y en otros ensayos de *trans*-activación (Soto *et al.*, 1994; Soto *et al.*, 1995; Andersen *et al.*, 2002; Lemaire *et al.*, 2006). Se observó también que, a dosis no efectivas individualmente (1μM), estos pesticidas sí que daban positivo en el E-Screen cuando se administraban en combinación, lo que indica que sus efectos estrogénicos son aditivos (Soto *et al.*, 1994; Soto *et al.*, 1995). Aunque inactivo en el ensayo E-Screen, el lindano ha demostrado tener afinidad por el sitio de unión del estradiol en el receptor de estrógenos, aunque ligeramente inferior a la de endosulfan y dieldrín (Scippo *et al.*, 2004). En cambio, su isómero β-HCH es capaz de inducir la proliferación de las células MCF-7, a pesar de no inhibir la unión de [^{3}H]-17β-estradiol al receptor de estrógenos, a través de un mecanismo no convencional (Coosen & van Velsen, 1989; Steinmetz *et al.*, 1996). Los efectos estrogénicos del lindano han sido atribuidos a su acción sobre ERβ, mientras que los de endosulfan y dieldrín parecen mediados por ERα, aunque todos ellos han mostrado afinidad por ambos receptores (Sumbayev *et al.*, 2005; Lemaire *et al.*, 2006; Maranghi *et al.*, 2007; Li *et al.*, 2008). Otros pesticidas organoclorados que interaccionan con ERα y ERβ, como DDT y metoxicloro, también parecen actuar sobre GPR30 (Thomas & Dong, 2006). Si bien los efectos genómicos de los disruptores endocrinos han sido ampliamente estudiados, sus acciones no genómicas se están empezando a conocer. Por ejemplo, algunos pesticidas como dieldrín y endosulfan son capaces de estimular de manera transitoria la fosforilación de ERK1/2 y Akt, la entrada de Ca^{2+} y la liberación de prolactina en diferentes líneas celulares (Li *et al.*, 2006; Watson *et al.*, 2007).

Además de sus efectos sobre los receptores de estrógenos, los pesticidas organoclorados han demostrado poseer muchas otras dianas farmacológicas dentro del sistema endocrino. Así, por ejemplo son capaces de activar la expresión génica mediada por el receptor del ácido retinoico y por el de pregnanos PXR (Coumoul *et al.*, 2002; Lemaire *et al.*, 2005) y también de inhibir *la trans*-activación del receptor de andrógenos y del de progesterona, aunque en el último caso no parece ser debido a un efecto directo (Jin *et al.*, 1997; Andersen *et al.*, 2002; Li *et al.*, 2008). Asimismo, endosulfan y lindano modulan la actividad de la aromatasa (Andersen *et al.*, 2002; Nativelle-Serpentini *et al.*,

2003). Por último, otro mecanismo de disrupción endocrina observado tras la exposición prolongada a estos compuestos es la alteración de los niveles de los receptores de estrógenos, ya sea por sí solos o en presencia de estradiol (Waters *et al.*, 2001; Grunfeld & Bonefeld-Jorgensen, 2004).

4.3. EFECTOS DE LOS PESTICIDAS ORGANOCLORADOS SOBRE EL SISTEMA NERVIOSO CENTRAL

A lo largo de la décadas de los años 70 y 80 varios estudios demostraron que la exposición aguda y crónica a los pesticidas organoclorados producía alteraciones en el sistema nervioso central. En la mayoría de casos se reportaron déficits en el aprendizaje y en la memoria así como alteraciones locomotoras y del comportamiento, en algunos de ellos (Smith *et al.*, 1976; Topinka *et al.*, 1984; Tilson *et al.*, 1987; Paul *et al.*, 1994; Schantz & Widholm, 2001; Mariussen & Fonnum, 2006) para revisión consultar. Sin embargo, los mecanismos implicados en tales efectos cognitivos no se han estudiado con detalle.

Los pesticidas organoclorados de la familia de los HCHs y de los ciclodienos actúan como antagonistas no competitivos del receptor $GABA_A$ sobre el sitio de unión de la picrotoxinina, desplazando al ligando radioactivo [^{35}S]-TBPS (Lawrence & Casida, 1984; Pomes *et al.*, 1993). La potencia de algunos de estos contaminantes sobre el receptor $GABA_A$ sigue el siguiente orden decreciente endrín > α-endosulfan ~ dieldrín > lindano > aldrín. Estos efectos farmacológicos se han correlacionado, además, con su acción inhibitoria sobre el flujo de Cl^- inducido por GABA (Gant *et al.*, 1987; Pomes *et al.*, 1994b) y se corresponden con su letalidad aguda en roedores (Lawrence & Casida, 1984; Bloomquist, 2002). Por el contrario, los isómeros del lindano β-HCH y δ-HCH tienen efectos potenciadores a dosis moderadas e inhibidores a dosis altas sobre el receptor $GABA_A$, teniendo en cualquier caso menor afinidad que el lindano (Pomes *et al.*, 1994b). Los pesticidas organoclorados bloquean también el receptor de glicina, aunque el dieldrín y el endosulfan con menor potencia que el receptor $GABA_A$ (Vale *et al.*, 2003), y los canales de Cl^- regulados por voltaje, siendo el lindano en este caso más potente que los ciclodienos (Bloomquist, 2002). Además, los HCHs estimulan los canales de Ca^{2+} de tipo L, lo que contribuye a sus efectos convulsivos, y liberación de Ca^{2+} de depósitos intracelulares a través de los receptores de rianodina (δ-HCH) u otros sensibles a dantroleno o a IP_3 (lindano) (Tusell *et al.*, 1992; Rosa *et al.*, 1997; Fujita *et*

al., 1998). En cambio, los efectos excitatorios del DDT se deben a su acción sobre los canales de Na^+ (Hille, 1968), aunque también actúa sobre los transportadores de dopamina (Hatcher *et al.*, 2008).

Aunque sus dianas farmacológicas conocidas en el cerebro son numerosas, la exposición crónica a los pesticidas organoclorados altera mayor número de proteínas, entra las que se incluyen, en el caso del dieldrín, enzimas antioxidantes, receptores y transportadores de determinados neurotransmisores, etc (Liu *et al.*, 1998; Richardson *et al.*, 2006; Slotkin & Seidler, 2008; Slotkin & Seidler, 2009). Otras dianas afectadas por la exposición prolongada a estos pesticidas son enzimas metabólicas, como la acetilcolinesterasa, o canales iónicos como las bombas de Mg^{2+}, Na^+/K^+ y Ca^{2+} ATPasas de la membrana plasmática y mitocondrial, aunque, en este caso, a concentraciones bastante altas (> 50μM) (Mehrotra *et al.*, 1982; Mehrotra *et al.*, 1989; Sahoo *et al.*, 1999; Jia & Misra, 2007a).

4.3.1. Efectos de los pesticidas organoclorados sobre la neurotransmisión monoaminérgica

Inicialmente, se describieron alteraciones en los niveles de ciertos neurotransmisores, como la serotonina, la dopamina y la noradrenalina, en diferentes regiones cerebrales en respuesta al tratamiento con lindano, endosulfan o dieldrín (Heinz *et al.*, 1980; Suñol *et al.*, 1988; Naqvi & Vaishnavi, 1993). Poco después, (Fleming *et al.*, 1994) detectaron niveles de dieldrín más altos en cerebros *post mortem* procedentes de enfermos de Parkinson. Este mismo grupo, descubrió que el dieldrín producía además una toxicidad selectiva sobre las neuronas dopaminérgicas mesencefálicas, lo que indicaba que la exposición a este pesticida podía suponer un riesgo potencial para el desarrollo de la enfermedad de Parkinson (Sanchez-Ramos *et al.*, 1998). Más adelante, (Corrigan *et al.*, 2000) detectaron también niveles mayores de dieldrín y lindano en la *substantia nigra* de pacientes de Parkinson con respecto a los de otras demencias o sujetos control. Estudios posteriores determinaron los mecanismos implicados en la toxicidad de dieldrín, endosulfan y lindano sobre las neuronas dopaminérgicas, en donde la producción de radicales libres y la activación de las caspasas tienen un papel determinante (Chun *et al.*, 2001; Kitazawa *et al.*, 2001; Jia & Misra, 2007b; Sharma *et al.*, 2010). La posterior degradación proteolítica de PKCδ por éstas desencadenará la fragmentación del ADN, proceso que puede ser prevenido con la sobre-expresión de la proteína mitocondrial *Bcl*-2 (Kanthasamy *et al.*, 2003). La liberación de dopamina y la

modulación de sus transportadores han sido también mecanismos implicados en la toxicidad del dieldrín sobre estas neuronas (Kitazawa *et al.*, 2001; Richardson *et al.*, 2006).

Por otro lado, se ha observado que la exposición prolongada a dieldrín modifica la expresión de los receptores de serotonina y tiene, además, efectos negativos sobre la diferenciación y la maduración de las neuronas serotoninérgicas, siendo estos últimos efectos mediados por su acción antagonista sobre el receptor $GABA_A$ (Liu *et al.*, 1997a; Slotkin & Seidler, 2008).

4.3.2. Efectos de los pesticidas organoclorados sobre la neurotransmisión aminoacidérgica

A pesar de saberse desde hace varios años que la principal diana de los pesticidas organoclorados en el sistema nervioso central es el receptor $GABA_A$, pocos estudios se han desarrollado para evaluar su efecto sobre las neuronas GABAérgicas. Por ejemplo, se ha observado que éstas son menos susceptibles que las neuronas monoaminérgicas, tanto durante el desarrollo como cuando son maduras, frente a la toxicidad del dieldrín (Liu *et al.*, 1997a; Sanchez-Ramos *et al.*, 1998). Además, la exposición crónica a dieldrín altera la expresión de algunas de las subunidades del receptor $GABA_A$, tanto *in vivo* como *in vitro* (Liu *et al.*, 1997b; Liu *et al.*, 1998).

Estudios realizados en nuestro laboratorio han demostrado que las células granulares de cerebelo, principalmente glutamatérgicas, también son sensibles a los efectos tóxicos de estos pesticidas. Así, por ejemplo, la exposición prolongada a dieldrín disminuye la funcionalidad y la expresión en la membrana de los receptores NMDA en estas neuronas (Babot *et al.*, 2007). Otro trabajo muestra que el dieldrín disminuye la expresión de NR2B, NR2C y NR3B en la línea celular PC12 (Slotkin & Seidler, 2009). Respecto al lindano, su toxicidad en células granulares de cerebelo se previene con los antagonistas del receptor $GABA_B$, mientras que en neuronas corticales está mediada mayoritariamente por su acción sobre los receptores $GABA_A$ (Vale *et al.*, 1998a; Vale *et al.*, 1998b).

II. OBJETIVOS

1. OBJETIVO GENERAL

El objetivo principal de este trabajo es estudiar los mecanismos implicados en la neurotoxicidad producida por la exposición crónica a los pesticidas organoclorados en cultivos primarios neuronales, y, en especial, la implicación de la neurotransmisión GABAérgica y glutamatérgica así como de la señalización mediada por los receptores de estrógenos en estos efectos tóxicos.

2. OBJETIVOS PARTICULARES

Con el fin de abordar este estudio, nos hemos propuestos los siguientes objetivos concretos:

2.1. Estudiar los efectos de la exposición prolongada a dieldrín sobre la expresión, localización y funcionalidad de los receptores de glutamato en cultivos primarios de neuronas corticales.

2.2. Estudiar los efectos de los neuroesteroides estradiol y alopregnanolona sobre los receptores de glutamato en neuronas corticales previamente tratadas con dieldrín.

2.3 Estudiar los efectos de los pesticidas organoclorados sobre los receptores de estrógenos así como sobre sus vías de señalización no genómicas asociadas en cultivos primarios de neuronas corticales y de células granulares de cerebelo.

2.4. Estudiar los mecanismos moleculares involucrados en la muerte neuronal causada por la exposición prolongada a los pesticidas organoclorados en neuronas corticales y en células granulares de cerebelo.

2.5 Estudiar la posible acción neuroprotectora del estradiol y la alopregnanolona frente a la toxicidad inducida por los pesticidas organoclorados en ambos cultivos neuronales.

III. RESULTADOS

TRABAJO Nº 1

Reduction of glutamatergic neurotransmission by prolonged exposure to dieldrin involves NMDA receptor internalization and metabotropic glutamate receptor 5 down-regulation

Víctor Briz, Mireia Galofré, Cristina Suñol

Departamento de Neuroquímica y Neurofarmacología, Instituto de Investigaciones Biomédicas de Barcelona, CSIC – IDIBAPS, Barcelona.

Toxicological Sciences 113(1): 138-149 (2010)

En el presente trabajo hemos utilizado cultivos primarios de neuronas corticales para estudiar los mecanismos implicados en la toxicidad del dieldrín sobre la neurotransmisión glutamatérgica. La exposición durante 2 y 6 DIV a una concentración no citotóxica (60nM) de dieldrín redujo el aumento de la concentración de calcio intracelular ($[Ca^{2+}]_i$) y la excitotoxicidad causada por el glutamato. Observamos que la exposición más prolongada a dieldrín produjo también la internalización de los receptores NMDA (NMDAR) y disminuyó los niveles del receptor metabotrópico de glutamato 5 (mGluR5). El marcaje inmunocitoquímico para NMDAR y mGluR5 mostró una menor co-localización de éstos en la membrana celular de las neuronas tratadas con dieldrín. Sin embargo, no se observaron cambios en la funcionalidad o en los niveles de estos receptores después de 2 DIV de exposición a dieldrín. En cambio, el aumento de $[Ca^{2+}]_i$ inducido por la co-activación de NMDAR y mGluR5 se redujo significativamente. Por lo tanto, estos resultados indican que la interacción funcional entre estos dos receptores parece jugar un papel importante en la excitotoxicidad inducida por glutamato. En este trabajo, confirmamos, además, que el bloqueo permanente de los receptores GABA por este plaguicida persistente provoca cambios homeostáticos que dan como resultado una reducción de la neurotransmisión glutamatérgica.

TOXICOLOGICAL SCIENCES 113(1), 138–149 (2010)
doi:10.1093/toxsci/kfp244
Advance Access publication October 7, 2009

Reduction of Glutamatergic Neurotransmission by Prolonged Exposure to Dieldrin Involves NMDA Receptor Internalization and Metabotropic Glutamate Receptor 5 Downregulation

Victor Briz,*,† Mireia Galofré,*,† and Cristina Suñol*,†,[1]

*Department of Neurochemistry and Neuropharmacology, Institut d'Investigacions Biomèdiques de Barcelona, Consejo Superior de Investigaciones Científicas, CSIC–IDIBAPS, Rosselló 161, E-08036 Barcelona, Spain; and †CIBER Epidemiología y Salud Pública (CIBERESP), Spain

[1] To whom correspondence should be addressed at Department of Neurochemistry and Neuropharmacology, Institut d'Investigacions Biomèdiques de Barcelona, Consejo Superior de Investigaciones Científicas, CSIC–IDIBAPS, Rosselló 161, Barcelona E-08036, Spain. Fax: +34 93 363 83 01. E-mail: csenqi@iibb.csic.es.

Received August 4, 2009; accepted September 23, 2009

Dieldrin was previously used as a pesticide. Although its use has been discontinued, humans are still exposed to it due to its high environmental persistence and because it accumulates in the adipose tissue of animals. Acute exposure to dieldrin provokes convulsions due to its antagonism on the gamma-aminobutyric acid-A ($GABA_A$) receptor. However, little is known about the effects of low chronic exposure to this pollutant. In the present work, we use primary cultures of cortical neurons to study the mechanisms involved in the toxic action of dieldrin. We found that 2 and 6 days *in vitro* (DIV) exposure to a subcytotoxic concentration (60nM) of dieldrin reduced the increase in intracellular calcium concentration ($[Ca^{2+}]_i$) and the excitotoxicity caused by glutamate. Exposure to dieldrin for 6 DIV induced N-methyl-D-aspartate receptor (NMDAR) internalization and reduced metabotropic glutamate receptor 5 (mGLUR5) levels. Double immunostaining for NMDAR and mGLUR5 showed that these receptors lose colocalization on the cell membrane in neurons treated with dieldrin. No changes were observed in receptor functionalities or receptor levels after 2 DIV of exposure to dieldrin. However, the increase in $[Ca^{2+}]_i$ induced by coactivation of NMDAR and mGLUR5 was significantly reduced. Thus, a functional interaction between the two receptors seems to play an important role in glutamate-induced excitotoxicity. We confirm that permanent blockade of the $GABA_A$ receptor by this persistent pesticide triggers adaptive neuronal changes consisting of a reduction of glutamatergic neurotransmission. This might explain the cognitive and learning deficits observed in animals after chronic treatment with dieldrin.

Key Words: dieldrin; neurotoxicity; *in vitro*; glutamate; receptor.

Dieldrin is an organochlorine pesticide that was extensively used in agriculture in the 1950's and 1960's. Its use was prohibited or restricted in many countries in the 1970's and 1980's. Despite this, humans are still exposed to dieldrin both due to its considerable persistence in the environment and more importantly because it accumulates in the adipose tissue of animals and so may be ingested. In farmed salmon, it has been detected at higher levels than in wild ones (Hites *et al.*, 2004). It has also been found in horticultural soils and the plants grown in them (Hilber *et al.*, 2008) and in the adipose tissue of 30% of women who live near agriculture areas (Botella *et al.*, 2004). For the last few years, dieldrin has been 1 of the 12 "persistent organic pollutants" assessed in accordance with the Stockholm Convention (http://www.pops.int). This organochlorine pesticide is also a derivate of the epoxidation of the cyclodiene pesticide aldrin. It blocks the chloride flux through both the gamma-aminobutyric acid-A ($GABA_A$) receptor (where it inhibits t-[^{35}S]butylbicyclophosphorothionate ([^{35}S]TBPS) from binding to it) and the glycine receptor (Pomés *et al.*, 1993; Vale *et al.*, 2003). Acute exposure to dieldrin produces convulsions in mammals (Bloomquist, 1992). However, the effects of chronic exposure to this compound have yet to be established.

Several studies suggest that dieldrin could be a risk factor in the development of Parkinson's disease (PD) (Kanthasamy *et al.*, 2005; Sanchez-Ramos *et al.*, 1998). Moreover, Corrigan *et al.* (2000) found higher levels of dieldrin in PD brains than in control brains. The pesticide causes mitochondrial damage in dopaminergic neurons in culture, and these neurons appear to be sensitive to dieldrin-induced oxidative stress and cell death (Kitazawa *et al.*, 2001; Sanchez-Ramos *et al.*, 1998). In addition, prenatal exposure to dieldrin had deleterious effects on the GABAergic-dependent development of the embryonic brainstem (Liu *et al.*, 1997).

The main target for acute lethal toxicity of cyclodienes is the $GABA_A$ receptor for which dieldrin presents a high affinity (Narahashi *et al.*, 1995; Pomés *et al.*, 1993; Vale *et al.*, 2003). We have previously reported that the blockade of the $GABA_A$ receptor reduces the synaptic release of glutamate from cerebellar granule cells and also protects these neurons from excitotoxic insults (Babot *et al.*, 2005). A link between inhibitory GABA and excitatory glutamate neurotransmission, which compensate each other to maintain brain electrical activity, is at present fully acknowledged. Furthermore, long

timescale changes in both neurotransmitter systems finally leads to regulation of receptor expression and trafficking (Pérez-Otaño and Ehlers, 2005; Turrigiano, 2008). In a previous study, long-term exposure of cerebellar granule cells to dieldrin resulted in a significant reduction of activity and in internalization of N-methyl-D-aspartate receptor (NMDAR) (Babot *et al.*, 2007).

Here, we study the effects of long-term exposure to dieldrin on cultured cortical neurons in order to further characterize the long-term effects of dieldrin on GABA and glutamate neurotransmission. It is accepted that both $GABA_A$ and glutamate receptor subunits are differently expressed in the cortex and cerebellum (Garrett *et al.*, 1990; Llansola *et al.*, 2005), and therefore, different effects of dieldrin could be envisaged. We use primary cultures of cortical neurons that are mainly composed of GABAergic neurons (around 40%) and also glutamatergic and cholinergic neurons, whereas primary cultures of cerebellar granule cells contain predominantly glutamatergic neurons (> 90%). Both the cell types express functional GABA and glutamate receptors (Sonnewald *et al.*, 2004; Suñol *et al.*, 2008). It is also well known that human cortex is responsible for higher brain functions, such as working memory, and that GABA and glutamate play a crucial role in these processes.

This work strongly supports the effects found in cerebellar granule cells by showing that partial inhibition of the $GABA_A$ receptor induced by continuous (but not acute) dieldrin exposure triggers adaptive neural changes consisting of NMDAR internalization. Furthermore, we report a novel target altered by dieldrin, the metabotropic glutamate receptor 5 (mGLUR5). Thus, the effect of dieldrin on GABAergic and glutamatergic neurotransmission in cortical neuronal cultures might indicate that the cortex is a highly susceptible region of the brain against chronic organochlorine pesticide exposure.

MATERIALS AND METHODS

Materials

Pregnant NMRI mice (16th gestational day) were obtained from Charles River, Iffa Credo (Saint Germain-sur-l'Arbreste, France). Plastic culture multiwell plates were from Nunc (Roskilde, Denmark). Fetal calf serum was obtained from Gibco (Invitrogen, Barcelona, Spain), and Dulbecco's modified Eagle's minimum essential medium (DMEM) was from Biochrom (Berlin, Germany). Trypsin, soybean trypsin inhibitor, DNAse, dimethyl sulfoxide (DMSO), L-glycine, L-glutamic acid (NMDA), kainic acid, MK-801, 6-methyl-2-(phenylethynyl)pyridine (MPEP), 7-(hydroxyimino)cyclopropa[b]chromen-1a-carboxylate ethyl ester (CPCOOEt), 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT), dieldrin, picrotoxinin (PTX), L-pyruvic acid, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) and β-nicotinamide adenine dinucleotide–reduced (NADH) disodium salt were from Sigma (St Louis, MO). 2,3-Dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[f]quinoxaline-7-sulfonamide disodium salt and (R,S)-3,5-dihydroxyphenylglycine (DHPG) were from Tocris Cookson (Bristol, UK). $^{36}Cl^-$ was from American Radiolabeled Chemicals, Inc. (St Louis, MO). [^{35}S]TBPS and [^{3}H]-MK-801 were from PerkinElmer (Boston, MA). Fluo-3 AM was from Molecular Probes (Leiden, The Netherlands).

Cell Cultures

Primary cultures of cortical neurons were prepared from the cerebral cortices of 16-day-old mice fetuses according to Frandsen and Schousboe (1990) with minor modifications. Briefly, pregnant animals were anesthetized with isofluorane (FORANE; Abbott Laboratories SA, Madrid, Spain), killed by cervical dislocation, and the fetuses extracted. Cortices were dissected with forceps, mechanically minced, and cells were then dissociated by mild trypsinization (0.02% [wt/vol]) at 37°C for 10 min followed by trituration in a DNAse solution (0.004% [wt/vol]) containing soybean trypsin inhibitor [0.05% (wt/vol]). The cells were then suspended in DMEM (5mM KCl, 31mM glucose, and 0.2mM glutamine) supplemented with *p*-aminobenzoate, insulin, penicillin, and 10% fetal calf serum. The cell suspension (1.5×10^6 cells/ml) was seeded in 6-, 24-, or 96-well plate precoated with poly-D-lysine and incubated for at least 8 days in a humidified 5% CO_2/95% air atmosphere at 36.8°C. A mixture of 5μM 5-fluoro-2′-deoxyuridine and 20μM uridine was added after 2 days *in vitro* (DIV) in culture to prevent glial proliferation.

Animals were handled in compliance with protocol DMA1852 of the University of Barcelona, approved by the Generalitat de Cataluña, Spain, following the European Union guidelines.

Dieldrin Treatment

A stock solution of dieldrin was prepared in DMSO and frozen in aliquots of 100 μl. The final concentration of DMSO in the culture medium was 0.5%. To avoid cross-contamination between different wells in the same plate, the different treatments, DMSO or dieldrin, were performed on separate plates. Cultures were treated at 2 or 6 DIV by adding the stock dieldrin solution in DMSO to the culture medium. The medium was not changed until the experiments were performed at 8 DIV (exposure for 6 or 2 DIV, respectively). The dieldrin concentration was stable in culture medium for at least 8 DIV at 37°C (Babot *et al.*, 2007). In some experiments, neurons were acutely exposed to dieldrin (less than 5 min).

Chloride Influx

Chloride influx was determined as $^{36}Cl^-$ uptake in intact cell cultures of cortical neurons grown in 24-well plates (Vale *et al.*, 2003). Briefly, the culture medium was replaced by prewarmed Earle's balanced salt solution (EBSS: 116mM NaCl, 1.8mM $CaCl_2$, 0.8mM $MgSO_4$, 1mM NaH_2PO_4, 15.2mM $NaHCO_3$, and 5.5mM glucose, adjusted to pH 7.4), and cell cultures were incubated in a humidified 5% CO_2/95% air atmosphere at 36.8°C. After 30-min incubation, the buffer was replaced by new EBSS solution and they were incubated for an additional 15 min. After this, the cells were incubated for 10 s with 225 μl HEPES buffered salt solution ([HBSS]: 136mM NaCl, 5.4mM KCl, 1.2mM $CaCl_2$, 1.4mM $MgCl_2$, 1.0mM NaH_2P0_4, 10mM HEPES, and 9mM glucose, adjusted to pH 7.3) containing $^{36}Cl^-$ (0.4 μCi/ml) and 30μM GABA. PTX (100μM) was used as a positive control of the assay. After removing the $^{36}Cl^-$ solution, each well was immediately rinsed four times with 1.5 ml cold HBSS solution. Cells were lysed by water-induced hyposmotic shock, and radioactivity was determined by liquid scintillation counting in a Wallac 1414 WinSpectral (GMI Inc., Ramsey, MN).

[^{35}S]TBPS Binding

The binding assay was performed on cells grown in 24-well plates according to García *et al.* (2008). In brief, the cells were washed three times with 0.5 ml of HBSS prewarmed to 37°C. The incubation system contained 1.5–3nM [^{35}S]TBPS in Tris-citrate–buffered saline solution (TCBSS: 50mM Tris-citrate and 200mM NaCl, adjusted at pH 7.4). After 30 min at 25°C, the solution was removed and the cells were rinsed three times with 0.5 ml of cold HBSS solution. Nonspecific binding was measured in the presence of 200μM PTX. Cells were collected in 0.25 ml 0.2M NaOH, and their radioactivity was determined by liquid scintillation counting (Optiphase 'Hisafe'2; PerkinElmer, Waltham, MA).

Glutamate Receptor Function

Ionotropic as well as metabotropic glutamate receptor function was evaluated by measuring the agonist-induced increase in Fluo-3 fluorescence

according to Babot *et al.* (2007). This is an indicator of changes in intracellular calcium. It is used to measure the Ca^{2+} influx through the ionotropic receptors or the metabotropic receptor–induced mobilization of intracellular Ca^{2+} stores in response to the interaction with glutamate or specific agonists. Supplementary figure 1 shows that the agonist-induced increase in intracellular Ca^{2+} is completely blocked by specific glutamate receptor antagonists. Cultured cells grown in 96-well plates were incubated with Fluo-3 AM (9μM) for 1 h at 37°C in Hank's solution (1.3mM CaCl, 5.4mM KCl, 0.4mM KH_2PO_4, 0.5mM MgCl, 0.4mM $MgSO_4$, 137mM NaCl, 4.2mM $NaHCO_3$, 0.3mM Na_2HPO_4, 8mM HEPES, and 5.5mM glucose, adjusted to pH 7.4). Excess Fluo-3 AM was rinsed away, and the cells were treated with different agonist concentrations (from 1μM to 1mM) in Hank's solution (or magnesium-free Hank's solution when using NMDA) in the presence of 5μM glycine (when using glutamate or NMDA). Fluorescence (F) was immediately determined in a fluorimetric plate reader (Em 485/Em 530, SpectraMax GeminisXS; Molecular Devices, Sunnyvale, CA). In order to calculate the intracellular calcium concentration ($[Ca^{2+}]_i$), the cells were incubated with the ionophore A23187 (10μM) for 30 min and thereafter with $CuSO_4$ (5mM) to obtain the maximum (F_{max}) and the minimum (F_{min}) fluorescence values, respectively. $[Ca^{2+}]_i$ was calculated for each well as: $[Ca^{2+}]_i = K_d\ (F - F_{min})/(F_{max} - F)$, where K_d is the dissociation constant of Fluo-3 AM/Ca^{2+} (320nM).

[^{3}H]-MK-801 Binding

The binding assay was performed as described by Babot *et al.* (2007) with modifications. Cultures grown in 24-well plates for at least 8 DIV were washed with PBS (137mM NaCl, 2.7mM KCl, 10mM Na_2HPO_4, and 2mM KH_2PO_4) and then exposed for 15 min at 37°C to 4–5nM [^{3}H]-MK-801 in PBS solution containing 100μM glutamate, 100μM glycine, and different concentrations of nonlabeled MK-801. After washing with cold PBS, cells were collected in 0.2M NaOH and then their radioactivity and protein content were measured. Apparent B_{max} and K_d were calculated by fitting the binding values to a competitive one-site binding curve. Nonspecific binding was determined in the presence of 100μM of nonlabeled MK-801.

Western Blot

Cultures grown in 6-well plates were washed twice with cold Hank's solution, and cells were harvested with 0.2 ml of loading buffer (62.5mM Tris-HCl [pH 6.8], 10% glycerol, 2% SDS, and 50mM dithiothreitol) and briefly sonicated. After boiling for 5 min and being centrifuged at 16,100 × g for 5 min, 25 μl of the homogenate was subjected to SDS-polyacrylamide gel electrophoresis using 8–10% polyacrylamide resolving gel at 60 mA for 1.5–2 h. Proteins were transferred into a nitrocellulose membrane and incubated with 5% nonfat dry milk in Tris-Buffered Saline Tween-20 (TBS-T) (20mM Tris-HCl [pH 7.6], 140mM NaCl, and 0.1% Tween-20). Membranes were incubated overnight at 4°C with a goat polyclonal anti-NMDAR1 (1:200; Santa Cruz Biotechnology, Santa Cruz, CA) or a rabbit polyclonal anti-mGLUR5 and anti-mGLUR1 (1:2000; Millipore, Temecula, CA) in TBS-T solution containing 5% bovine serum albumin (BSA). After the membranes were washed, they were incubated with anti-goat and anti-rabbit, respectively, horseradish peroxidase–conjugated (HRP) secondary antibodies (1:4000; Molecular Probes). On all the membranes, a monoclonal anti-actin (1:10,000; Sigma) and a secondary HRP-linked anti-rabbit (1:10,000) antibodies were used as a control of the amount of protein loaded. The membranes were washed and incubated for 4 min in a chemiluminescent solution (Immun-Star HRP kit; Bio-Rad, Hercules, CA). Luminescence was quantified with a Versadoc Imagine System (Bio-Rad). Digital images were then quantified in the Quantity One software (Bio-Rad).

Immunocytochemistry

Cells were seeded in Permanox chamber slides (Nunc) treated with poly-D-lysine, and immunostaining was performed as previously described by Babot *et al.* (2007) with minor modifications. Cultures were rinsed with PBS and fixed with methanol at −20°C for 10 min. Cells were incubated with 0.03% Triton X-100 in PBS for 5 min, and after three rinses with PBS, they were blocked in 5% BSA in PBS for 5 min at room temperature. Subsequently, cultures were incubated overnight at 4°C together with the primary anti-NMDAR1 (1:50) and anti-mGLUR5 (1:500) antibodies in a solution containing 5% BSA in PBS, rinsed three times with PBS for 5 min, and incubated for 1 h at room temperature with both secondary antibodies, chicken anti-goat Alexa 488 (1:1000; Molecular Probes) and donkey anti-rabbit Alexa 594 (1:1000; Molecular Probes) simultaneously. After rinsing with PBS, the slides were coverslipped with Mowiol. The immunostained cells were examined in a confocal fluorescence microscope (Leica Microsystems Inc., Bannockburn, IL) using the same excitation laser intensity for control and dieldrin-treated cells.

Cell Viability and Cytotoxicity

Cell viability was determined by measuring the reduction of MTT, whereas cytotoxicity was assessed by quantifying the release of the cytosolic enzyme lactate dehydrogenase (LDH). The methods were used simultaneously for every experiment.

The treatment with glutamate was always performed at 7–8 DIV when functional ionotropic glutamate receptors are completely expressed (Frandsen and Schousboe, 1990). Cells grown in 96-well plates were exposed to different concentrations of glutamate (from 10μM to 10mM) in Hank's solution for 1 h. After rinsing twice with Hank's solution, fresh DMEM supplemented with gentamicin (1 mg/ml) was added. Cell viability/cytotoxicity was determined 24 h later.

MTT assay. The substrate MTT, when exposed to cells, is reduced by mitochondrial activity to a colored formazan salt, which can be quantified spectrophotometrically. The cells were incubated with MTT (500 μg/ml) dissolved in Hank's solution at 37°C for 1 h protected from light. After washing off the excess MTT, the cells were disaggregated with 5% SDS and the amount of colored formazan salt formed was measured at 560 nm in a spectrophotometer plate reader (iEMS Reader MF; Lab Systems, Helsinki, Finland).

LDH assay. The measurement of LDH released by damaged cells was performed as described previously by Rosa *et al.* (1997) with slight modifications. Briefly, after glutamate treatment, the solution was replaced with fresh DMEM in order to determine only the LDH released in the 24 h after glutamate exposure. Therefore, 50 μl of the medium from each well was placed into another 96-well plate and mixed with 200 μl of Sorensen's phosphate buffer solution (53.4mM Na_2HPO_4 and 13.4mM KH_2PO_4, adjusted to pH 7.4) containing 2.08mM pyruvic acid, 0.375mM NADH, and 3.97mM $NaHCO_3$. Immediately, the mixture was mixed and incubated for 1 min at 37°C before reading the absorbance. Twenty readings of the absorbance of the reaction mixture at 340 nm, an index of NADH concentration, taken every 15 s using a spectrophotometer plate reader (iEMS Reader MF; Lab Systems), were recorded as the kinetic curve of the coenzyme's disappearance. LDH changes were automatically calculated from the slope of the absorbance curve, fitted by linear regression to the initial portion of the curve. We used 50 μl of DMEM plus 200 μl of the NADH solution as a blank for this measure. Cytotoxicity was determined as % of released LDH with respect to control cells. Total death value (100% of LDH release) was obtained from cells treated with 0.3% Triton for 30 min.

Data Analysis

Data are shown as mean ± SE. Unless otherwise stated, at least three experiments from independent culture batches were performed, each one in triplicate. Statistical comparisons were made by *t*-test when comparing control and dieldrin-exposed groups (E_{max} values from Figs. 3–5 and 8), one-way ANOVA followed by Dunnett's multiple comparison test when comparing more than two groups (different exposure time or dieldrin concentrations; Fig. 1, Tables 1 and 2), and two-way ANOVA followed by Bonferroni posttest when comparing two factors (dieldrin and glutamate/antagonists exposures; Figs. 2 and 7) using GraphPad Prism version 4.0 (GraphPad Software, San Diego, CA).

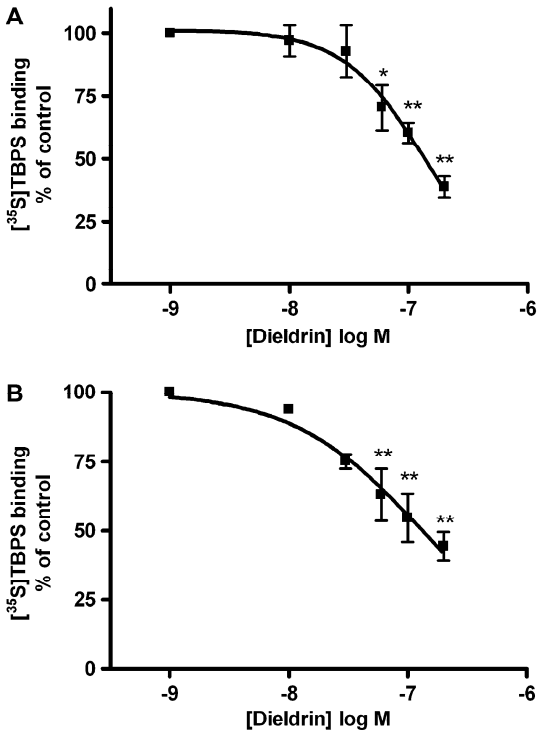


FIG. 1. Concentration-dependent inhibition of [^{35}S]TBPS binding after long-term exposure to dieldrin. Primary cultures of cortical neurons were exposed to dieldrin at concentrations ranging from 0 to 200nM for 2 DIV (A) and 6 DIV (B). Thereafter, the cells were incubated with [^{35}S]TBPS (1.5–3nM in TCBSS) for 30 min at 25°C. The data correspond to mean ± SE of three independent experiments determined in triplicate. (A) *p < 0.05 and **p < 0.01. (B) **p < 0.01 with respect to control.

RESULTS

Effects of Dieldrin Exposure on GABA$_A$ Receptor and Cell Viability in Cortical Neurons

Exposure to dieldrin for 2 DIV inhibited [^{35}S]TBPS binding in intact cultured cortical neurons with an inhibitory concentration 50 (IC50) value of 140 ± 9nM (N = 3) (Fig. 1A). Similar results were obtained after 6 DIV of exposure (IC50 = 145 ± 46nM, N = 3) (Fig. 1B). Long-term exposure to 60nM dieldrin inhibited the 30μM GABA-induced $^{36}Cl^-$ uptake by 55 ± 11% (N = 3, p < 0.05). Since 60nM of dieldrin was the lowest-observed-effect concentration in the [^{35}S]TBPS binding for both 2 and 6 DIV of treatment (Fig. 1), we decided to choose this concentration for all subsequent experiments. This is a noncytotoxic concentration since exposure to up to 200nM dieldrin for 6 DIV did not produce a reduction in cell viability (MTT value was 91 ± 6% with respect to control, N = 4), while exposure to 600nM dieldrin significantly reduced cell viability by 28 ± 8% (p < 0.01).

TABLE 1
Time Course for Glutamate-Induced Reduction of Cell Viability

Time	15 min (3)	30 min (5)	1 h (9)	2 h (2)	24 h (2)
LC50 (mM)	12.1 ± 0.6***	6.6 ± 2.2*	2.7 ± 0.5	0.9 ± 0.8	0.6 ± 0.3

Note. Cell viability was measured 24 h after transient exposure to glutamate for the specified time using MTT assay. Values are at least mean ± SE of (N) experiments performed in triplicate.

*p < 0.05 and ***p < 0.001 versus 1 h treatment.

TABLE 2
Apparent K_d and B_{max} Values for [^{3}H]-MK-801 Binding in Cultured Cortical Neurons

	K_d (μM)	B_{max} (pmol/mg protien)
Control	6.9 ± 1.6	180.0 ± 26.2
Dieldrin 60nM	1.9 ± 0.4*	92.3 ± 12.9*
Dieldrin 200nM	2.4 ± 0.8*	75.5 ± 11.5*

Note. Values represent the mean ± SE of three independent experiments, each performed in triplicate. The values were obtained by adjusting data from each experiment to a one-site competition curve.

*p < 0.05 with respect to control.

Effects of Dieldrin on Glutamate-Induced Excitotoxicity and Intracellular Calcium Increase

We previously reported that a short pulse of high K^+ concentration (> 70mM) releases glutamate ([glutamate]$_o$ > 2μM) from cultured cerebellar granule neurons producing excitotoxic cell death (Babot *et al.*, 2005). Treatment of primary cultures of cortical neurons with 100mM KCl for 15 min induced a lower release of endogenous glutamate ([Glutamate]$_o$ = 0.85 ± 0.13μM, after the insult) without producing cell death (data not shown). Therefore, we developed a model of excitotoxicity using different concentrations of glutamate (from 10μM to 10mM) at different exposure times (from 15 min to 24 h). Table 1 shows LC_{50} values for glutamate-induced loss of cell viability. Treatment with glutamate for 15 or 30 min was significantly less toxic than exposure for 1 h, whereas exposures for 2 or 24 h were not significantly different with respect to 1-h exposure. Therefore, an exposure time of 1 h was selected for the subsequent studies of glutamate-induced excitotoxicity in cultured cortical neurons.

Cells were exposed to 60nM dieldrin for 2 or 6 DIV and subsequently exposed to glutamate. The glutamate-induced reduction in cell viability led to cell membrane damage, as determined by the significant release of LDH. This effect was significantly attenuated by exposure to dieldrin for 2 and 6 DIV (Fig. 2). Similar results were obtained from the MTT assay (Supplementary fig. 2).

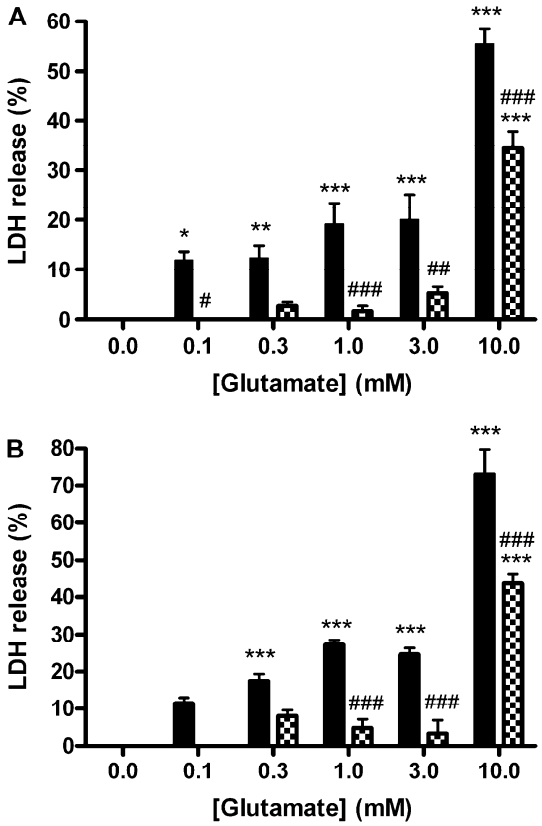


FIG. 2. Effect of long-term exposure to dieldrin on glutamate-induced excitotoxicity in cultured cortical neurons. LDH release was determined 24 h after transient glutamate treatment for 1 h, for neurons exposed to DMSO (black bars) or dieldrin (squared bars) for 2 DIV (A) or 6 DIV (B). Results are expressed as % of LDH release corresponding to the total cell death (mean ± SE, $N = 3$–4). Two-way ANOVA shows statistical differences between glutamate and control ($^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$). Exposure to dieldrin significantly reversed the effect of glutamate: (A) $^{\#}p < 0.05$ and $^{\#\#\#}p < 0.001$; (B) $^{\#\#\#}p < 0.001$.

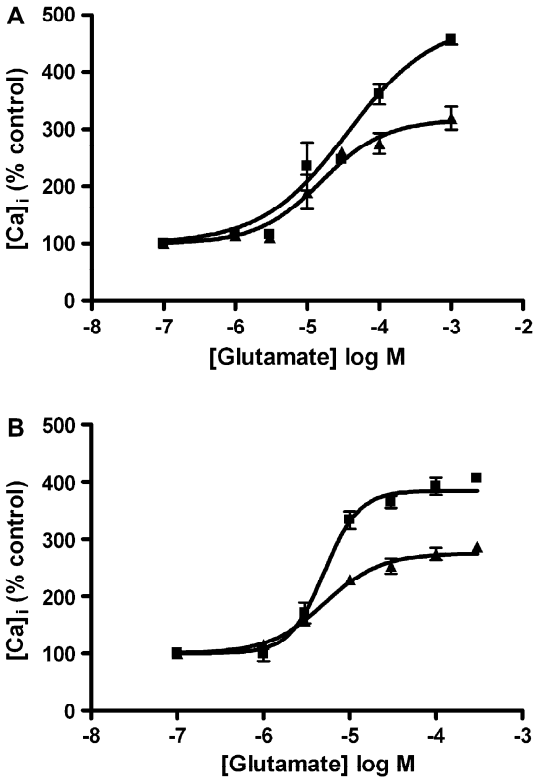


FIG. 3. Effect of exposure to dieldrin on the glutamate-induced increase in $[Ca^{2+}]_i$ in cultured cortical neurons. Fluo-3 fluorescence was measured immediately after glutamate treatment, for neurons exposed to DMSO (■) or dieldrin (▲) for 2 DIV (A) or 6 DIV (B). Values obtained are expressed as % with respect to cells incubated with Hank's solution and are mean ± SE of three to four experiments, each in triplicate. Nonlinear regression fit shows statistical differences between the E_{max} values of the curves. (A) One-tailed t-test, $p < 0.05$ and (B) two-tailed t-test, $p < 0.01$.

We further analyzed whether the increase in $[Ca^{2+}]_i$ caused by glutamate was affected by long-term exposure to dieldrin. Six DIV of treatment with dieldrin did not modify the basal intracellular calcium (values were 56.6 ± 5.9nM and 71.2 ± 6.9nM for control and dieldrin-exposed cells, respectively, $N = 12$) but significantly reduced glutamate-induced increase in $[Ca^{2+}]_i$. Figure 3 shows the concentration-response curves for the increase in $[Ca^{2+}]_i$ due to glutamate. Exposure to dieldrin for 2 DIV led to a significant reduction of the E_{max} values (463 ± 46% and 307 ± 48% for control and dieldrin-exposed cells, respectively, $N = 3$, $p < 0.05$) (Fig. 3A). Likewise, longer exposure to dieldrin up to 6 DIV resulted in reduction of E_{max} values (403 ± 14% and 261 ± 23% for control and dieldrin-exposed cells, respectively, $N = 4$, $p < 0.01$) (Fig. 3B). To corroborate that this effect was mediated by inhibition of the $GABA_A$ receptor as a result of the cells being exposed to dieldrin, we treated primary cultures of cortical neurons with the $GABA_A$ receptor antagonist PTX (100μM) for 6 DIV. PTX mimicked the effect of dieldrin on the glutamate-induced rise in $[Ca^{2+}]_i$ and cell death (Supplementary fig. 3).

Effects of Dieldrin on Ionotropic Glutamate Receptors

The next step was to elucidate which glutamatergic target was altered by exposure to dieldrin in order to explain the reduced responsiveness to the glutamate excitotoxic insult in these neurons. We first studied the function of the NMDAR, which is known to be the main receptor involved in glutamate-triggered excitotoxicity (Choi, 1987; Michaels and Rothman, 1990). Surprisingly, we did not observe a reduction in NMDAR functionality after 2 DIV of exposure to dieldrin (Fig. 4A). However, prolonging exposure to dieldrin up to 6 DIV resulted in a significant reduction in the Ca^{2+} influx induced by NMDA (E_{max} values were 286 ± 40% and 195 ± 12% for control and dieldrin-exposed cells, respectively, $N = 3$, $p < 0.05$) (Fig. 4B). This reduction in NMDAR function was confirmed by a significant reduction of the B_{max} value for

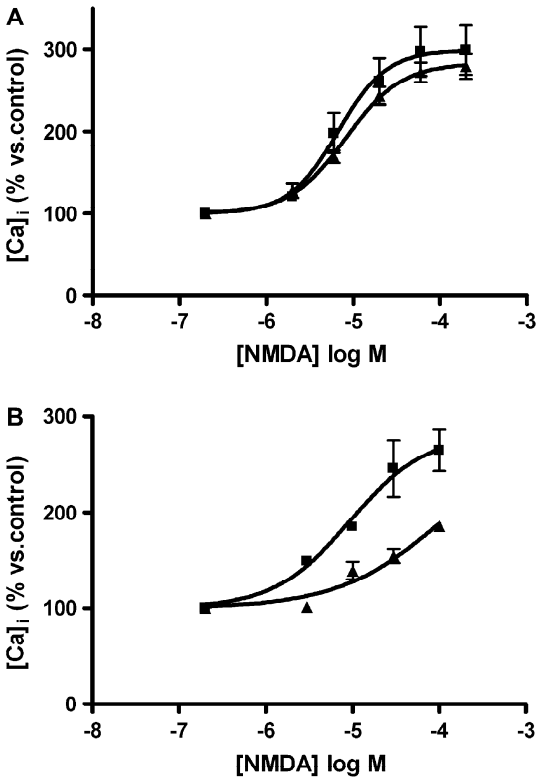


FIG. 4. Effect of exposure to dieldrin on the NMDA-induced increase in $[Ca^{2+}]_i$ in cultured cortical neurons. Fluo-3 fluorescence was measured immediately after NMDA treatment, for neurons exposed to DMSO (■) or dieldrin (▲) for 2 DIV (A) or 6 DIV (B). Values obtained are expressed as % with respect to cells incubated with Hank's solution and are mean ± SE of three to five independent experiments, each in triplicate. Nonlinear regression fit shows statistical differences between the E_{max} values of the curves shown in (B) two-tailed *t*-test, $p < 0.05$.

[^{3}H]-MK-801 binding in intact cells (Table 2). To reject the possibility that dieldrin could be acting as an antagonist at the NMDAR, we evaluate NMDA-induced Ca^{2+} influx in the presence of dieldrin. However, NMDAR functionality was not inhibited by acute exposure to dieldrin (data not shown). We next wished to verify whether the reduction in NMDAR function and the reduced binding of [^{3}H]-MK-801 after long-term exposure to this pesticide was accompanied by a reduction in the total amount of NMDAR protein. To do this, we performed an immunoblot against the obligatory NR1 subunit of NMDAR. No differences were observed in the ratio of NR1/actin between control and dieldrin-treated cells (Fig. 5A). In contrast, immunocytochemistry studies revealed that the NR1 subunit, which is predominantly located on the cell membrane in control neurons, was internalized after exposure to dieldrin for 6 DIV (Figs. 6A and 6G). This effect was less evident in cultures exposed to dieldrin for 2 DIV (Fig. 6D).

We further studied the involvement of the non-NMDARs in our model of excitotoxicity by using the noncompetitive NMDAR antagonist MK-801. As expected, 10μM MK-801 significantly reduced the cytotoxicity caused by 10mM glutamate but exposure to dieldrin for 6 DIV further reduced the glutamate-induced excitotoxicity in the presence of MK-801 (Fig. 7A). This effect was confirmed by measuring the glutamate-induced rise in $[Ca^{2+}]_i$ in the presence of this NMDAR antagonist. Whereas MK-801 reduced the increase in $[Ca^{2+}]_i$ caused by glutamate in control cells (E_{max} values were 353 ± 32% and 242 ± 8% in control and MK-801-treated cells, respectively, $p < 0.05$), this reduction was even greater in dieldrin-treated cells ($E_{max} = 219 \pm 10\%$, $p < 0.01$), although not statistically different from cells treated with MK-801 (Fig. 7B). Taking into account that the excitotoxic response to glutamate when all NMDARs were blocked by MK-801 was reduced to a higher extent in cells exposed to dieldrin with respect to control cells, we wonder if long-term exposure to this pollutant might be affecting other glutamate receptors.

In addition, the reduced glutamate excitotoxicity observed after 2 DIV of exposure to dieldrin was not accompanied with a reduced NMDAR function. Therefore, we sought to determine whether AMPA/kainate receptors could be targeted by dieldrin exposure. However, neither the kainate-induced increase in $[Ca^{2+}]_i$ nor the kainate-induced depolarization were affected by 2 DIV of exposure to dieldrin (Supplementary fig. 4).

Effects of Dieldrin on Metabotropic Glutamate Receptors

Next, we studied the group-I ionotropic glutamate receptors (mGLUR1 and mGLUR5) since their activation increases $[Ca^{2+}]_i$ through the mobilization of intracellular Ca^{2+} stores (Nakanishi, 1994). The antagonists for both mGLUR5 and mGLUR1 reduced the increase in $[Ca^{2+}]_i$ induced by glutamate with similar efficacy (E_{max} values were 361 ± 13%, 255 ± 18%, and 266 ± 25% for control, MPEP-treated, and CPCOOEt-treated cells, respectively; $p < 0.05$) (Supplementary fig. 5A). However, only the antagonist of mGLUR5, MPEP, significantly prevented glutamate-induced excitotoxicity ($p < 0.05$, $N = 3$, for MPEP with respect to control) (Supplementary fig. 5B). When cortical neurons were exposed to dieldrin for 6 DIV, the increase of $[Ca^{2+}]_i$ induced by the specific agonist of group-I metabotropic glutamate receptor, DHPG, was significantly reduced (E_{max} values were 157 ± 10% and 132 ± 4% in control and dieldrin-exposed cells, respectively; $p < 0.05$, $N = 3$) (Fig. 8A). However, this effect was not observed neither after exposure to dieldrin for 2 DIV nor after acute exposure (data not shown). Since exposure to dieldrin for 2 DIV reduced the glutamate-induced rise in $[Ca^{2+}]_i$ (Fig. 3A) but not that induced by NMDA (Fig. 4A) or DHPG, we wondered whether coexposure to both agonists would be needed to observe a similar reduction to that observed with glutamate on the increase of $[Ca^{2+}]_i$. The simultaneous treatment with 100μM of NMDA and 100μM of DHPG induced an increase in $[Ca^{2+}]_i$ in control cells ($[Ca^{2+}]_i = 213 \pm 7\%$), which was significantly

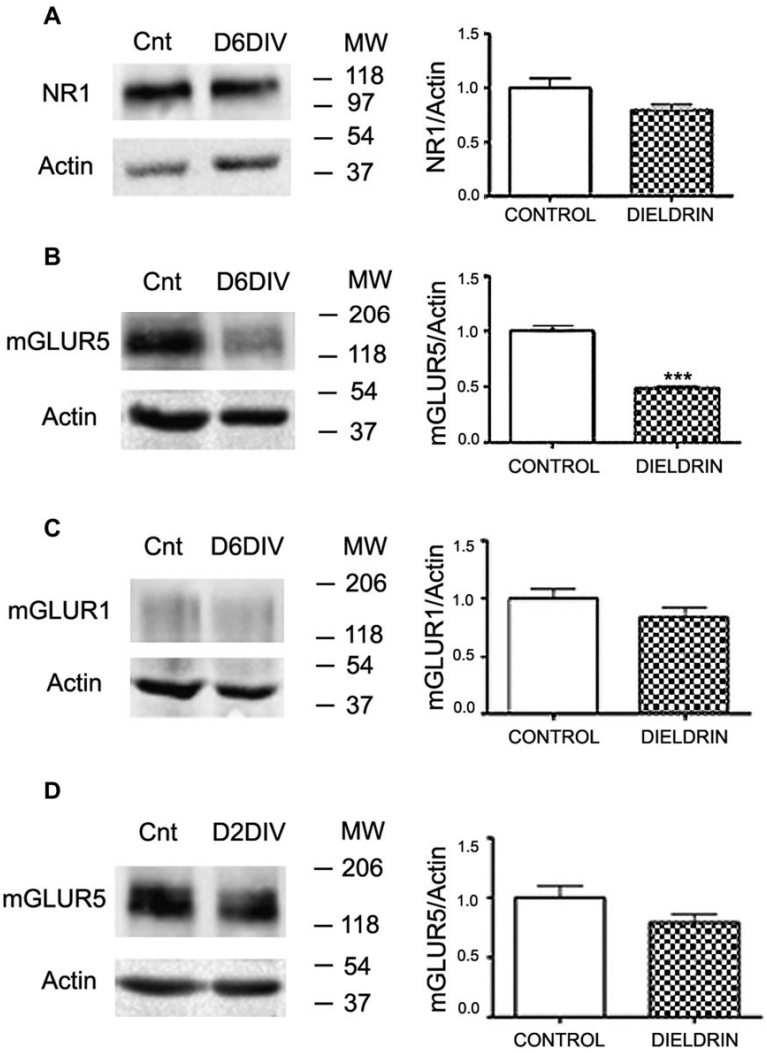


FIG. 5. Long-term exposure to dieldrin decreased mGLUR5 protein but not mGLUR1 or NMDAR. Left: representative immunoblots for the indicated proteins from control cultures (Cnt) and cultures treated with dieldrin for 6 DIV (A–C) or 2 DIV (D). The corresponding immunoblot for actin is shown at the bottom. Molecular weights are given on the right. Right: densitometric quantification of the immunoblots. Data are mean ± SE of three to four independent experiments. (B) Exposure to dieldrin for 6 DIV reduced the levels of mGLUR5 protein with respect to control ($p < 0.001$, $N = 4$).

reduced in cells exposed to dieldrin for 2 DIV ($[Ca^{2+}]_i = 177 \pm 10\%$, $N = 3$, $p < 0.05$) (Fig. 8B).

In order to discriminate which of the group-I metabotropic glutamate receptors was affected by the exposure to dieldrin, we used specific antibodies against mGLUR1 and mGLUR5. Quantification of the immunoblots showed a reduction in the total amount of mGLUR5 protein after 6 DIV of exposure to dieldrin (Fig. 5B). The reduction in the amount of mGLUR5 was confirmed by immunostaining. mGLUR5 colocalizes with the NR1 subunit of NMDAR on the cell membrane of the soma in control cultures (Fig. 6A–C). In contrast, in neurons exposed to dieldrin for 6 DIV, the mGLUR5 immunostaining was reduced overall (Fig. 6H) and the colocalization with the NR1 subunit disappeared (Fig. 6I). No significant changes were observed in the mGLUR1 protein; however, the immunoblot signal was very faint in our cultures (Fig. 5C). Contrarily, exposure to dieldrin for 2 DIV did not modify either mGLUR5 (Fig. 5D) or mGLUR1 protein levels (data not shown), which is in agreement with the lack of effect on the functionality of these receptors after 2 DIV of exposure to dieldrin. Nevertheless, mGLUR5 immunostaining was mainly located at the cell membrane in control cells (Fig. 6B), as mentioned, whereas in cells treated with dieldrin for 2 DIV, a diffuse distribution was observed (Fig. 6E) and moreover the colocalization with NMDAR almost completely disappeared (Fig. 6F). This effect may explain why after 2 DIV of dieldrin exposure the individual

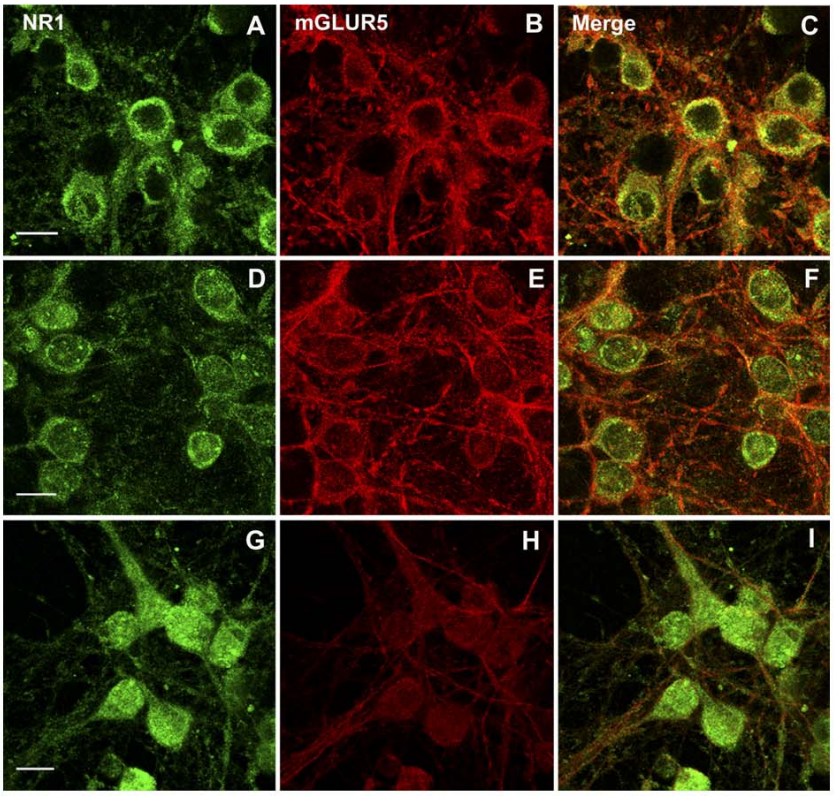


FIG. 6. Confocal micrograph of the double immunostaining for the NR1 subunit of NMDAR (left) and mGLUR5 (middle) and a merge of the two (right), of a representative culture of control (A–C) and cells treated with dieldrin for 2 DIV (D–F) and 6 DIV (G–I). Scale bar = 10 μm.

functionalities of both NMDAR and mGLUR5 were not significantly different from control, but the physiological consequences of their simultaneous activation were affected.

DISCUSSION

In this work, we show that long-term exposure to a non-cytotoxic concentration of dieldrin produced both an internalization of NMDAR and a downregulation of mGLUR5 in primary cultures of cortical neurons. These effects were secondary and a consequence of the continuous blockade of $GABA_A$ receptor by dieldrin. This compensatory balance between excitation and inhibition explains the reduced responsiveness to glutamate in long-term dieldrin-treated cells, as measured by both the increase in $[Ca^{2+}]_i$ and the excitotoxicity induced by glutamate (Figs. 3 and 4). Swann *et al.* (2007) have recently found that chronic inhibition of $GABA_A$ receptor with bicuculline reduces glutamate receptor–mediated synaptic activity and several components of the glutamatergic synapse, such as the post synaptic density-95 (PSD-95) and Homer. As opposed to toluene, which is known to acutely enhance glycine and $GABA_A$ receptor–mediated currents, NMDA-mediated (but not AMPA/Kainate-mediated) neurotransmission is enhanced after chronic exposure to this solvent (Bale *et al.*, 2005). Moreover, inhibition of synaptic activity using NMDAR antagonists also induces upregulation of NMDAR expression (Slikker *et al.*, 2007). Thus, increasing evidence shows an activity-dependent trafficking and expression of membrane receptors that accurately regulate synaptic excitability.

The concentration of dieldrin used in this work (60nM) inhibited the binding of $[^{35}S]$TBPS after 2 and 6 DIV of exposure to a similar extent to that observed after acute exposure (Cole and Casida, 1986; Pomés *et al.*, 1993). Accordingly, we also report that long-term exposure to a noncytotoxic concentration of dieldrin inhibited the GABA-induced chloride influx by around 50%. Again, this effect is similar to that observed after acute exposure in neuronal cultures (Vale *et al.*, 2003), which is consistent with the reported stability of this compound in serum-containing culture medium (Babot *et al.*, 2007). Some work reports transient effects on $[Ca^{2+}]_i$ fluxes and prolactin release at very low concentrations (picomolar to nanomolar) of dieldrin in a pituitary tumor cell line (Wozniak *et al.*, 2005). However, to the best of our knowledge, this is the first study in which such low concentrations of dieldrin are shown to cause

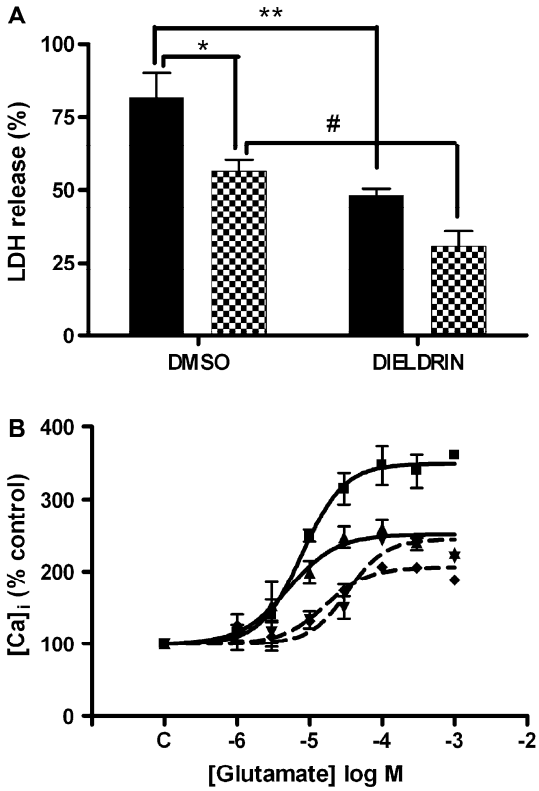


FIG. 7. Effect of long-term exposure to dieldrin on glutamate-induced excitotoxicity (A) and on $[Ca^{2+}]_i$ increase (B), in the presence of MK-801. (A) LDH release values were obtained 24 h after 10mM glutamate treatment for neurons exposed for 6 DIV to DMSO or dieldrin in the absence (black bars) or presence (squared bars) of 10μM MK-801. Results are expressed as % of LDH release with respect to the total cell death (mean ± SE, $N = 3$). $*p < 0.05$ for MK-801-treated cells versus control, $**p < 0.01$ for dieldrin versus DMSO in the absence of MK-801, and $^{\#}p < 0.05$ for dieldrin versus DMSO in the presence of MK-801. (B) Treatment with glutamate was performed, in the absence of MK-801 (continuous lines), on neurons exposed for 6 DIV to DMSO (■) or dieldrin (▲) and, in the presence of 10μM MK-801 (dashed lines), on neurons exposed to DMSO (▼) and dieldrin (♦). Nonlinear regression fit shows statistical differences between the E_{max} values of the curves ($p < 0.01$ for MK-801-treated cells versus control; $p < 0.05$ for dieldrin versus control in the absence of MK-801).

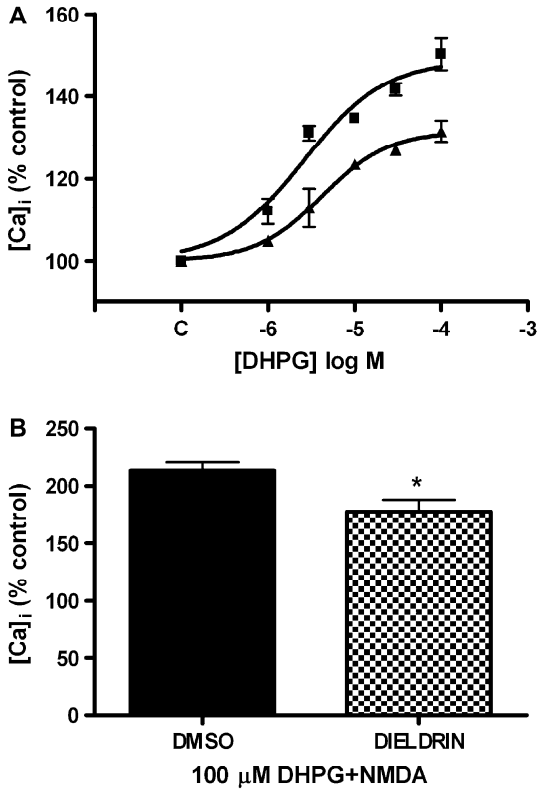


FIG. 8. Effect of long-term exposure to dieldrin on the $[Ca^{2+}]_i$ increase induced by DHPG (A) and by coadministration of DHPG and NMDA (B). Fluo-3 fluorescence was measured immediately after agonist administration, for neurons exposed to DMSO (■) or dieldrin (▲) for 6 DIV (A) or 2 DIV (B). Values obtained are expressed as % with respect to cells incubated with Hank's solution (mean ± SE, $N = 3$). (A) Nonlinear regression fit shows statistical differences between the E_{max} values of the curves ($p < 0.05$, one-tailed t-test). (B) $*p < 0.05$, two-tailed t-test.

prolonged alterations to synaptic transmission. A similar reduction of NMDAR function was observed in cultured cerebellar granule cells exposed to dieldrin (Babot *et al.*, 2007), however, the concentration used in that work was much higher than here. Other works have also found that micromolar concentrations of dieldrin strongly inhibit serotonergic neurons (Liu *et al.*, 1997) and cause oxidative stress and apoptosis in dopaminergic neurons (Kitazawa *et al.*, 2001; Sanchez-Ramos *et al.*, 1998). Our results show that cortical neurons are highly sensitive to prolonged exposure to dieldrin and therefore probably to other permanent organochlorine pesticides that share a similar mechanism of acute toxicity.

Glutamate-induced neuronal damage is predominantly not only Ca dependent but also Cl dependent (Babot *et al.*, 2005; Choi, 1987). It is also accepted that activation of NMDAR is the main cause of glutamate-triggered cytotoxicity (Michaels and Rothman, 1990). For these reasons, the effect of long-term exposure to dieldrin on glutamate-mediated cell death found in the present study cannot be accounted for only by partial inhibition of $GABA_A$ receptor. It probably indirectly affects some of the components of the glutamatergic system. Therefore, we first studied the possible action of long-term exposure to dieldrin on NMDAR. As shown in Figure 4, 2 DIV of exposure to dieldrin is not long enough to significantly affect the functionality of NMDAR; longer treatments are needed to observe an effect on this receptor. This is at variance with the rapid changes in the expression or localization of the NR1 subunit of NMDAR that has been found after excitotoxic stimulation in cortical neurons (Gascón *et al.*, 2005; Nakamichi and Yoneda 2006). However, in our context, we expect that

partial blockade of the $GABA_A$ receptor would produce slight but continuous hyperactivation of the glutamatergic system, the so-called "slow excitotoxicity", that probably requires longer periods of time to cause changes in NMDAR cell surface expression.

It has been demonstrated that several proteins are needed for cell membrane localization of NMDAR, such as PSD-95 (Kornau *et al.*, 1995). For instance, the administration of NMDAR antagonists upregulates SAP90/PSD-95 and SAP97 in the rat cortex (Linden *et al.*, 2001), whereas treatment with bicuculline reduces the expression of PSD-95 and Homer proteins (Swann *et al.*, 2007). Therefore, the fact that prolonged exposure to dieldrin (> 2 DIV) is needed to modify NMDAR function suggests that this pesticide may affect the expression of some transport or anchorage proteins needed for NMDAR clustering, which in turn can result in NMDAR internalization. Further studies are required to determine which of these proteins are primarily affected by dieldrin exposure.

Prolonged exposure to dieldrin specifically induced mGLUR5 downregulation, as observed by Western blot and immunocytochemistry, whereas mGLUR1 remained unaffected. This selective effect on mGLUR5 has also been observed in hippocampal slices after transient global ischemia and after status epilepticus (Kirschstein *et al.*, 2007; Yeh and Wang, 2005). Although there is a consensus concerning the major contribution of NMDAR to glutamate-induced cell death, the involvement of metabotropic glutamate receptors is still controversial. Dual role in neurotoxicity and neuroprotection has been proposed for type-I mGLUR (Nicoletti *et al.*, 1999). For instance, the activation of type-I mGLUR causes neurotoxic damage and enhances NMDA- (but not AMPA-) mediated degeneration in rat brain and in cultured cortical neurons (Bruno *et al.*, 1995; McDonald and Schoepp, 1992). Although the mGLUR1 antagonist CPCOOEt is neuroprotective against NMDA toxicity by enhancing GABA release (Battaglia *et al.*, 2001), it fails to prevent cell death induced by coadministration of NMDA and DHPG in cortical neurons, whereas MPEP is effective in the same conditions (Bruno *et al.*, 2001). In agreement with these data, here we show that both CPCOOEt and MPEP reduced the glutamate-induced $[Ca^{2+}]_i$ increase but only MPEP was able to prevent the neuronal loss induced by glutamate (Supplementary fig. 5). The lower expression of mGLUR1 with respect to mGLUR5 in cortical neurons (Fig. 5, this work; Bruno *et al.*, 1995) might explain these observations. However, a specific involvement of mGLUR5 in NMDAR-mediated neurotransmission cannot be ruled out since several studies show a direct association between mGLUR5 and NMDAR. For instance, it has been demonstrated that mGLUR5 knock-out (K.O.) mice show impaired NMDAR-dependent long-term potentiation (LTP) but not NMDAR-independent LTP in the hippocampus (Lu *et al.*, 1997). Furthermore, bicuculline-induced persistent bursting from the hippocampus to the entorhinal cortex is mediated by both mGLUR5 and NMDAR activation (Stoop *et al.*, 2003). In addition, Yang *et al.* (2004) have recently proved a synergistic effect on extracellular regulated kinase phosphorylation and gene expression by simultaneous mGLUR5 and NMDAR activation via PSD-95 and Homer-1b/c. This in turn could directly cross-link the two receptors, through the Shank family of proteins, to their respective intracellular signaling pathways (Tu *et al.*, 1999). After just 2 DIV of exposure to dieldrin, reduced colocalization in the cell soma and a more diffused pattern in the immunolabeling of mGLUR5 and NMDAR were observed. This may explain the reduced responsiveness to glutamate in these cells since at this time dieldrin was able to reduce both neuronal death and $[Ca^{2+}]_i$ increase that were induced by glutamate. These observations lend weight to the importance of simultaneous activation of the two receptors in glutamate excitotoxity.

Activation of type-I mGLURs has been shown to promote NMDAR trafficking to the cell membrane (Lan *et al.*, 2001). We therefore hypothesize that the internalization of the NMDAR observed after 6 DIV of dieldrin exposure might be explained at least in part as a consequence of the reduced expression of mGLUR5 in these cells. Nevertheless, a more detailed time-course monitoring study is needed to confirm this hypothesis.

It is well known that NMDAR plays a crucial role in learning and memory, whereas the involvement of type-I mGLURs is currently not so clear. Increasing evidence suggests that there is a functional interaction between NMDAR and mGLUR5 in these processes (Gravius *et al.*, 2006; Homayoun *et al.*, 2004). It has also been found that K.O. mice lacking NMDAR1 or mGLUR5 show impaired learning (Cheli *et al.*, 2006; Lu *et al.*, 1997). Thus, the reduced functionality of NMDAR and mGLUR5 found in the present study might explain the impairment of learning and cognition observed in animals after chronic treatment with dieldrin (Gesell and Robel, 1979; Smith *et al.*, 1976; Topinka *et al.*, 1984).

In conclusion, here we report that long-term exposure to a noncytotoxic concentration of dieldrin causes NMDAR internalization and mGLUR5 downregulation in cultured cortical neurons. Therefore, neuronal hyperactivation, such as that produced by $GABA_A$ receptor antagonists, may disrupt normal network activity and maturation of glutamatergic neurotransmission (Stoop *et al.*, 2003; Swann *et al.*, 2007; this work), and it is expected that these neurons have reduced responsiveness to physiological glutamate. Rather than presenting this pollutant as a neuroprotective drug against diseases in which glutamate excitotoxicity is involved, such as cerebral ischemia or Alzheimer's disease, here we emphasize the neurophysiologic alterations that may underlie the behavioral and learning deficits observed after prolonged exposure to persistent pesticides such as dieldrin (Gesell and Robel, 1979; Smith *et al.*, 1976; Topinka *et al.*, 1984).

SUPPLEMENTARY DATA

Supplementary data are available online at http://toxsci.oxfordjournals.org/.

FUNDING

This work was funded by the Instituto de Salud Carlos III, Ministry of Health, Spain (grant number PI 061212); Generalitat de Catalunya (grant number 2005/SGR/00826). V.B. is recipient of a predoctoral fellowship from Institut d'Investigacions Biomèdiques August Pi i Sunyer.

ACKNOWLEDGMENTS

The authors would like to thank Sara Sánchez-Redondo for her technical assistance.

REFERENCES

Babot, Z., Cristòfol, R., and Suñol, C. (2005). Excitotoxic death induced by released glutamate in depolarized primary cultures of mouse cerebellar granule cells is dependent on $GABA_A$ receptors and niflumic acid-sensitive chloride channels. *Eur. J. Neurosci.* **21,** 103–112.

Babot, Z., Vilaró, M. T., and Suñol, C. (2007). Long-term exposure to dieldrin reduces gamma-aminobutyric acid type A and N-methyl-D-aspartate receptor function in primary cultures of mouse cerebellar granule cells. *J. Neurosci. Res.* **85,** 3687–3695.

Bale, A. S., Tu, Y., Carpenter-Hyland, E. P., Chandler, L. J., and Woodward, J. J. (2005). Alterations in glutamatergic and gabaergic ion channel activity in hippocampal neurons following exposure to the abused inhalant toluene. *Neuroscience* **130,** 197–206.

Battaglia, G., Bruno, V., Pisani, A., Centonze, D., Catania, M. V., Calabresi, P., and Nicoletti, F. (2001). Selective blockade of type-1 metabotropic glutamate receptors induces neuroprotection by enhancing gabaergic transmission. *Mol. Cell. Neurosci.* **17,** 1071–1083.

Bloomquist, J. R. (1992). Intrinsic lethality of chloride-channel-directed insecticides and convulsants in mammals. *Toxicol. Lett.* **60,** 289–298.

Botella, B., Crespo, J., Rivas, A., Cerrillo, I., Olea-Serrano, M. F., and Olea, N. (2004). Exposure of women to organochlorine pesticides in Southern Spain. *Environ. Res.* **96,** 34–40.

Bruno, V., Battaglia, G., Copani, A., Cespedes, V. M., Galindo, M. F., Cena, V., Sanchez-Prieto, J., Gasparini, F., Kuhn, R., Flor, P. J., *et al.* (2001). An activity-dependent switch from facilitation to inhibition in the control of excitotoxicity by group I metabotropic glutamate receptors. *Eur. J. Neurosci.* **13,** 1469–1478.

Bruno, V., Copani, A., Knopfel, T., Kuhn, R., Casabona, G., Dell'Albani, P., Condorelli, D. F., and Nicoletti, F. (1995). Activation of metabotropic glutamate receptors coupled to inositol phospholipid hydrolysis amplifies NMDA-induced neuronal degeneration in cultured cortical cells. *Neuropharmacology* **34,** 1089–1098.

Cheli, V., Adrover, M., Blanco, C., Ferrari, C., Cornea, A., Pitossi, F., Epstein, A. L., and Jerusalinsky, D. (2006). Knocking-down the NMDAR1 subunit in a limited amount of neurons in the rat hippocampus impairs learning. *J. Neurochem.* **97**(Suppl. 1), 68–73.

Choi, D. W. (1987). Ionic dependence of glutamate neurotoxicity. *J. Neurosci.* **7,** 369–379.

Cole, L. M., and Casida, J. E. (1986). Polychlorocycloalkane insecticide-induced convulsions in mice in relation to disruption of the GABA-regulated chloride ionophore. *Life Sci.* **39,** 1855–1862.

Corrigan, F. M., Wienburg, C. L., Shore, R. F., Daniel, S. E., and Mann, D. (2000). Organochlorine insecticides in substantia nigra in Parkinson's disease. *J. Toxicol. Environ. Health A* **59,** 229–234.

Frandsen, A., and Schousboe, A. (1990). Development of excitatory amino acid induced cytotoxicity in cultured neurons. *Int. J. Dev. Neurosci.* **8,** 209–216.

García, D. A., Vendrell, I., Galofré, M., and Suñol, C. (2008). GAB_A released from cultured cortical neurons influences the modulation of t-[(35)S]butyl-bicyclophosphorothionate binding at the $GABA_A$ receptor Effects of thymol. *Eur. J. Pharmacol.* **600,** 26–31.

Garrett, K. M., Saito, N., Duman, R. S., Abel, M. S., Ashton, R. A., Fujimori, S., Beer, B., Tallman, J. F., Vitek, M. P., and Blume, A. J. (1990). Differential expression of gamma-aminobutyric acidA receptor subunits. *Mol. Pharmacol.* **37,** 652–657.

Gascón, S., Deogracias, R., Sobrado, M., Roda, J. M., Renart, J., Rodríguez-Peña, A., and Díaz-Guerra, M. (2005). Transcription of the NR1 subunit of the N-methyl-D-aspartate receptor is down-regulated by excitotoxic stimulation and cerebral ischemia. *J. Biol. Chem.* **280,** 35018–35027.

Gesell, G. G., and Robel, R. J. (1979). Effects of dieldrin on operant behavior of bobwhites. *J. Environ. Sci. Health B* **14,** 153–170.

Gravius, A., Pietraszek, M., Schmidt, W. J., and Danysz, W. (2006). Functional interaction of NMDA and group I metabotropic glutamate receptors in negatively reinforced learning in rats. *Psychopharmacology (Berl.)* **185,** 58–65.

Hilber, I., Mader, P., Schulin, R., and Wyss, G. S. (2008). Survey of organochlorine pesticides in horticultural soils and there grown Cucurbitaceae. *Chemosphere* **73,** 954–961.

Hites, R. A., Foran, J. A., Schwager, S. J., Knuth, B. A., Hamilton, M. C., and Carpenter, D. O. (2004). Global assessment of polybrominated diphenyl ethers in farmed and wild salmon. *Environ. Sci. Technol.* **38,** 4945–4949.

Homayoun, H., Stefani, M. R., Adams, B. W., Tamagan, G. D., and Moghaddam, B. (2004). Functional interaction between NMDA and mGlu5 receptors: effects on working memory, instrumental learning, motor behaviors, and dopamine release. *Neuropsychopharmacology* **29,** 1259–1269.

Kanthasamy, A. G., Kitazawa, M., Kanthasamy, A., and Anantharam, V. (2005). Dieldrin-induced neurotoxicity: relevance to Parkinson's disease pathogenesis. *Neurotoxicology* **26,** 701–719.

Kirschstein, T., Bauer, M., Muller, L., Ruschenschmidt, C., Reitze, M., Becker, A. J., Schoch, S., and Beck, H. (2007). Loss of metabotropic glutamate receptor-dependent long-term depression via downregulation of mGluR5 after status epilepticus. *J. Neurosci.* **27,** 7696–7704.

Kitazawa, M., Anantharam, V., and Kanthasamy, A. G. (2001). Dieldrin-induced oxidative stress and neurochemical changes contribute to apoptopic cell death in dopaminergic cells. *Free Radic. Biol. Med.* **31,** 1473–1485.

Kornau, H. C., Schenker, L. T., Kennedy, M. B., and Seeburg, P. H. (1995). Domain interaction between NMDA receptor subunits and the postsynaptic density protein PSD-95. *Science* **269,** 1737–1740.

Lan, J. Y., Skeberdis, V. A., Jover, T., Zheng, X., Bennett, M. V., and Zukin, R. S. (2001). Activation of metabotropic glutamate receptor 1 accelerates NMDA receptor trafficking. *J. Neurosci.* **21,** 6058–6068.

Linden, A. M., Vasanen, J., Storvik, M., Lakso, M., Korpi, E. R., Wong, G., and Castren, E. (2001). Uncompetitive antagonists of the N-methyl-D-aspartate (NMDA) receptors alter the mRNA expression of proteins associated with the NMDA receptor complex. *Pharmacol. Toxicol.* **88,** 98–105.

Liu, J., Morrow, A. L., Devaud, L., Grayson, D. R., and Lauder, J. M. (1997). $GABA_A$ receptors mediate trophic effects of GABA on embryonic brainstem monoamine neurons in vitro. *J. Neurosci.* **17,** 2420–2428.

Llansola, M., Sanchez-Perez, A., Cauli, O., and Felipo, V. (2005). Modulation of NMDA receptors in the cerebellum. 1. Properties of the NMDA receptor that modulate its function. *Cerebellum* **4,** 154–161.

Lu, Y. M., Jia, Z., Janus, C., Henderson, J. T., Gerlai, R., Wojtowicz, J. M., and Roder, J. C. (1997). Mice lacking metabotropic glutamate receptor 5 show impaired learning and reduced CA1 long-term potentiation (LTP) but normal CA3 LTP. *J. Neurosci.* **17,** 5196–5205.

McDonald, J. W., and Schoepp, D. D. (1992). The metabotropic excitatory amino acid receptor agonist 1S,3R-ACPD selectively potentiates N-methyl-D-aspartate-induced brain injury. *Eur. J. Pharmacol.* **215,** 353–354.

Michaels, R. L., and Rothman, S. M. (1990). Glutamate neurotoxicity in vitro: Antagonist pharmacology and intracellular calcium concentrations. *J. Neurosci.* **10,** 283–292.

Nakamichi, N., and Yoneda, Y. (2006). Maturation-dependent reduced responsiveness of intracellular free Ca^{2+} ions to repeated stimulation by N-methyl-D-aspartate in cultured rat cortical neurons. *Neurochem. Int.* **49,** 230–237.

Nakanishi, S. (1994). Metabotropic glutamate receptors: Synaptic transmission, modulation, and plasticity. *Neuron* **13,** 1031–1037.

Narahashi, T., Carter, D. B., Frey, J., Ginsburg, K., Hamilton, B. J., Nagata, K., Roy, M. L., Song, J. H., and Tatebayashi, H. (1995). Sodium channels and $GABA_A$ receptor-channel complex as targets of environmental toxicants. *Toxicol. Lett.* **82–83,** 239–245.

Nicoletti, F., Bruno, V., Catania, M. V., Battaglia, G., Copani, A., Barbagallo, G., Cena, V., Sanchez-Prieto, J., Spano, P. F., and Pizzi, M. (1999). Group-I metabotropic glutamate receptors: hypotheses to explain their dual role in neurotoxicity and neuroprotection. *Neuropharmacology* **38,** 1477–1484.

Pérez-Otaño, I., and Ehlers, M. D. (2005). Homeostatic plasticity and NMDA receptor trafficking. *Trends Neurosci.* **28,** 229–238.

Pomés, A., Rodríguez-Farré, E., and Suñol, C. (1993). Inhibition of t-[35S]butylbicyclophosphorothionate binding by convulsant agents in primary cultures of cerebellar neurons. *Brain Res. Dev. Brain Res.* **73,** 85–90.

Rosa, R., Sanfeliu, C., Suñol, C., Pomés, A., Rodríguez-Farré, E., Schousboe, A., and Frandsen, A. (1997). The mechanism for hexachlorocyclohexane-induced cytotoxicity and changes in intracellular Ca^{2+} homeostasis in cultured cerebellar granule neurons is different for the gamma- and delta-isomers. *Toxicol. Appl. Pharmacol.* **142,** 31–39.

Sanchez-Ramos, J., Facca, A., Basit, A., and Song, S. (1998). Toxicity of dieldrin for dopaminergic neurons in mesencephalic cultures. *Exp. Neurol.* **150,** 263–271.

Slikker, W., Jr., Zou, X., Hotchkiss, C. E., Divine, R. L., Sadovova, N., Twaddle, N. C., Doerge, D. R., Scallet, A. C., Patterson, T. A., Hanig, J. P., *et al.* (2007). Ketamine-induced neuronal cell death in the perinatal rhesus monkey. *Toxicol. Sci.* **98,** 145–158.

Smith, R. M., Cunningham, W. L., Jr., van Gelder, G. A., and Karas, G. G. (1976). Deldrin toxicity and successive discrimation reversal in squirrel monkeys (Saimiri sciureus). *J. Toxicol. Environ. Health* **1,** 737–747.

Sonnewald, U., Olstad, E., Qu, H., Babot, Z., Cristòfol, R., Suñol, C., Schousboe, A., and Waagepetersen, H. (2004). First direct demonstration of extensive GABA synthesis in mouse cerebellar neuronal cultures. *J. Neurochem.* **91,** 796–803.

Stoop, R., Conquet, F., and Pralong, E. (2003). Determination of group I metabotropic glutamate receptor subtypes involved in the frequency of epileptiform activity in vitro using mGluR1 and mGluR5 mutant mice. *Neuropharmacology* **44,** 157–162.

Suñol, C., Babot, Z., Fonfría, E., Galofré, M., García, D., Herrera, N., Iraola, S., and Vendrell, I. (2008). Studies with neuronal cells: From basic studies of mechanisms of neurotoxicity to the prediction of chemical toxicity. *Toxicol. In Vitro* **22,** 1350–1355.

Swann, J. W., Le, J. T., Lam, T. T., Owens, J., and Mayer, A. T. (2007). The impact of chronic network hyperexcitability on developing glutamatergic synapses. *Eur. J. Neurosci.* **26,** 975–991.

Topinka, M. A., Eisenstein, E. M., Siegel, D., Trofeli, R., and Barraco, D. (1984). The effects of dieldrin and chlordimeform on learning and memory in the cockroach, Periplaneta americana: a study in behavioral toxicology. *J. Toxicol. Environ. Health* **13,** 705–719.

Tu, J. C., Xiao, B., Naisbitt, S., Yuan, J. P., Petralia, R. S., Brakeman, P., Doan, A., Aakalu, V. K., Lanahan, A. A., Sheng, M., *et al.* (1999). Coupling of mGluR/Homer and PSD-95 complexes by the Shank family of postsynaptic density proteins. *Neuron* **23,** 583–592.

Turrigiano, G. G. (2008). The self-tuning neuron: synaptic scaling of excitatory synapses. *Cell* **135,** 422–435.

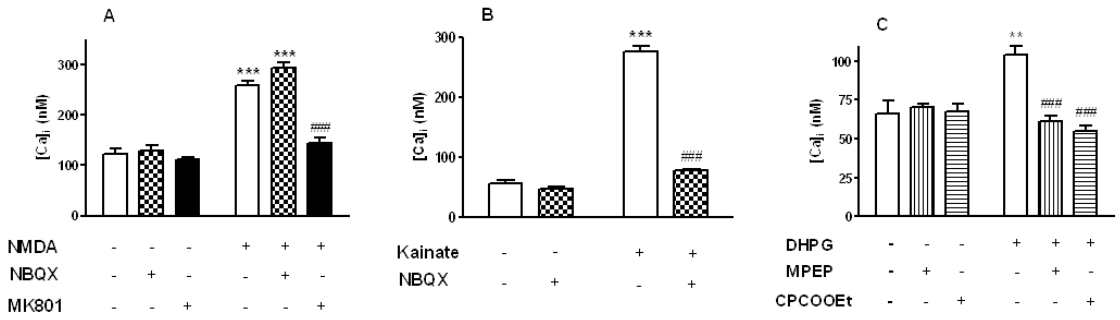
Vale, C., Fonfría, E., Bujons, J., Messeguer, A., Rodríguez-Farré, E., and Suñol, C. (2003). The organochlorine pesticides gamma-hexachlorocyclohexane (lindane), alpha-endosulfan and dieldrin differentially interact with GABA(A) and glycine-gated chloride channels in primary cultures of cerebellar granule cells. *Neuroscience* **117,** 397–403.

Wozniak, A. L., Bulayeva, N. N., and Watson, C. S. (2005). Xenoestrogens at picomolar to nanomolar concentrations trigger membrane estrogen receptor-alpha-mediated Ca^{2+} fluxes and prolactin release in GH3/B6 pituitary tumor cells. *Environ. Health Perspect.* **113,** 431–439.

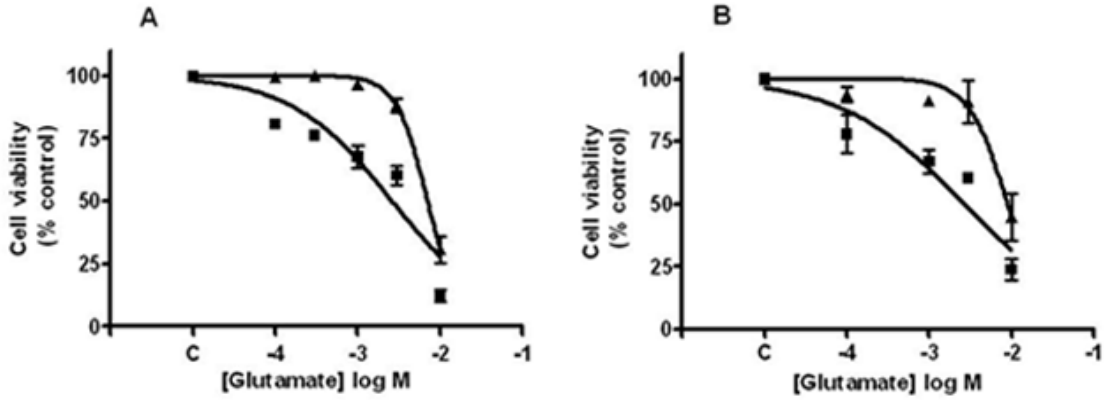
Yang, L., Mao, L., Tang, Q., Samdani, S., Liu, Z., and Wang, J. Q. (2004). A novel Ca^{2+}-independent signaling pathway to extracellular signal-regulated protein kinase by coactivation of NMDA receptors and metabotropic glutamate receptor 5 in neurons. *J. Neurosci.* **24,** 10846–10857.

Yeh, T. H., and Wang, H. L. (2005). Global ischemia downregulates the function of metabotropic glutamate receptor subtype 5 in hippocampal CA1 pyramidal neurons. *Mol. Cell. Neurosci.* **29,** 484–492.

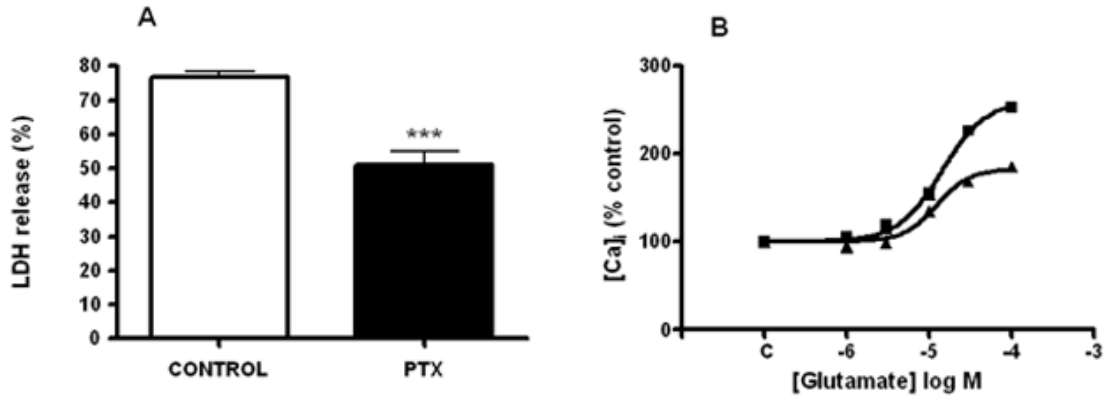
SUPPLEMENTARY DATA



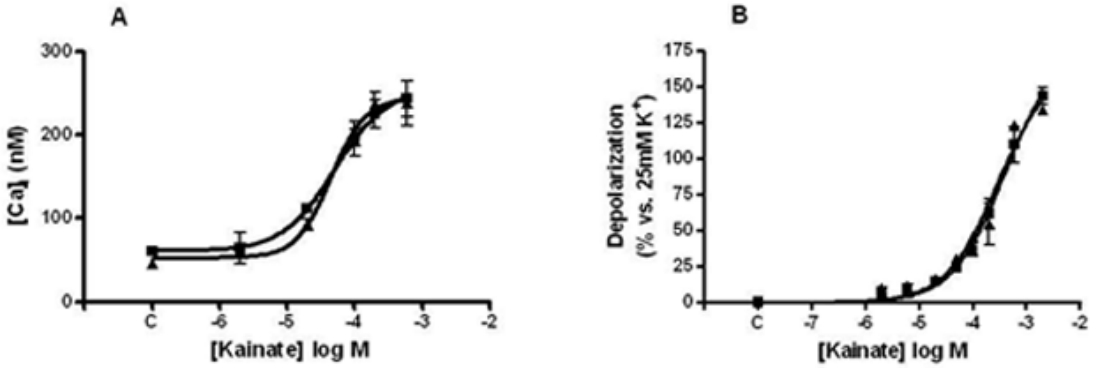
Supplementary Fig. 1. Effect of glutamate receptor antagonists on the increase in $[Ca^{2+}]_i$ induced by 100µM NMDA (A), 200µM kainate (B) and 100µM DHPG (C) in cultured cortical neurons. Fluo-3 fluorescence was measured immediately after agonist/antagonist treatment and values obtained are expressed as $[Ca^{2+}]_i$ (mean ± SE, N = 3). Antagonist concentrations were 10µM for MK-801 and NBQX; and 30µM for MPEP and CPCOOEt. ** $p < 0.01$, *** $p < 0.001$ vs. absence of agonist; ### $p < 0.001$ vs. agonist alone.



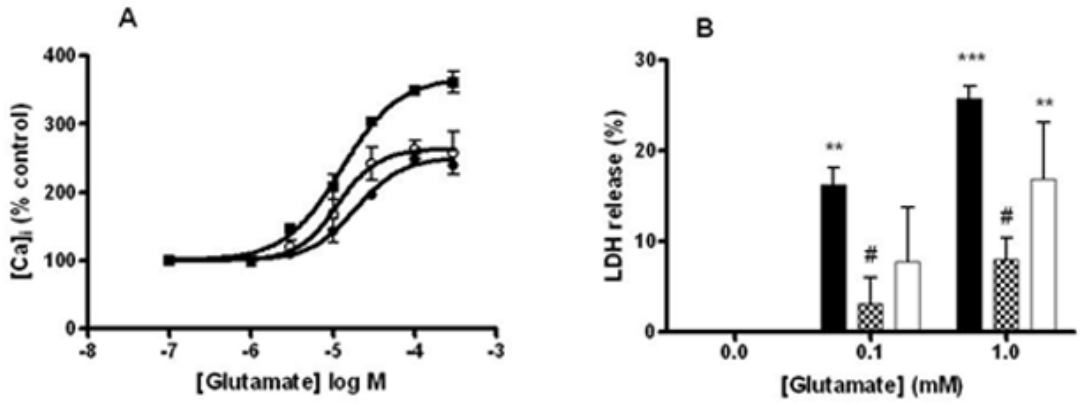
Supplementary Fig. 2. Effect of long-term exposure to dieldrin on the glutamate-induced reduction of cell viability in cultured cortical neurons. MTT values were obtained 24 h after transient glutamate treatment for 1 h, from neurons exposed to DMSO (■) or dieldrin (▲) for: 48 h (A); or 6 DIV (B). Results are expressed as % with respect to cells incubated with Hank's solution (mean ± SE, N = 3-4). Non-linear fit regression shows statistical differences between the EC50 values of the curves: A) 2.8 ± 0.8mM and 7.2 ± 1.2mM for control and dieldrin-treated cells respectively ($p < 0.05$). B) 2.6 ± 0.6mM and 8.3 ± 1.7mM for control and dieldrin-treated cells respectively ($p < 0.05$).



Supplementary Fig. 3. Effect of long-term exposure to 100µM picrotoxinin (PTX) on glutamate-induced cytotoxicity (A), and $[Ca^{2+}]_i$ increase (B), in cultured cortical neurons. A) LDH release values were obtained 24 h after transient treatment with 10mM glutamate for 1 h, from neurons exposed to DMSO (black bars) or PTX (white bars) for 6 DIV. Results are expressed as % of LDH release corresponding to the total cell death (mean ± SE, N = 4). ***$p < 0.001$ vs. control. B) Fluo-3 fluorescence was measured immediately after glutamate treatment, for neurons exposed to DMSO (■) or PTX (▲) for 6 DIV. Values are expressed as % with respect to cells incubated with Hank's solution (mean ± SE, N = 2). Non-linear regression fit shows statistical differences between the E_{max} values of the curves (262 ± 10% and 178 ± 11% for control and PTX-treated cells respectively, $p < 0.05$)



Supplementary Fig. 4. Effect of long-term exposure to dieldrin on Kainate-induced $[Ca^{2+}]_i$ increase (A), and cell membrane depolarization (B), in cultured cortical neurons. A) Fluo-3 fluorescence was measured immediately after kainate treatment, for neurons exposed to DMSO (■) or dieldrin (▲) for 48 h. Values are expressed as $[Ca^{2+}]_i$ and are mean ± SE of 5 independent experiments. B) FMP fluorescence was measured 5 min after kainate treatment, for neurons exposed to DMSO (■) or dieldrin (▲) for 48 h. Values obtained are expressed as % with respect to 25mM KCl and are mean ± SE of 2 independent experiments.



Supplementary Fig. 5. Effect of group-I metabotropic glutamate receptor antagonists on the glutamate-induced increase in $[Ca^{2+}]_i$ (A), and cytotoxicity (B), in cultured cortical neurons. A) Fluo-3 fluorescence was measured immediately after glutamate treatment and values obtained are expressed as % with respect to cells incubated with Hank's solution (mean ± SE, N = 3). The treatment with glutamate was performed in the absence of antagonists (■) or in the presence of 30μM MPEP (○) or 30μM CPCOOEt (●). Non-linear regression fit shows statistical differences between the E_{max} values with respect to control (Two-tail *t*-test, $p < 0.01$ for MPEP; $p < 0.05$ for CPCOOEt). B) LDH assay was performed 24 h after glutamate treatment, on neurons treated with 1mM glutamate alone (black bars) or in combination with 30μM MPEP (squared bars) or 30μM CPCOOEt (white bars). Values are expressed as % with respect to those corresponding to total cell death (mean ± SE, N = 3). $^{**}p < 0.01$, $^{***}p < 0.001$ for glutamate vs. control; MPEP significantly reverses the effect of glutamate ($^{\#}p < 0.001$).

Supplementary methods

Cell membrane potential

The Fluorescent Membrane Potential Assay kit-BLUE (FMP®) (Molecular Devices Corporation, Sunnyvale, CA, USA) was used to detect changes in the voltage across cell membranes. Cultured cortical neurons grown in 96-well plates were rinsed with pre-warmed Hanks solution and incubated with Hanks solution for 20 min at 37ºC in darkness. After this, cells were loaded with FMP and incubated for 30 min at 37ºC. After measuring basal fluorescence, Hanks solution containing different concentrations of kainate (5x) was added and fluorescence read 5 min later. Fluorescence was determined in a bottom-reader fluorimeter plate reader (Cytofluor 2350, Millipore) at an Ex/Em of 530/590 nm. 30mM KCl was used as a positive control of cell membrane depolarization and to normalize fluorescence values.

TRABAJO Nº 2

Differential estrogenic effects of the persistent organochlorine pesticides dieldrin, endosulfan and lindane in primary neuronal cultures

Víctor Briz[1,4], José-Manuel Molina-Molina[2,4], Sara Sánchez-Redondo[1,4], Marieta F. Fernández[2,4], Joan O. Grimalt[3], Nicolás Olea [2,4], Eduard Rodríguez-Farré[1,4], Cristina Suñol[1,4]

[1]Instituto de Investigaciones Biomédicas de Barcelona, CSIC-IDIBAPS, Barcelona. [2]Laboratorio de Investigaciones Médicas, Hospital Universitario San Cecilio, Universidad de Granada. [3]Departmento de Química Ambiental, Instituto de Diagnóstico Ambiental y Estudios del Agua (IDÆA-CSIC), Barcelona. [4]CIBER Epidemiología y Salud Pública (CIBERESP), Barcelona.

Toxicological Sciences 120(2): 413-427 (2011)

En este trabajo, hemos estudiado los efectos de dieldrín, endosulfán y lindano sobre el ER en cultivos primarios de neuronas corticales (NC) y de células granulares de cerebelo (CGC). Todos ellos inhiben la unión de [^{3}H]-estradiol en ambos tipos neuronales, siendo el dieldrín en CGC aquel con mayor afinidad sobre el ER. También se determinaron los efectos de los plaguicidas sobre la fosforilación de las proteínas Akt y ERK1/2. Dieldrín y endosulfán causaron un aumento de la fosforilación de Akt en NC, que fue inhibido por el antagonista de ERβ, PHTPP. En cambio, la fosforilación de Akt y ERK1/2 inducida por dieldrín en CGC fue mediada por la activación múltiple de ERα, ERβ, y GPR30. Por el contrario, el lindano no tuvo ningún efecto la activación de Akt o ERK1/2, pero inhibió el efecto del estradiol sobre estas kinasas. En NC, todos los pesticidas activaron ERK1/2 a través de un mecanismo que implica los receptores $GABA_A$ y de glutamato. La exposición a largo plazo a estos pesticidas redujo los niveles de ERα pero no de ERβ. Por otra parte, extractos de NC previamente expuestas a endosulfán, dieldrín o lindano indujeron la proliferación celular de las células MCF-7 derivadas de cáncer de mama humano, mientras que solamente extractos de CGC tratadas con dieldrín causaron la proliferación de estas células. El presente estudio muestra que los pesticidas organoclorados interaccionan con los receptores de estrógenos neuronales y son, además, capaces de mimetizar o inhibir algunas de las acciones no genómicas del estradiol y de disminuir los niveles de ERα, lo que puede interferir con la señalización fisiológica de esta hormona en el cerebro.

TOXICOLOGICAL SCIENCES **120(2)**, 413–427 (2011)
doi:10.1093/toxsci/kfr019
Advance Access publication January 27, 2011

Differential Estrogenic Effects of the Persistent Organochlorine Pesticides Dieldrin, Endosulfan, and Lindane in Primary Neuronal Cultures

Víctor Briz,*,‡ José-Manuel Molina-Molina,†,‡ Sara Sánchez-Redondo,*,‡ Mariana F. Fernández,†,‡ Joan O. Grimalt §, Nicolás Olea,†,‡ Eduard Rodríguez-Farré,*,‡ and Cristina Suñol*,‡,[1]

*Department of Neurochemistry and Neuropharmacology, Institut d'Investigacions Biomèdiques de Barcelona, Consejo Superior de Investigaciones Científicas (CSIC), Institut d'investigacions Biomèdiques August Pi i Sunyer (IDIBAPS), E-08036 Barcelona, Spain; †Laboratory of Medical Investigations, San Cecilio University Hospital, University of Granada, Granada, E-18071, Spain; ‡Biomedical Research Centre Network for Epidemiology and Public Health (CIBERESP), Spain; and §Department of Environmental Chemistry, Institute of Environmental Assessment and Water Research (IDÆA) CSIC, E-08034 Barcelona, Spain

[1]To whom correspondence should be addressed at Department of Neurochemistry and Neuropharmacology, Institut d'Investigacions Biomèdiques de Barcelona, CSIC-IDIBAPS, Rosselló 161, E-08036 Barcelona, Spain. Fax: +34-93-363-83-01. E-mail: csenqi@iibb.csic.es.

Received September 27, 2010; accepted January 19, 2011

The organochlorine chemicals endosulfan, dieldrin, and γ-hexachlorocyclohexane (lindane) are persistent pesticides to which people are exposed mainly via diet. Their antagonism of the γ-aminobutyric acid-A ($GABA_A$) receptor makes them convulsants. They are also endocrine disruptors because of their interaction with the estrogen receptor (ER). Here, we study the effects of dieldrin, endosulfan, and lindane on ERs in primary cultures of cortical neurons (CN) and cerebellar granule cells (CGC). All the compounds tested inhibited the binding of [^{3}H]-estradiol to the ER in both CN and CGC, with dieldrin in CGC showing the highest affinity. We also determined the effects of the pesticides on protein kinase B (Akt) and extracellular-regulated kinase 1 and 2 (ERK1/2) phosphorylation. Dieldrin and endosulfan increased Akt phosphorylation in CN, which was inhibited by the ERβ antagonist 4-[2-phenyl-5,7-*bis*(trifluoromethyl)pyrazolo[1,5-*a*]pyrimidin-3-yl]phenol. Instead, Akt and ERK1/2 phosphorylation induced by dieldrin in CGC was mediated by multiple activation of ERα, ERβ, and G protein–coupled receptor 30. Lindane did not activate these pathways, but it inhibited estradiol-mediated Akt and ERK1/2 activation. In CN, all the chemicals activated ERK1/2 through a mechanism involving $GABA_A$ and glutamate receptors. Long-term exposure to these pesticides reduced the levels of ERα, but not of ERβ. Moreover, extracts of CN treated with endosulfan, dieldrin, or lindane induced cell proliferation in MCF-7 human breast cancer–derived cells, whereas only extracts of CGC treated with dieldrin induced MCF-7 cell proliferation. Overall, the observed alterations on ER-mediated signaling and ER levels in neurons might contribute to the neurotoxicity of these organochlorine pesticides.

Key Words: **pesticides; estrogen receptor; protein kinase B (Akt); extracellular-regulated kinase; $GABA_A$ receptor; neuronal cultures.**

Dieldrin, endosulfan, and hexachlorocyclohexane (HCH) are organochlorine chemicals that have been used in agriculture and pharmaceutical applications for many years. They all persist in the environment and bioaccumulate in animals and plants, leading to instances of food contamination (Gonzalez *et al.*, 2003; Hites *et al.*, 2004) and eventually dietary exposure in humans (Mariscal-Arcas *et al.*, 2010). Because of their persistence and toxicity, dieldrin and γ-HCH (lindane) are listed in the Stockholm Convention on Persistent Organic Pollutants (http://www.pops.int), whereas the status of endosulfan is currently under review. Despite the uses and production of many organochlorine pesticides (OCPs) are banned in most developed countries, the general population is still exposed to them (Bouvier *et al.*, 2006). Furthermore, dieldrin and lindane have been detected in postmortem human brain from Parkinson disease patients at higher levels than in control ones (Corrigan *et al.*, 2000; Fleming *et al.*, 1994).

Estrogens represent an important class of hormones that can promote the development, maturation, and function of the central nervous system (CNS). The wide distribution of estrogen receptors (ERs) in brain tissue supports the view that estrogens are important to the functioning of the CNS. For instance, 17β-estradiol (E2) plays a crucial role in synaptogenesis, reproductive behavior, and neuronal survival (Garcia-Segura *et al.*, 2001; Micevych and Dominguez, 2009), and different roles of ERα and ERβ in mediating these effects have been reported (Morissette *et al.*, 2008; Zhao and Brinton, 2007). The effects of E2 have often been said to be mediated via ERs located in the cytoplasm or the nuclear membrane, and thus, the effect of E2 on the regulation of target genes has been extensively studied. However, recent findings indicate that E2 also acts on plasma membrane–bound ERs to initiate intracellular signaling pathways and regulate cellular functions; these are known as nongenomic actions. Mitogen-activated protein kinase (MAPK) and phosphoinositide 3-kinase (PI3K)/protein kinase B (Akt) pathways are believed to be involved in

such ER-mediated neuroendocrine events and neuroprotection (Mendez *et al.*, 2005; Morissette *et al.*, 2008; Ogiue-Ikeda *et al.*, 2008; Zhao and Brinton, 2007). In addition, the membrane G protein–coupled receptor 30 (GPR30) has been described to mediate some of the nongenomic actions of E2 in nonneuronal cells, such as activation of MAPK (Filardo *et al.*, 2000). Although GPR30 is widely distributed in the rodent brain (Hazell *et al.*, 2009), its involvement in the neuroendocrine effects of estrogens is poorly understood.

The estrogenic activity of dieldrin, endosulfan, and lindane has been demonstrated *in vitro* in human breast cancer–derived cells and recombinant cell lines (Lemaire *et al.*, 2006; Maranghi *et al.*, 2007; Soto *et al.*, 1994, 1995). Moreover, the three OCPs have been shown to act as antagonists of the androgen receptor, whereas endosulfan and lindane inhibit aromatase activity (Andersen *et al.*, 2002; Li *et al.*, 2008; Nativelle-Serpentini *et al.*, 2003), the enzyme responsible for E2 synthesis. Prolonged exposure to these pollutants modifies the expression of both ERα and ERβ in MCF-7 human-derived breast cancer cells (Grunfeld and Bonefeld-Jorgensen, 2004). Studies of endocrine disruptors have largely focused on the genomic pathways. However, there is growing concern about the nongenomic responses to these compounds. For example, OCPs have been reported to increase extracellular-regulated kinase 1 and 2 (ERK1/2) and Akt phosphorylation by activating ER in cell lines (Bulayeva and Watson, 2004; Li *et al.*, 2006). Furthermore, some endocrine disruptors (including OCPs) have been described to activate GPR30 in cell lines (Thomas and Dong, 2006). However, whether dieldrin, endosulfan, or lindane binds and activates neuronal ERs remains to be determined.

Several studies have reported that animals chronically exposed to OCPs have learning and behavioral deficits and alterations in locomotor activity (Paul *et al.*, 1994; Schantz and Widholm, 2001; Tilson *et al.*, 1987; Topinka *et al.*, 1984). Also, they can affect the development of several neurotransmitter systems, including aminoacidergic (γ-aminobutyric acid [GABA]ergic and glutamatergic) and monoaminergic (serotonergic) (Briz *et al.*, 2010; Cabaleiro *et al.*, 2008; Liu *et al.*, 1997). Some of the latest effects have been attributed to their inhibitory action at the $GABA_A$ receptor (Pomés *et al.*, 1994; Vale *et al.*, 2003). However, the molecular mechanisms underlying such cognitive impairments are not yet fully understood. Ogiue-Ikeda *et al.* (2008) recently reported that some xenoestrogens are able to modulate synaptic plasticity and spinogenesis in hippocampal neurons. Nevertheless, little attention has been paid on their endocrine-disrupting activity in the CNS (Bulayeva and Watson, 2004; Schantz and Widholm, 2001).

The objective of the present work was to examine the potential activity of dieldrin, endosulfan, and lindane on neuronal ERs in order to evaluate them as possible targets of OCPs in the CNS. Two different neuronal cultures were used: cerebellar granule cells (CGC) and cortical neurons (CN), because the cortex and cerebellum are known to be involved in important brain functions such as memory processes and motor coordination. Furthermore, ERα and ERβ are present in CGC and in the mice cortex and show different patterns of expression during development (Belcher, 1999; Prewitt and Wilson, 2007). We aimed to study the effects of these OCPs on two of the major intracellular signaling pathways associated with ER activation: MAPK and PI3K/Akt, both in the absence and presence of E2. We also wanted to address the involvement of the different ERs in these estrogenic actions of OCPs by using specific antagonists of ERα, ERβ, and GPR30. The effects of prolonged exposure to these pollutants on ERα and ERβ levels were also studied. Finally, to further characterize the short- and long-term estrogenic effects on neurons of the chemicals tested, we used the E-Screen assay, in which MCF-7 human breast cancer–derived cells were incubated with extracts of CGC and CN that had previously been exposed to these pollutants.

MATERIALS AND METHODS

Materials

Pregnant NMRI mice (16th gestational day) and mice pups (seventh postnatal day) were obtained from Charles River, Iffa Credo (Saint Germain-sur-l'Arbreste, France). Plastic multiwell plates were from Nunc (Rockilde, Denmark). Fetal bovine serum (FBS) was obtained from Gibco (Invitrogen, Barcelona, Spain). Dulbecco's modified Eagle's minimum essential medium (DMEM) was from Biochrom (Berlin, Germany), and phenol red–free DMEM was from Thermo Scientific HyClone (Logan, UT). Isofluorane (FORANE) was from Abbot Laboratories (Madrid, Spain). Trypsin, soybean trypsin inhibitor, DNAse, bovine serum albumin (BSA), charcoal, EDTA, dimethyl sulfoxide (DMSO), dieldrin (97.9% of purity), α-endosulfan (99.6% of purity), E2, methyl-piperidino-pyrazole (MPP) dihydrochloride, picrotoxinin (PTX), (+)-MK-801 hydrogen maleate (MK-801), and sulforhodamine-B (SRB) were from Sigma (St Louis, MO). β-HCH (99% of purity) was from LGC (Teddington Middlesex, UK). Lindane (99% of purity) was from the Institute of Industrial Organic Chemistry (Warsaw, Poland). ICI182780 (ICI), (3a*S**,4*R**,9b*R**)-4-(6-bromo-1,3-benzodioxol-5-yl)-3a,4,5,9b-3*H*-cyclopenta [*c*]quinoline (G-15), 4-[2-phenyl-5,7-*bis*(trifluoromethyl)pyrazolo[1,5-*a*]pyrimidin-3-yl]phenol (PHTPP), and 2,3-dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[f]quinoxaline-7-sulfonamide (NBQX) were from Tocris Cookson (Bristol, UK). Dextran T-70 was from Pharmacia-LKB (Uppsala, Sweden). [2,4,6,7-^{3}H]-E2 ([^{3}H]-E2, 88 Ci/mmol) was from Amersham Biosciences (GE Healthcare, Buckinghamshire, UK). Optiphase "Hisafe"2 liquid scintillation cocktail was from Wallac Oy (Turku, Finland).

Neuronal Cultures

Primary cultures of CN and of CGC were prepared from cerebral cortices of 16th gestational day mice fetuses and from cerebellum of 7-day-old mice pups, respectively, as previously described (Babot *et al.*, 2007; Briz *et al.*, 2010). For CN, pregnant animals were anesthetized with isofluorane, killed by cervical dislocation, and the fetuses extracted. Cortices were dissected with forceps and mechanically minced, and cells were then dissociated by mild trypsinization (0.02% [wt/vol]) at 37°C for 10 min followed by trituration in a DNAse solution (0.004% [wt/vol]) containing soybean trypsin inhibitor (0.05% [wt/vol]). The cells were then suspended in DMEM containing 5mM KCl, 31mM glucose, and 0.2mM glutamine supplemented with *p*-aminobenzoate, insulin, penicillin, and 10% fetal calf serum. The cell suspension (1.5×10^6 cells per milliliter) was seeded in 6- or 24-well plates precoated with poly-D-lysine and incubated for at least 8 days in a humidified 5% CO_2/95% air atmosphere at 37°C. A mixture of 5μM 5-fluoro-2′-deoxyuridine and 20μM uridine was

added after 1–2 days *in vitro* (DIV) to prevent glial proliferation. In the case of CGC, the cerebellum was removed after decapitation and cells were dissociated by mild trypsinization (0.025% [wt/vol]) at 37°C for 15 min followed by trituration in a DNAse solution (0.004% [wt/vol]) containing soybean trypsin inhibitor (0.06% [wt/vol]). From this step on, the procedure is the same as for CN, but the DMEM contains 25mM KCl. Animals were handled in compliance with protocols approved by the Generalitat de Catalunya Spain, following the European Union (EU) guidelines.

MCF-7 Cell Line

Cloned MCF-7 human breast cancer cells were grown in DMEM supplemented with 10% FBS in an atmosphere of 5% CO_2/95% air under saturating humidity at 37°C. The cells were subcultivated at weekly intervals using a mixture of 0.05% trypsin and 0.01% EDTA.

Charcoal-Dextran Treatment of Serum to Remove Sex Steroids

Sex steroids were removed from FBS by charcoal-dextran (CD) stripping. Briefly, a suspension of 5% charcoal with 0.5% dextran T-70 was prepared. Aliquots of the CD suspension of a volume similar to the serum aliquot to be processed were centrifuged at 1000 × g for 10 min. Supernatants were aspirated, and the serum aliquots were mixed with the charcoal pellets. This CD-serum mixture was maintained in suspension by rolling (6 cycles per minute) at 37°C for 1 h. The suspension was centrifuged at 2000 × g for 20 min, and the supernatant was then filtered through a 0.22-mm filter (Millipore). CD-treated FBS (CD-FBS) was stored at −20°C until needed. For some experiments, a commercial CD-treated serum was used (Gibco).

Chemical Treatments

Stock solutions for each compound were prepared in DMSO and frozen in aliquots of 100 µl. The final concentration of DMSO in the culture medium was < 0.5%. To avoid cross-contamination between different wells in the same plate, DMSO or OCPs were each always added to separate plates. Cultured neurons were exposed for short periods of time (≤ 5 h) to OCPs alone or in combination with the ER agonist/antagonist in Hank's solution (1.3mM CaCl, 5.4mM KCl, 0.4mM KH_2PO_4, 0.5mM MgCl, 0.4mM $MgSO_4$, 137mM NaCl, 4.2mM $NaHCO_3$, 0.3mM Na_2HPO_4, 8mM 4-[2-hydroxyethyl]-1-pyperazineethanesulfonic acid, and 5.5mM glucose, adjusted to pH 7.4), unless otherwise stated. The concentrations of OCPs used in these experiments were generally chosen ranging from the lowest observed effect concentration (LOECs) up to the half-inhibitory concentration (IC_{50}), both obtained from the [^{3}H]-E2 binding assay (Table 1). In contrast, for prolonged exposure periods, cells were treated after 1–2 DIV by adding the stock pesticide solution or DMSO to the culture medium. The medium was not changed until the experiments were performed, generally at 7–8 DIV. In this case, the concentrations used were those previously reported to have long-term effects in neuronal endpoints in these cultures (Babot *et al.*, 2007; Briz *et al.*, 2010). In order to examine the effects of the cultured neuronal cells exposed to OCPs on MCF-7 cell proliferation, CGC and CN were grown in 6-well plates and the medium was replaced for phenol red–free DMEM containing 5% CD (CD-DMEM) after 1–2 DIV. CGC and CN were treated with the chemicals for different exposure times. At the end of the exposure time (5 or 48 h), the cells were washed three times with 1.5 ml of sterile PBS (137mM NaCl, 2.7mM KCl, 10mM Na_2HPO_4, and 2mM KH_2PO_4), scrapped, and collected in 200 microliters per well of sterile PBS. They were stored at −80°C until used.

[^{3}H]-E2 Binding Assay

[^{3}H]-E2 binding experiments were performed on intact cultured neurons grown for at least 8 DIV in 24-well plates. Cells were washed three times with Hank's solution and incubated with [^{3}H]-E2 in Hank's solution for 5 h at 37°C. Cells were washed three times with 1.5 ml of cold Hank's solution and then incubated with 0.2 ml of ethanol for 30 min at room temperature. Ethanol extracts were collected, and then their radioactivity was measured by liquid scintillation counting (with Optiphase "Hisafe"2 cocktail). Saturation curves were obtained by using different [^{3}H]-E2 concentrations (0.02–4nM), whereas a fixed [^{3}H]-E2 concentration (0.5–1nM) and six to eight concentrations of OCPs were used to obtain the competition curves. Nonspecific binding was determined in the presence of 50µM of unlabeled E2. Specific binding was calculated by subtracting the nonspecific binding. Apparent K_d and B_{max} parameters and IC_{50} of each pesticide were determined by adjusting specific [^{3}H]-E2 binding into a one-site saturation and competition binding curve, respectively.

TABLE 1
IC_{50} and LOEC Values of OCPs on [^{3}H]-E2 Binding in Primary Cultures of CGC and CN

Culture	CGC	CN	CGC	CN
Test compound	IC_{50} (µM)		LOEC (µM)	
Dieldrin	2.2 ± 0.5*,##	34.0 ± 4.4	1**	3**
Endosulfan	21.7 ± 4.2	35.0 ± 8.3	3*	10*
Lindane	24.8 ± 5.4	36.2 ± 2.4	10**	30**

Note. IC_{50} values were calculated from the concentration-dependent inhibition of [^{3}H]-E2 binding and are mean ± SE of three independent experiments. Statistical differences between IC_{50} values were obtained for dieldrin with respect to the other pesticides in CGC (*$p < 0.05$, two-way ANOVA) and for dieldrin comparing CGC and CN (##$p < 0.01$, two-way ANOVA). LOEC values were the lowest concentrations used that resulted in statistically different [^{3}H]-E2 binding with respect to control (*$p < 0.05$, **$p < 0.01$, one-way ANOVA).

Western Blot

Neuronal cultures grown in six-well plates were washed twice with cold Hank's solution, and cells were harvested with 0.2 ml of loading buffer (62.5mM Tris-HCl [pH 6.8] 10% glycerol, 2% SDS, and 50mM dithiothreitol) and briefly sonicated. After boiling for 5 min and centrifugation at 16,100 × g for 5 min, 15–25 µg of protein from each sample were subjected to SDS-polyacrilamide gel electrophoresis using 10–12% polyacrylamide resolving gel at 60 mA for 1.5–2 h. Proteins were transferred into a nitrocellulose membrane and incubated with 5% nonfat dry milk in Tris-buffered saline Tween-20 (TBS-T: 20mM Tris-HCl [pH 7.6] 140mM NaCl, and 0.1% Tween-20). Membranes were incubated overnight at 4°C with the following primary antibodies: rabbit polyclonal anti-Akt, rabbit monoclonal anti-p44/42 MAPK, anti-phospho-Akt (Ser473), anti-phospho-p44/42 MAPK (Thr202/Tyr204) (All 1:2000, Cell Signaling, Danvers, MA), and rabbit polyclonal anti-ERα or anti-ERβ (both 1:500, Santa Cruz Biotechnology, Santa Cruz, CA). All primary antibodies were diluted in TBS-T containing 5% BSA, except anti-ERβ which was diluted in TBS-T containing 5% nonfat dry milk. After the membranes were washed, they were incubated for 1 h with anti-rabbit horseradish peroxidase–conjugated (HRP) secondary antibody (1:4000, Jackson ImmunoResearch, West Grove, PA). On all the membranes, a monoclonal anti-actin (1:10,000, Sigma) or anti-glyceraldehyde 3-phosphate dehydrogenase (1:4000, Assay Designs, Ann Arbor, MI) and a secondary HRP-linked anti-mouse (1:8000, Jackson InmunoResearch) antibodies were used as a control of the amount of protein loaded. The membranes were washed and incubated for 4 min in a chemiluminescent solution (Immun-Star HRP Kit, Bio-Rad, Hercules, CA). Luminescence was quantified with a Versadoc Imagine System (Bio-Rad). Digital images were then quantified by using the Quantity One software (Bio-Rad).

E-Screen Bioassay

MCF-7 cells were used in the test of estrogenicity according to a technique slightly modified from that originally described in Soto *et al.* (1994). Briefly, MCF-7 cells were trypsinized and seeded in 24-well plates at initial concentrations of 1×10^4 cells per well in 10% FBS in DMEM. Cells were allowed to attach for 24 h; then, the seeding medium was removed and replaced

by the experimental medium (10% CD-FBS–supplemented phenol red–free DMEM). Extracts of CGC and CN, treated as described above with the chemicals for different exposure times, were sonicated using a Sonopuls HD 200 sonicator (Bandelin Electronic, Berlin, Germany) three times for 4-s bursts each at 50% power on ice. Extracts were resuspended in 4 ml of experimental medium, vigorously shaken, left at rest for 10 min, then filtered through a 0.22-μm filter, and tested on MCF-7 cells. In each experiment, a dose-response curve (0.1–1000pM) of E2 and a negative control (cell treated only with hormone-free medium) were included. The bioassay was terminated on day 6 (late exponential phase) by removing the media from the wells, fixing the cells, and staining them with SRB. In brief, the cells were treated with cold 10% trichloracetic acid (TCA) and incubated at 4°C for 30 min, washed five times with tap water, and left to dry. TCA-fixed cells were stained for 10 min with 0.4% (wt/vol) SRB dissolved in 1% acetic acid. Wells were rinsed with 1% acetic acid and air-dried. Bound dye was solubilized with 10mM Tris base (pH 10.5) in a shaker for 20 min. Finally, aliquots were transferred to a 96-well plate and read in Titertek Multiscan apparatus (Flow, Irvine, CA) at 492 nm. Linearity of the SRB assay with cell number was verified prior to the cell growth experiments. The proliferative effect was calculated as the ratio between the highest cell yield obtained with 100pM of E2 and the proliferation of hormone-free control cells (MCF-7 cell proliferation [fold-over control]).

Gas Chromatography and Electron Capture Detector of OCPs

Cells were grown in six-well plates and treated with OCPs for 6 DIV as indicated previously. After rinsing three times with PBS, the cells were scrapped in PBS and briefly sonicated. The homogenates were then extracted by gentle agitation with n-hexane (200 μl) for 10 min and stored at −20°C until use.

Cleanup. The extracts were cleaned up by elution through 15-ml chromatographic columns packed with 2 g of ISOLUTE Florisil (Biotage AB, Uppsala, Sweden). The columns were preconditioned with 7 ml of n-hexane. The solvent in the column was removed under a low vacuum (−0.1 bar) to dry by extraction with a vacuum manifold station (J.T. Baker, Deventer, The Netherlands). The eluates were disposed in hazardous waste. The extracts for analysis (200 μl) were loaded onto the column and eluted with 5 ml n-hexane, followed by 5 ml n-hexane-ethyl acetate (4:1, vol/vol). The eluates were collected in 40 ml reservoirs and concentrated under low vacuum (−0.1 bar). These extracts were concentrated to 0.5 ml and transferred to gas chromatography vials where they were further concentrated under a gentle stream of nitrogen (the last drop solution). These mixtures were reconstituted to 200 μl with isooctane. Recoveries were calculated with three spiking concentrations, 5, 50, and 500 ng/ml, respectively. Mean recoveries were 70, 85.6, and 85.5% for lindane, endosulfan, and dieldrin, respectively. These values were considered when calculating OCP concentrations. Unspiked Florisil solid phase extraction columns were also prepared and analyzed following the same cleanup method. These samples were analyzed as blanks. No trace levels of these chemicals were observed.

Instrumental analysis. Instrumental analysis was performed on a 6890N Agilent Gas Chromatograph coupled to a 63Ni Electron Capture Detector (Agilent Technologies, Avondale, PA) with a 7683 Agilent Autosampler. The samples were injected (2 μl) in splitless mode at 280°C into a 30 m × 0.25 mm HP-5 MS UI capillary column containing 5% phenyl methyl siloxane (0.25 μm film thickness). The temperature program was from 90°C (held for 2 min) to 130°C at 15°C/min and then from 130°C to 290°C (held for 18 min) at 4°C/min. The carrier gas was helium and was kept at 2 ml/min constant flow. Detector temperature was 320°C. Nitrogen was used as makeup gas at 60 ml/min constant flow. Solvent standards were prepared in isooctane in a concentration range between 0.5 and 500 ng/ml. These standards were used to create calibration curves for each compound. Linearity in the concentration range studied was $r^2 > 0.999$. Analyte concentrations in the samples were determined by the external standard method using these curves. Limits of detection (LODs) and quantification (LOQs) were calculated as the minimum amounts of analytes, which produce peaks with signal-to-noise ratios equal to 3 and 10, respectively. LODs were 0.17, 0.13, and 0.24 injected picograms and LOQs 0.93, 0.78, and 1.55 injected picograms for lindane, endosulfan, and dieldrin, respectively.

Data Analysis

Data are shown as mean ± SE. Unless otherwise stated, at least three experiments from independent culture batches were performed, each one in triplicate. For MCF-7 cell proliferation assays, mean cell numbers from each experiment were normalized to the steroid-free control cultures to correct for differences in the initial seeding density. Individual dose-response curves were fitted using the sigmoid dose-response function of a graphics and statistics software package (Graph-Pad Prism, version 4.0, Graph-Pad Software Inc., San Diego, CA). Statistical comparisons were made by one-way ANOVA followed by Dunnett's postcomparison test when comparing more than two groups and two-way ANOVA followed by the Bonferroni posttest when comparing two factors.

RESULTS

OCPs Inhibit [^{3}H]-E2 Binding in Primary Neuronal Cultures

Saturation curves of [^{3}H]-E2 binding were derived from primary cultures of CGC and CN in order to characterize the binding parameters (K_d and B_{max}) of these cells. Values for apparent K_d were 1.1 ± 0.4 and 2.5 ± 0.8nM, respectively, and those for B_{max} were 479 ± 93 fmol/mg protein and 282 ± 38 fmol/mg protein, respectively (N = 3–5). However, these cultures differed in the relative expression of the two ER isoforms. ERα was expressed more in CGC than in CN, whereas the opposite occurred with ERβ (Supplementary fig. 1).

The OCPs dieldrin, endosulfan, and lindane caused a concentration-dependent inhibition of [^{3}H]-E2 binding in both CGC and CN (Fig. 1). Table 1 summarizes the IC_{50} and LOEC values of the three pesticides in each culture. Dieldrin was the inhibitory agent with highest affinity for ER in CGC ($p < 0.05$ vs. endosulfan and lindane), whereas no differences were observed between the three compounds in CN. Moreover, dieldrin showed greater affinity for ER in CGC than in CN ($p < 0.01$). In contrast, β-HCH up to 50μM did not displace [^{3}H]-E2 from binding to ER in CN (Fig. 1C) or CGC (data not shown).

Dieldrin, Endosulfan, and Lindane Differently Activate Akt and ERK1/2 Phosphorylation in Neurons

We aimed to test whether OCPs could activate two of the major intracellular signaling pathways associated with the ER-dependent nongenomic effects of E2: the MAPK and PI3K/Akt pathways. Exposure to dieldrin or endosulfan for 5 h enhanced Akt phosphorylation at concentrations close to their IC_{50} values against the ER in CGC and CN (Figs. 2A and 2B). Likewise, ERK1/2 phosphorylation was increased in CN after exposure to these compounds; however, only dieldrin produced a significant effect on CGC (Figs. 2C and 2D). In contrast, lindane induced ERK1/2 phosphorylation in CN but not in CGC after 5 h of exposure. Nevertheless, a higher

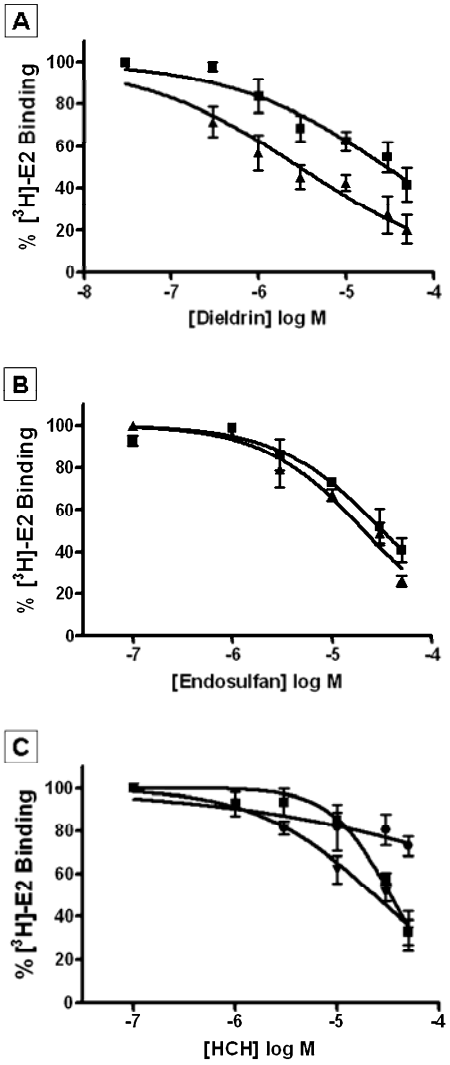


FIG. 1. Concentration-dependent inhibition of [^{3}H]-E2 binding by OCPs in primary neuronal cultures. Competition curves of specific [^{3}H]-E2 binding for dieldrin (A), endosulfan (B), and HCH isomers (C). Different concentrations of OCPs were incubated with a fix [^{3}H]-E2 concentration both in CN (■) and in CGC (▲). (C) β-HCH competition curve (•) was performed in CN.

concentration of lindane than of dieldrin or endosulfan was required to observe a similar effect (Fig. 2).

Next, we studied the effects of OCPs on E2-induced activation of ERK1/2 and Akt. Because E2 induces a rapid activation of both kinases that peaks after 30–60 min (Mannella and Brinton, 2006; Minano *et al.*, 2007), cells were treated with 10nM E2 both in the absence and in the presence of the OCPs for 1 h. E2 increased ERK1/2 phosphorylation in CGC and CN and Akt phosphorylation in CN but not in CGC (Fig. 3). Under the above conditions, dieldrin and endosulfan activated Akt phosphorylation in both cultures both in the presence and absence of E2. Instead, lindane did not modify phospho-Akt levels within 1 h, but it inhibited E2-induced Akt activation in CN (Figs. 3A and 3B). Dieldrin enhanced phospho-ERK1/2 levels to a similar extent as 10nM E2 did; moreover, no additive effects were observed when the two compounds were present. In contrast, endosulfan and lindane did not affect ERK1/2 phosphorylation within 1 h of treatment; however, they inhibited E2-mediated ERK1/2 activation in both cell types (Figs. 3C and 3D).

OCP-Induced Akt and ERK1/2 Phosphorylation Is Inhibited by ER Antagonists

In order to confirm that the effects of the pesticides on Akt and ERK1/2 phosphorylation are mediated through ER activation, we used the ER antagonist ICI182780 and specific antagonists for ERα (MPP), ERβ (PHTPP), and GPR30 (G-15). Cells were previously treated with the respective ER antagonists (all at 1μM) for 30 min and then exposed to OCPs for 5 h in the presence of the ER antagonists. ICI prevented dieldrin-induced Akt phosphorylation in both CGC and CN (Figs. 4A and 4B). Similar results were obtained with endosulfan (Figs. 4C and 4D). Likewise, the increase in phospho-ERK1/2 caused by dieldrin treatment in CGC was significantly reduced by the ER antagonist ICI (Fig. 5A). In contrast, dieldrin-induced ERK1/2 activation in CN was unaffected by ICI (Fig. 5B). The $GABA_A$ receptor antagonist bicuculline has been shown to increase ERK1/2 phosphorylation through an activity-dependent glutamate receptor activation (Chen *et al.*, 2007). Therefore, it is possible that OCPs can also activate the MAPK pathway as a consequence of their blockade of the $GABA_A$ receptor (Pomés *et al.*, 1994). In order to test this hypothesis in our cultures, we used the noncompetitive $GABA_A$ receptor antagonist PTX. Exposure to 100μM PTX increased ERK1/2 phosphorylation in CN after 5 h of exposure (Fig. 5B). In contrast, PTX did not affect phospho-ERK levels in CGC (Fig. 5A), suggesting different mechanisms in OCP-mediated activation of the MAPK pathway between the cultures studied. Moreover, PTX- (Supplementary fig. 2) and dieldrin (Fig. 5D)-induced activation of ERK1/2 was inhibited by a cocktail of glutamate receptor antagonists.

Finally, we used MPP, PHTPP, and G15 (alone and combined) to find out which ER is responsible for the effects of OCPs on Akt and ERK1/2. All the ER antagonists reduced the increase on Akt and ERK phosphorylation induced by dieldrin in CGC, but statistical significance was only reached with G-15. Furthermore, combinations of these antagonists completely abolished dieldrin-induced Akt (Fig. 4A) and ERK1/2 (Fig. 5C) activation. In contrast, the activation of Akt induced by this OCP in CN was only blocked by PHTPP (Fig. 4B). On the other hand, the effects of endosulfan on Akt were selectively inhibited by PHTPP in both CGC and CN

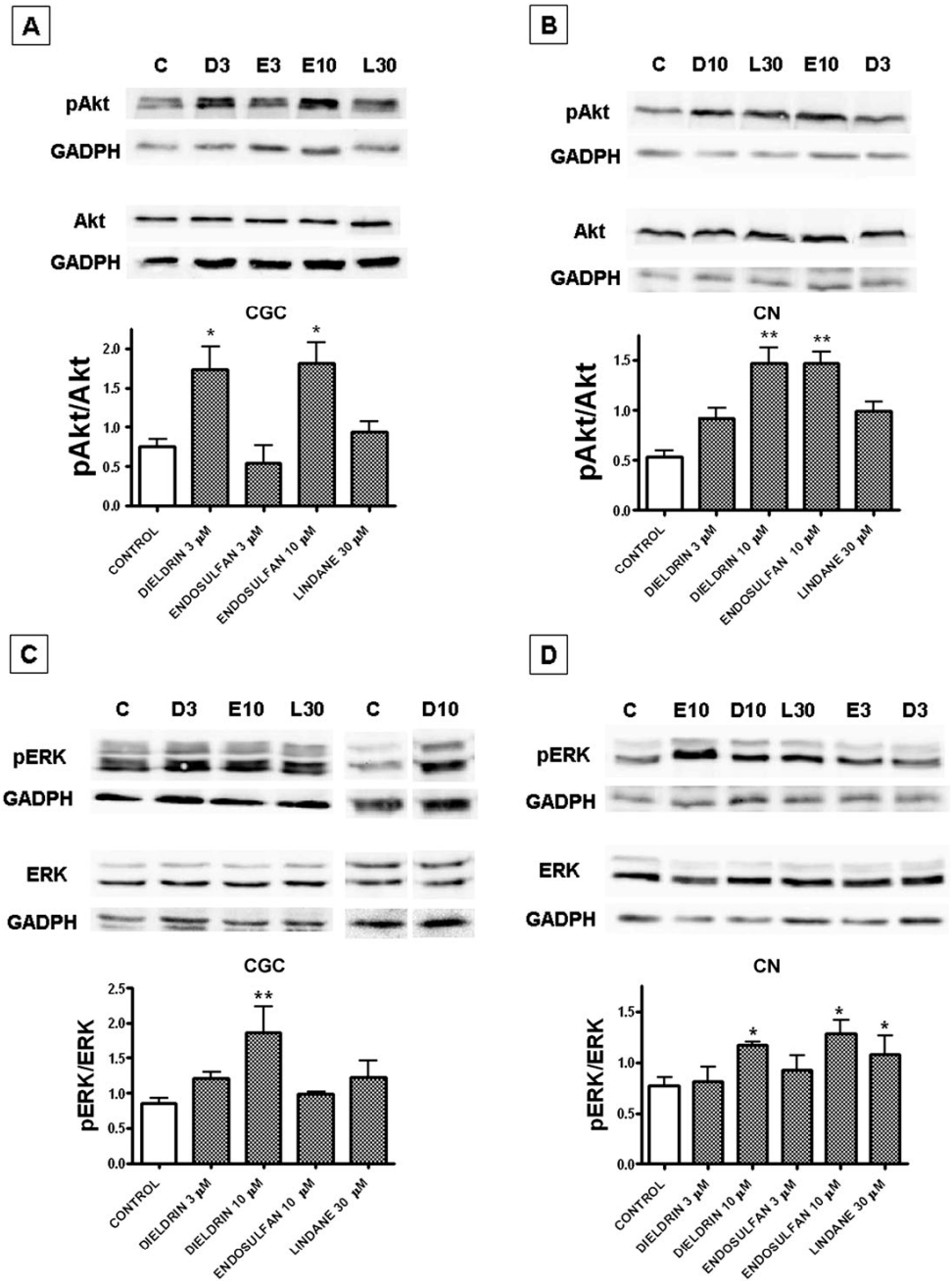


FIG. 2. Effect of OCPs on Akt and ERK1/2 phosphorylation in primary neuronal cultures. CGC and CN were exposed to DMSO (control or C), 3 or 10µM dieldrin (D3 or D10), 3 or 10µM endosulfan (E3 or E10), and 30µM lindane (L30) for 5 h and then the levels of total and phosphorylated Akt, ERK1, and ERK2 were quantified by Western blot. Densitometric quantification of the immunoblots is shown on the bottom, and representative immunoblots for the indicated proteins are shown on the top of each panel. Data are mean ± SE of three to four independent experiments. Statistical comparisons were made by one-way ANOVA: $*p < 0.05$, $**p < 0.01$ versus control.

(Figs. 4C and 4D), indicating a specific action of this OCP on ERβ. Instead, none of these ER antagonists reversed the effects of dieldrin (data not shown), endosulfan, or lindane (Supplementary fig. 3) on ERK1/2 phosphorylation in CN. These results rule out the involvement of the ERs on OCP-induced MAPK activation in CN.

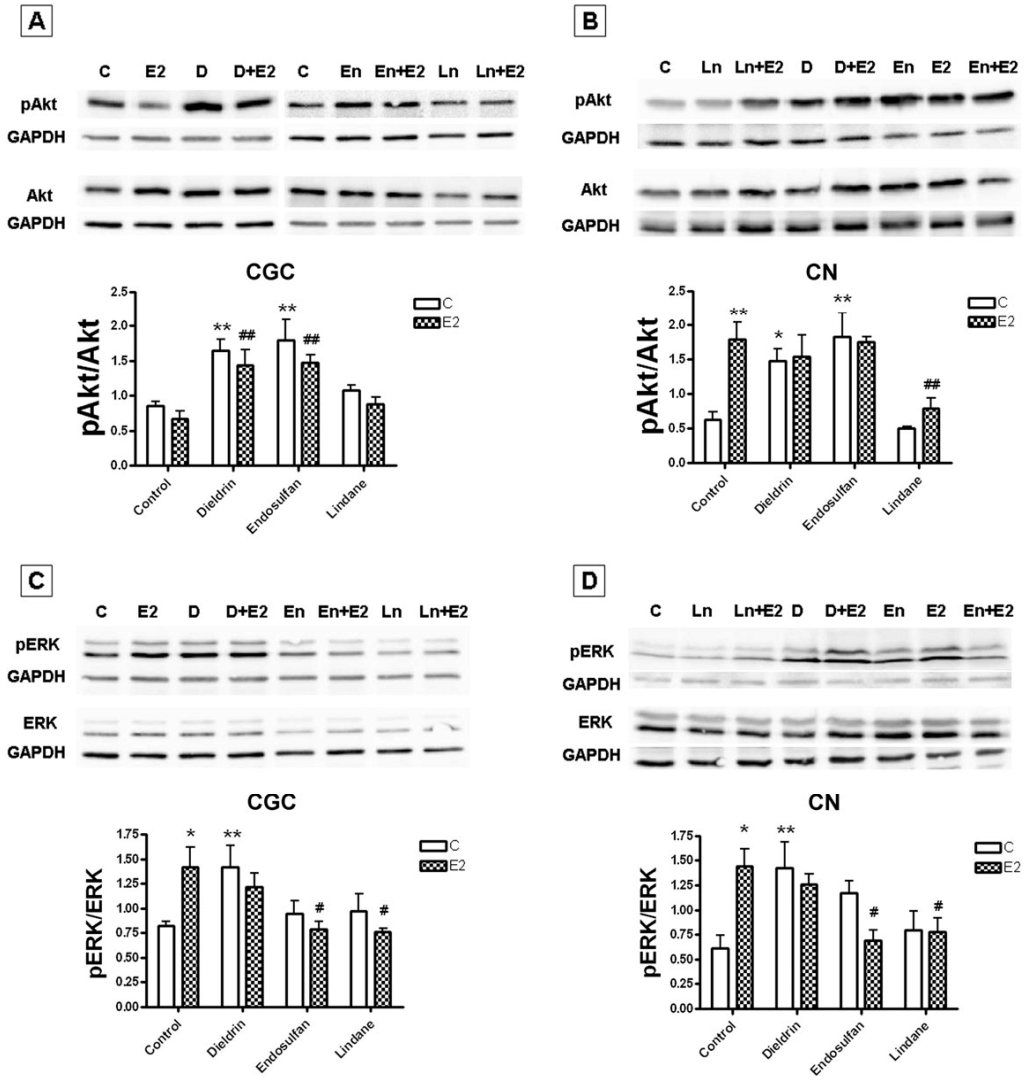


FIG. 3. Effect of OCPs on E2-mediated Akt and ERK1/2 phosphorylation. Cultures were exposed to DMSO (Control or C), 10μM dieldrin (D), 10μM endosulfan (En), or 30μM lindane (Ln) for 1 h both in the absence and presence of 10nM E2. Densitometric quantification of the immunoblots is shown on the bottom, and representative immunoblots for the indicated proteins are shown on the top of each panel. Data are mean ± SE of three independent experiments. Statistical comparisons were made by two-way ANOVA: $*p < 0.05$, $**p < 0.01$ versus control; $\#p < 0.05$, $\#\#p < 0.01$ versus E2-treated cells.

Estrogenic Effects of CGC and CN Extracts in the E-Screen Bioassay

We used the E-Screen bioassay, a method widely used to assess the estrogen-like activity of environmental pollutants (Soto *et al.*, 1994, 1995), to estimate the potential estrogenicity of primary cultures of CGC and CN pre-exposed to OCPs for short and long periods of time (5 and 48 h, respectively). This test is based on the proliferation of the human-derived breast cancer cell line MCF-7 in response to chemicals that activate ERα. Extracts of naive and DMSO-treated neurons similarly increased basal proliferation of MCF-7 cells (data not shown). Therefore, the statistical comparisons for OCPs were made with respect to neuronal cultures treated with DMSO. Extracts of CGC pre-exposed to dieldrin for short and long periods of time significantly enhanced the proliferation of MCF-7 cells (with respect to DMSO-treated neurons) (Figs. 6A–C). Furthermore, pre-exposure of extracts of CN to dieldrin, endosulfan, or lindane for 48 h increased the proliferative effect of DMSO-treated neurons (Fig. 6D). However, this effect was not observed at shorter exposure times (Fig. 6B).

We also studied the direct effects of dieldrin, endosulfan, and lindane on MCF-7 cell proliferation to evaluate the potential endocrine-disrupting activity of these compounds. In

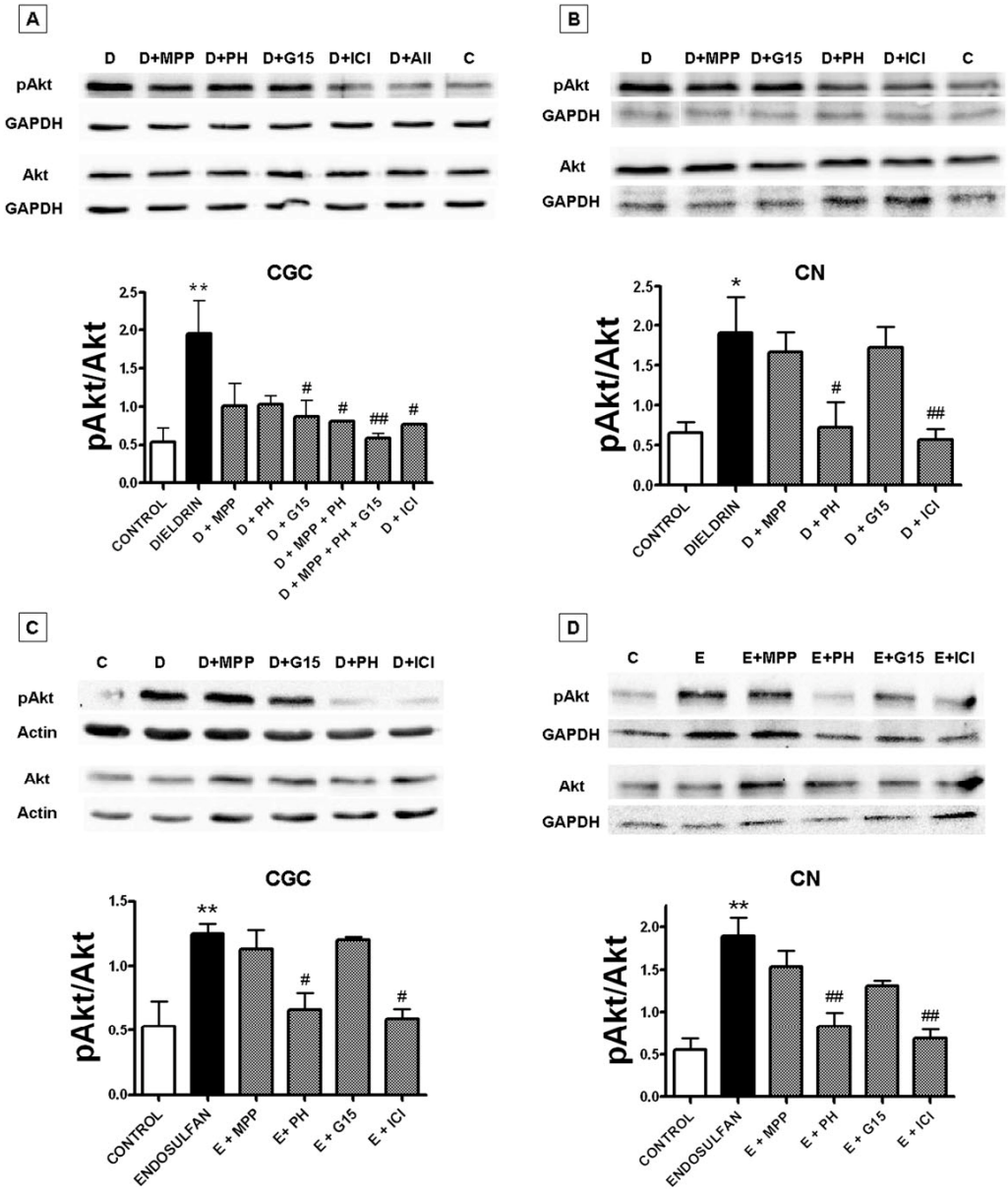


FIG. 4. Effect of ER antagonists on OCP-induced Akt phosphorylation. Cells were exposed to DMSO (Control or C), 10μM dieldrin (D), or 10μM endosulfan (E) for 5 h both in the absence and in the presence of ICI, MPP, PHTPP (PH), and G-15 (all at 1μM) and combinations of them (D + All represent D + MPP + PH + G-15). Densitometric quantification of the immunoblots is shown on the bottom and representative immunoblots for the indicated proteins are shown on the top of each panel. Data are mean ± SE of three to four independent experiments. Statistical comparisons were made by one-way ANOVA: $*p < 0.05$, $**p < 0.01$ versus control; $\#p < 0.05$, $\#\#p < 0.01$ versus OCP-treated cells.

this cell line, 5μM of either dieldrin or endosulfan significantly induced cell proliferation. Conversely, lindane did not have any effect up to 50μM (Supplementary fig. 4). These effects are consistent with our previous observations, but now we show that the LOEC for both dieldrin and endosulfan on the E-screen bioassay is 5μM (Supplementary fig. 4) instead of 10μM (Soto *et al.*, 1994).

Long-Term Exposure to OCPs Reduces ERα but Not ERβ Levels in CGC and CN

Because prolonged treatment with E2 and several xenoestrogens, including endosulfan and dieldrin, differently modulates the expression of ERα and ERβ in epithelial cells (Grunfeld and Bonefeld-Jorgensen, 2004), we addressed the question of whether this effect also occurred in our neuronal

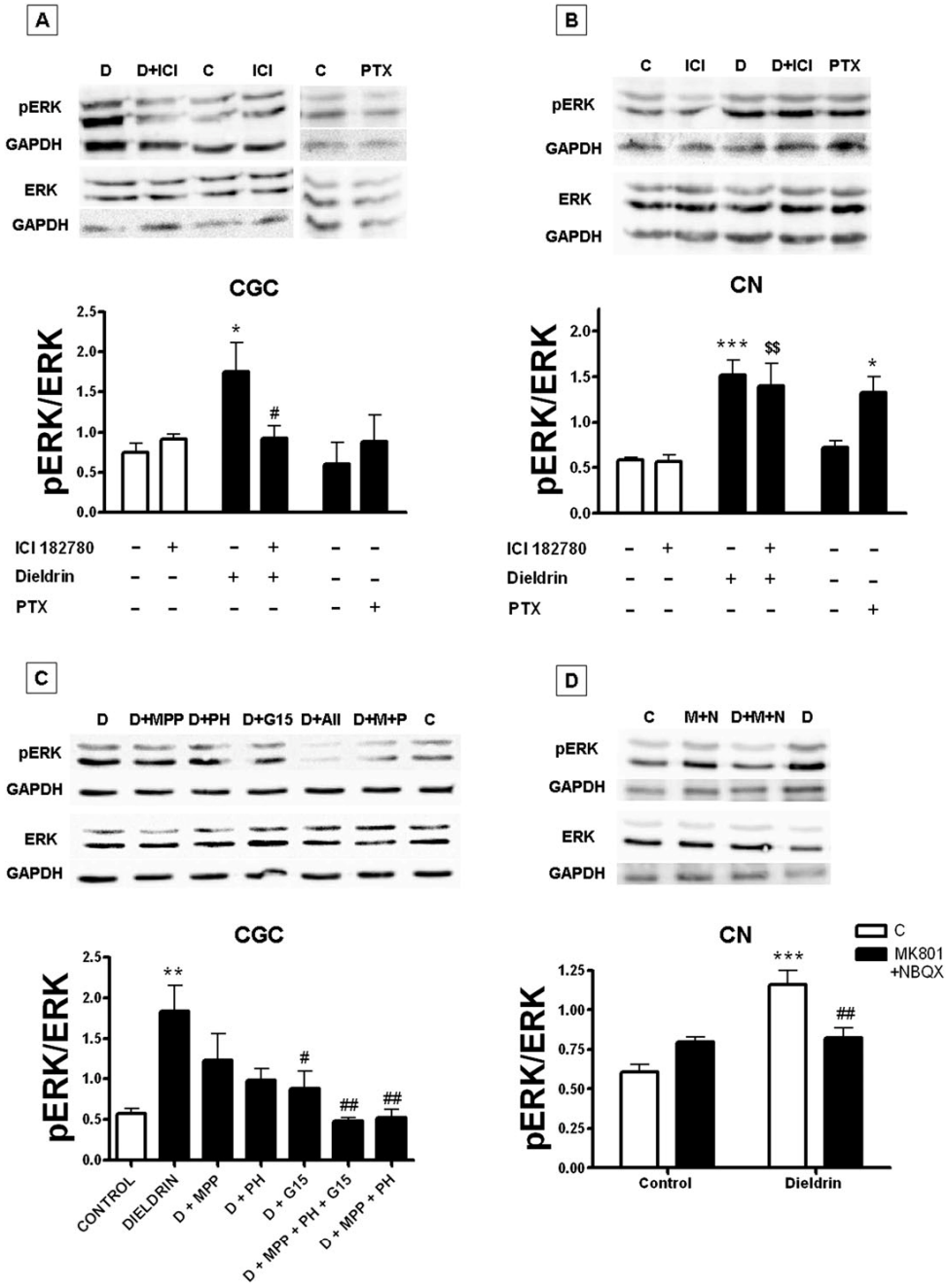


FIG. 5. Effect of ER antagonists on OCP-induced ERK1/2 phosphorylation. Cultures were exposed to DMSO (Control or C) or 10μM dieldrin (D) for 5 h alone or in the presence of the respective ER antagonists. (A and B) Cultures were pretreated with 1μM ICI for 30 min and then exposed to dieldrin. The treatment with PTX was performed at 100μM for 5 h. (C) Cultures were pretreated with MPP, PHTPP (PH), G-15 (all at 1μM), and combinations of them (D + All represent D + MPP + PH + G-15) for 30 min and then exposed to dieldrin. (D) Cells were treated with the glutamate receptor antagonists MK-801 and NBQX (M + N, both at 10μM) together with DMSO or dieldrin. Densitometric quantification of the immunoblots is shown on the bottom, and representative immunoblots for the indicated proteins are shown on the top of each panel. Data are mean ± SE of three independent experiments. Statistical comparisons were made by *t*-test (for PTX), one-way ANOVA (panel C), or two-way ANOVA (panels A, B, and D): $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ versus control; $\#p < 0.05$, $\#\#p < 0.01$ versus dieldrin-treated cells; \$\$ $p < 0.01$ versus ICI-treated cells.

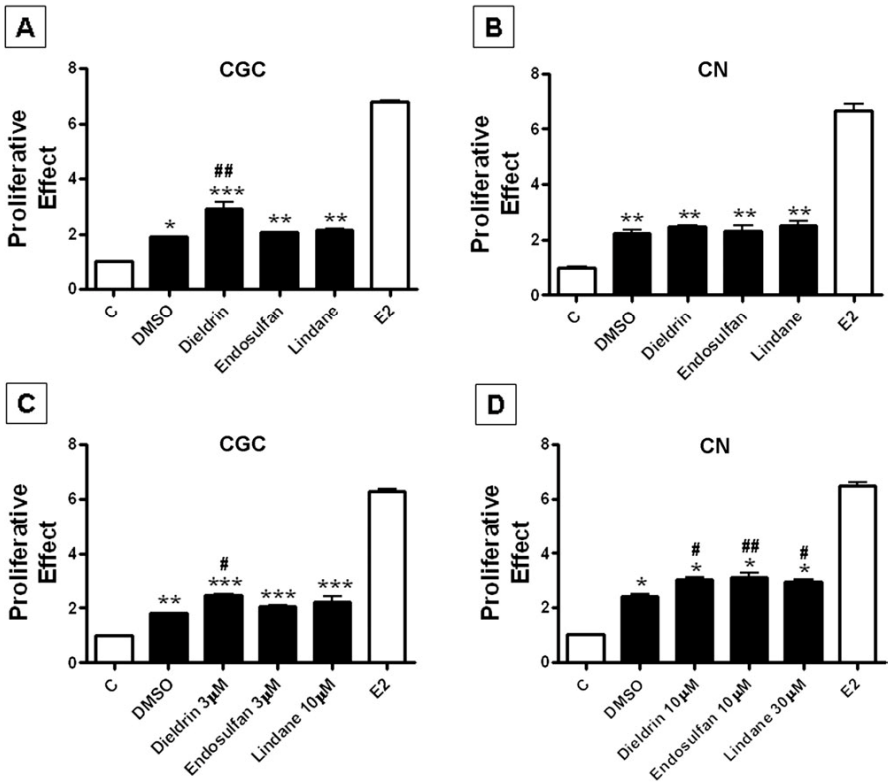


FIG. 6. Proliferative effect of extracts from primary neuronal cultures pre-exposed to OCPs in MCF-7 cells. (A and B) Cultured neurons were exposed to DMSO or OCPs (all at 30μM) for 5 h. (C and D) Neurons were exposed to DMSO or the indicated concentrations of OCPs for 48 h. MCF-7 cells were incubated for 6 DIV in the presence of extracts of CN and of CGC. Results are expressed as proliferative effect (calculated as the ratio between the highest cell yield obtained with the chemical and the proliferation of hormone-free control cells). Data are mean ± SE of three independent experiments, each one performed in triplicates. Statistical comparisons were made by one-way ANOVA: $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ versus control; $\#p < 0.05$, $\#\#p < 0.01$ versus DMSO-treated neurons.

cultures. Long-term exposure to dieldrin for 6 DIV reduced the levels of ERα in both CGC and CN. Nevertheless, this effect was evident at 0.06μM dieldrin in CN, whereas a higher dieldrin concentration (3μM) was required to see a similar reduction in CGC (Figs. 7A and 7B). Accordingly, long-term exposure to 0.2μM dieldrin in CN reduced apparent ER B_{max} from 321 ± 49 fmol/mg protein to 169 ± 29 fmol/mg protein ($p < 0.05, N = 3$) without affecting apparent K_d (3.7 ± 0.7 and 2.9 ± 0.9nM, respectively). Similarly, long-term exposure to endosulfan and lindane reduced ERα protein levels in CGC and CN to a similar extent as 1nM E2 did (Figs. 7A and 7B). None of the OCPs tested significantly modified ERβ protein levels (Figs. 7C and 7D).

We have previously reported that long-term exposure of CN and CGC to dieldrin concentrations like those used here results in decreased functionality of the $GABA_A$ receptor (Babot *et al.*, 2007; Briz *et al.*, 2010). Again, the effects of OCPs on CN were mimicked by the $GABA_A$ receptor antagonist PTX. Long-term exposure to PTX reduced ERα levels (Fig. 7B) without affecting ERβ levels (data not shown). In contrast, exposure to PTX for 6 DIV did not modify ERα protein levels in CGC (Fig. 7A). Finally, we aimed to determine the intracellular incorporation in our cultures after 6 DIV of exposure to OCPs. Table 2 shows the actual intracellular concentration of OCPs in CN. A similar yield of accumulation (around 10%) was found for all the pesticides.

DISCUSSION

The present study shows that dieldrin, endosulfan, and lindane have endocrine-disrupting activity in two different neuronal populations, CGC and CN, through their direct interaction with neuronal ERs. All three OCPs inhibited [^{3}H]-E2 binding to ER in CGC and CN, whereas the β-HCH isomer did not affect it. We found that dieldrin possesses greater affinity to ER than endosulfan and lindane in CGC. In addition, dieldrin was a significantly more potent inhibitor of [^{3}H]-E2 binding in CGC than in CN. The binding affinities for E2 (apparent K_d) and the IC_{50} values for the OCPs are similar to those observed in previous studies using recombinant ERs from different species, including humans (Gale *et al.*, 2004;

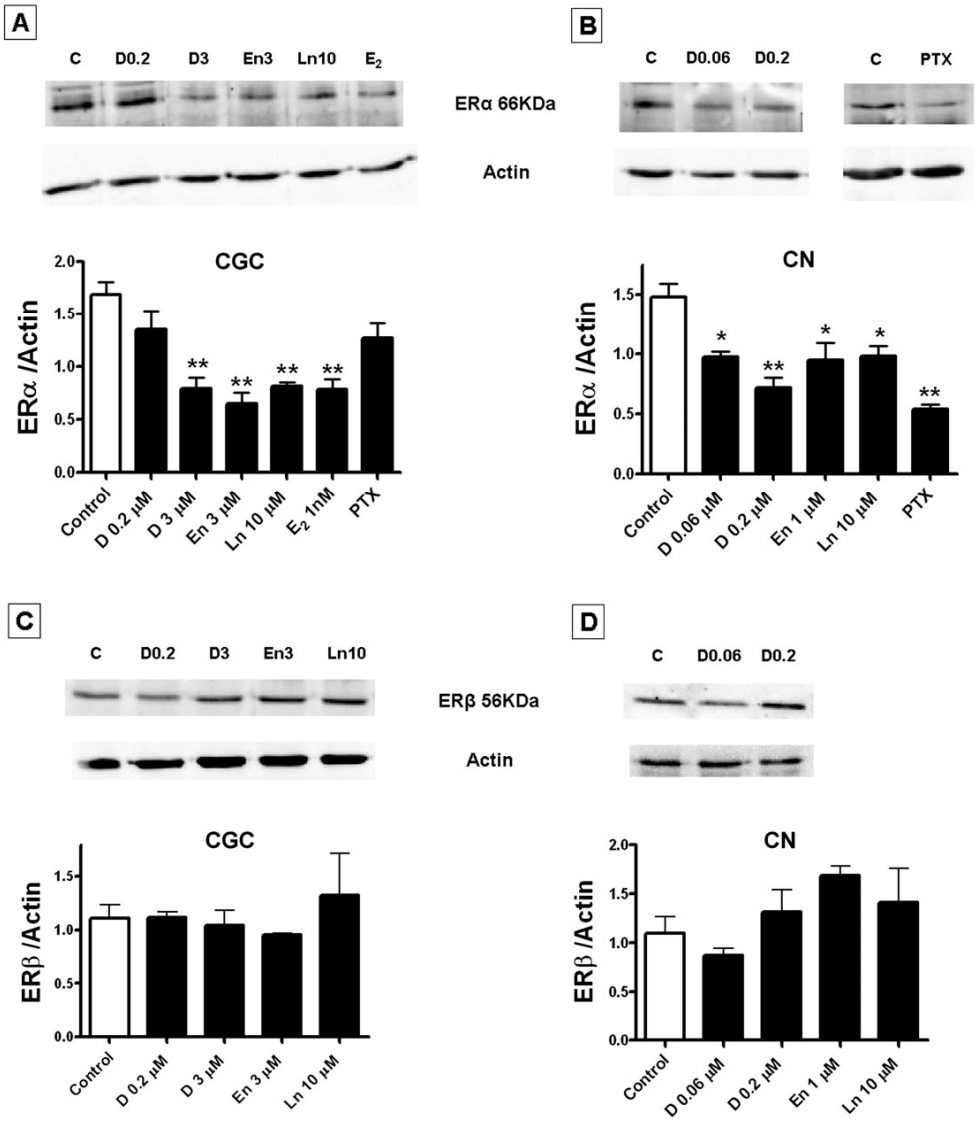


FIG. 7. Effect of long-term exposure to OCPs on ERα (panels A and B) and ERβ (panels C and D) levels in primary neuronal cultures. CGC and CN were exposed to DMSO (Control or C), 100µM PTX, or the indicated concentrations (µM) of dieldrin (D), endosulfan (En), and lindane (Ln) for 6 DIV. The treatment with E2 was performed at 1nM for 48 h. Densitometric quantification of the immunoblots is shown on the bottom, and representative immunoblots for the indicated proteins are shown on the top of each panel. Data are mean ± SE of three to six independent experiments. Statistical comparisons were made by one-way ANOVA: $^{*}p < 0.05$, $^{**}p < 0.01$ versus control.

Scippo *et al.*, 2004). Furthermore, IC_{50} values for dieldrin and endosulfan are of the same order of magnitude as the concentrations of pesticides that elicit positive effects on proliferation and transactivation assays in estrogen-sensitive cell lines (this work, Supplementary fig. 4; Andersen *et al.*, 2002; Lemaire *et al.*, 2006; Soto *et al.*, 1994). Dieldrin has been shown to possess greater affinity for ERα than endosulfan and lindane (Scippo *et al.*, 2004; Sumbayev *et al.*, 2005), and it also has a higher affinity for ERα than for ERβ (Gale *et al.*, 2004). Thus, the different relative expression of the two ER isoforms in the cultures studied (higher for ERα in CGC and for ERβ in CN; Supplementary fig. 1) might explain the observed differences in IC_{50} values (Table 1). In addition, we found that dieldrin is able to activate intracellular signaling pathways in CGC through a multiple action on ERα, ERβ, and GPR30 (Fig. 5). This promiscuity can also explain the highest inhibitory potency of dieldrin in the [^{3}H]-E2 binding assay with respect to endosulfan and lindane. Few studies have examined

TABLE 2
Intracellular Levels of OCPs in CN after 6 DIV of Exposure

OCP	Treatment concentration (μM)	Intracellular concentration (ng/mg protein)	Intracellular accumulation yield (%)
Dieldrin	0.1	35.1 ± 2.7	11.5 ± 0.9
Endosulfan	1	326.1 ± 13.5	10.0 ± 0.4
Lindane	10	2517 ± 174.2	10.8 ± 0.7

Note. Primary cultures of CN were treated for 6 DIV with OCPs. Chemicals from the intracellular extracts were separated by gas chromatography and chemical concentration was determined by using an Electron Capture Detector. Values represent mean ± SD of two independent measures.

the effects of lindane on ER. The results showed here are consistent with recent findings that attribute potential endocrine-disrupting activity to lindane through its action on either ERα or ERβ (Li *et al.*, 2008; Maranghi *et al.*, 2007). In contrast, β-HCH did not displace E2 from its binding to ER in our cultures, as previously reported in MCF-7 cells (Coosen and van Velsen, 1989; Steinmetz *et al.*, 1996). Nevertheless, this pollutant is considered an endocrine disruptor in human breast cancer cells through a nonclassic ER-dependent mechanism (Steinmetz *et al.*, 1996).

Among the nongenomic effects of E2, the activation of MAPK and PI3K/Akt pathways has been shown to be crucial in most of the neuronal functions regulated by estrogens (Mendez *et al.*, 2005; Ogiue-Ikeda *et al.*, 2008). In our experimental conditions, E2 enhanced both Akt and ERK1/2 phosphorylation in CN, whereas ERK1/2 was activated by E2 treatment in CGC, but Akt was not (Fig. 3, Table 2). These effects are in agreement with those previously described in the same cultures (Belcher *et al.*, 2005; Mannella and Brinton, 2006; Minano *et al.*, 2007). E2-mediated ERK1/2 activation in CGC has been reported to involve protein kinase A, Src-kinase, and a GPR (Belcher *et al.*, 2005). Accordingly, dieldrin-induced ERK1/2 activation was inhibited by the GPR30 antagonist G-15. The inability of endosulfan (whose effects appear to be mediated just through ERβ) to activate ERK1/2 in CGC supports the involvement of GPR30 in ERK1/2 activation in these cells. Nevertheless, ERα and ERβ also contribute to these effects because their respective antagonists further reduced dieldrin-induced ERK1/2 activation when combined with G-15. Likewise, the effects of dieldrin on Akt phosphorylation in CGC were only completely suppressed when all ERs were blocked by their respective antagonists. In contrast, dieldrin increases Akt phosphorylation in CN as a result of its interaction with ERβ. Similarly, activation of both ERK1/2 and Akt induced by E2 in CN has been described to be dependent on the interaction of ER with PI3K (Mannella and Brinton, 2006). Thus, the fact that ERβ is more expressed in CN than in CGC may underlie the observed differences regarding Akt activation by dieldrin.

Although ICI182780 is a commonly used ER antagonist, it has been reported to transiently activate ERK1/2 phosphorylation in CGC and other cells through a GPR (Belcher *et al.*, 2005; Filardo *et al.*, 2000). However, in our experimental conditions, this compound did not affect the MAPK pathway. Moreover, it inhibited rather than enhanced the effects of OCPs in Akt and ERK1/2. In contrast, OCP-mediated ERK1/2 activation in CN was not inhibited by ER antagonists. $GABA_A$ receptor antagonists are able to increase ERK1/2 phosphorylation in hippocampal neurons, being this effect inhibited by N-methyl-D-aspartate (NMDA) receptor antagonists (Chen *et al.*, 2007). Although OCPs does not acutely interfere with NMDA receptor activity (Babot *et al.*, 2007; Briz *et al.*, 2010), exposure to OCPs for 5 h could increase the activation of glutamate receptors as a consequence of $GABA_A$ receptor blockade. The results obtained here using the $GABA_A$ receptor antagonist PTX and a cocktail of glutamate receptor antagonists support the involvement of $GABA_A$ and glutamate receptors in OCP-induced ERK1/2 activation in these cells. These observations confirm that the mechanisms underlying the activation of the MAPK pathway by OCPs differ depending on the neuronal cell type.

The effects of endosulfan on Akt and those of dieldrin on Akt and ERK1/2 were unaltered by cotreatment with E2. In addition, the lack of additive effects on Akt phosphorylation when co-exposed to E2 suggests that dieldrin and endosulfan act as agonists at the ER ligand-binding site. Nongenomic effects of several OCPs (including dieldrin and endosulfan) have been linked to ERα activation in a pituitary cell line (Bulayeva and Watson, 2004). In the present work, we found that dieldrin was also able to activate ERβ. Instead, endosulfan had specific ERβ agonism without any activity on ERα, at least regarding Akt phosphorylation. This may be accounted for different expression levels and/or localization of ERα between cell lines and primary neuronal cultures. On the other hand, lindane has been proposed as ERα antagonist and ERβ agonist (Li *et al.*, 2008; Maranghi *et al.*, 2007). In the present work, lindane acts as ER antagonist rather than an agonist because it not only failed to activate Akt and ERK1/2 but also actually inhibited the effects of E2 on these protein kinases. Nevertheless, it would seem controversial to claim that dieldrin and endosulfan activated Akt through an ER-dependent mechanism in CGC, whereas E2 did not. Altogether, these results suggest that the classic view of endocrine disruptors as agonists or antagonists of ER may be too simplistic to describe the estrogenic effects of OCPs, at least in systems in which different ERs are present. In this sense, it has been demonstrated that OCPs induce a unique pattern of conformational changes in both ERα and ERβ, which is a combination of the patterns induced by E2 and the partial ER antagonist 4-hydroxy-tamoxifen (Sumbayev *et al.*, 2005). In turn, this would allow the ligand-activated ER to bind different coregulators or associated proteins, and therefore different effects on the signaling pathways associated can be expected depending on the relative expression of ERα, ERβ, and GPR30

and on the specific affinity for each ER that a given OCP has. For this reason, environmental estrogens could potentially affect brain development and behavior in very different ways (Schantz and Widholm, 2001).

E2 is *de novo* synthesized in the brain through the rate-limiting enzyme aromatase, which is present in cortex and cerebellum at moderate levels (Amateau *et al.*, 2004; Roselli *et al.*, 1984). Although it is believed that Purkinje cells are the main source of E2 in the cerebellum (Sakamoto *et al.*, 2003), active aromatase has been detected in a cerebellar granule progenitor cell line (Gottfried-Blackmore *et al.*, 2007). Therefore, it is not surprising that neuronal extracts from naive and DMSO-treated CN and CGC were positive in the E-Screen bioassay. Interestingly, neuronal cultures previously exposed to OCPs for 48 h showed an additional proliferative effect in MCF-7 cells (Fig. 6). It is unlikely that these effects are because of alterations in the intracellular levels of endogenous estrogens because endosulfan slightly inhibits rather than activates aromatase and dieldrin has no effect on aromatase activity (Andersen *et al.*, 2002). Instead, we observed that long-term exposure to OCPs causes them to accumulate inside the neurons during the time in culture (Table 2), and as a result of their persistence, they probably preserve their estrogen-like activity for days after the treatment. However, it is controversial to claim that neurons exposed to lindane had a significantly higher proliferative effect than nontreated cultures if we consider that this compound was inactive in the E-Screen assay (this work, Supplementary fig. 3; Soto *et al.*, 1995). Nevertheless, it has been reported that lindane enhances aromatase activity at short exposure times (< 6 h) followed by mild inhibition at longer times in cell lines (Nativelle-Serpentini *et al.*, 2003). In addition, aromatase activity has been shown to be dependent on neuronal excitability (Hojo *et al.*, 2004), which can be increased by OCPs. Therefore, we can expect fluctuations in E2 levels in our cultures that could eventually lead to increased estrogenicity after 48 h of lindane exposure. According to that observed in MCF-7 cells (Grunfeld and Bonefeld-Jorgensen, 2004), we found that prolonged exposure to OCPs specifically reduced the expression of ERα without affecting that of ERβ. In our study, these effects seemed to be mediated through their interaction with ER rather than with the $GABA_A$ receptor in CGC because PTX exposure did not modify ERα levels in these cells. In contrast, the effects of OCPs on cortical ERα were observed at concentrations more than 100 times lower than their respective affinities to ER. Moreover, prolonged exposure to PTX in CN significantly reduced ERα but not ERβ, suggesting that the effects of OCPs on ER levels are because of the blockade of $GABA_A$ receptor in CN. Similar changes in ERα expression have been observed in the rat hippocampus after status epilepticus and in the brains of patients with temporal lobe epilepsy (Killer *et al.*, 2009; Tokuhara *et al.*, 2005). It is worth noting that the ER dependence of both ERK1/2 activation and ERα levels differs in a similar way between the cultures studied. A cross talk between ERK1/2 activation and the genomic regulation of several ER targets (including ER) in MCF-7 cells after treatment with E2 or OCPs has been recently suggested (Silva *et al.*, 2010). In addition, the PI3K/Akt pathway has been shown to regulate ERα protein stability (Mendez and Garcia-Segura, 2006). However, further studies are needed to confirm a direct correlation between the activation of these signaling pathways by OCPs and their regulation of ER protein levels.

In summary, the present study shows that OCPs have estrogenic effects in primary cultures of CGC and CN through interaction with neuronal ERs, but more interestingly that these effects persist during the time in culture and may represent an important issue of OCP-induced neurotoxicity. Although the concentrations described here to have estrogenic effects suggest that OCPs are mild xenoestrogens however, we observed that long-term exposure to dieldrin reduced ERα levels at concentrations close to those found in human brain (Corrigan *et al.*, 2000), suggesting that this OCP could potentially act as endocrine disruptor in the CNS. Environmental estrogens have been reported to rapidly modulate synaptic plasticity and affect cognition (Ogiue-Ikeda *et al.*, 2008; Schantz and Widholm, 2001). However, the mechanisms underlying such effects are not completely understood. The PI3K/Akt and MAPK pathways are well known to be involved in synaptic plasticity and synaptogenesis. Therefore, the alterations in the physiological activation of these signaling pathways or on ER levels caused by OCPs and by other endocrine disruptors (Bulayeva and Watson, 2004; Fan *et al.*, 2010) might contribute to their cognitive and behavioral effects (Paul *et al.*, 1994; Schantz and Widholm, 2001; Tilson *et al.*, 1987; Topinka *et al.*, 1984). A better understanding of the molecular mechanism of OCP action in the CNS may be useful to predict and eventually prevent the neurotoxicity of OCPs. Therefore, future investigations should be designed to determine the specific role of the different ERs in the neurological alterations associated with OCP exposure.

SUPPLEMENTARY DATA

Supplementary data are available online at http://toxsci.oxfordjournals.org/.

FUNDING

Ministry of Health(FIS 061212, FIS 10/0453); CIBERESP (AA08-001); Generalitat de Catalunya (2009/SGR/214); Consejería de Innovación, Ciencia y Empresa de la Junta de Andalucía (P09-CTS-5488).

ACKNOWLEDGMENTS

The authors wish to thank Ms Esther Marco from IDÆA-CSIC for her skilful technical assistance.

REFERENCES

Amateau, S. K., Alt, J. J., Stamps, C. L., and McCarthy, M. M. (2004). Brain estradiol content in newborn rats: sex differences, regional heterogeneity, and possible de novo synthesis by the female telencephalon. *Endocrinology* **145,** 2906–2917.

Andersen, H. R., Vinggaard, A. M., Rasmussen, T. H., Gjermandsen, I. M., and Bonefeld-Jorgensen, E. C. (2002). Effects of currently used pesticides in assays for estrogenicity, androgenicity, and aromatase activity in vitro. *Toxicol. Appl. Pharmacol.* **179,** 1–12.

Babot, Z., Vilaró, M. T., and Suñol, C. (2007). Long-term exposure to dieldrin reduces gamma-aminobutyric acid type A and N-methyl-D-aspartate receptor function in primary cultures of mouse cerebellar granule cells. *J. Neurosci. Res.* **85,** 3687–3695.

Belcher, S. M. (1999). Regulated expression of estrogen receptor alpha and beta mRNA in granule cells during development of the rat cerebellum. *Brain Res. Dev. Brain Res.* **115,** 57–69.

Belcher, S. M., Le, H. H., Spurling, L., and Wong, J. K. (2005). Rapid estrogenic regulation of extracellular signal-regulated kinase 1/2 signaling in cerebellar granule cells involves a G protein- and protein kinase A-dependent mechanism and intracellular activation of protein phosphatase 2A. *Endocrinology* **146,** 5397–5406.

Bouvier, G., Blanchard, O., Momas, I., and Seta, N. (2006). Pesticide exposure of non-occupationally exposed subjects compared to some occupational exposure: a French pilot study. *Sci. Total Environ.* **366,** 74–91.

Briz, V., Galofré, M., and Suñol, C. (2010). Reduction of glutamatergic neurotransmission by prolonged exposure to dieldrin involves NMDA receptor internalization and metabotropic glutamate receptor 5 down-regulation. *Toxicol. Sci.* **113,** 138–149.

Bulayeva, N. N., and Watson, C. S. (2004). Xenoestrogen-induced ERK-1 and ERK-2 activation via multiple membrane-initiated signaling pathways. *Environ. Health Perspect.* **112,** 1481–1487.

Cabaleiro, T., Caride, A., Romero, A., and Lafuente, A. (2008). Effects of in utero and lactational exposure to endosulfan in prefrontal cortex of male rats. *Toxicol. Lett.* **176,** 58–67.

Chen, Q., He, S., Hu, X. L., Yu, J., Zhou, Y., Zheng, J., Zhang, S., Zhang, C., Duan, W. H., and Xiong, Z. Q. (2007). Differential roles of NR2A- and NR2B-containing NMDA receptors in activity-dependent brain-derived neurotrophic factor gene regulation and limbic epileptogenesis. *J. Neurosci.* **27,** 542–552.

Coosen, R., and van Velsen, F. L. (1989). Effects of the beta-isomer of hexachlorocyclohexane on estrogen-sensitive human mammary tumor cells. *Toxicol. Appl. Pharmacol.* **101,** 310–318.

Corrigan, F. M., Wienburg, C. L., Shore, R. F., Daniel, S. E., and Mann, D. (2000). Organochlorine insecticides in substantia nigra in Parkinson's disease. *J. Toxicol. Environ. Health A* **59,** 229–234.

Fan, C. Y., Besas, J., and Kodavanti, P. R. (2010). Changes in mitogen-activated protein kinase in cerebellar granule neurons by polybrominated diphenyl ethers and polychlorinated biphenyls. *Toxicol. Appl. Pharmacol.* **245,** 1–8.

Filardo, E. J., Quinn, J. A., Bland, K. I., and Frackelton, A. R., Jr. (2000). Estrogen-induced activation of Erk-1 and Erk-2 requires the G protein-coupled receptor homolog, GPR30, and occurs via trans-activation of the epidermal growth factor receptor through release of HB-EGF. *Mol. Endocrinol.* **14,** 1649–1660.

Fleming, L., Mann, J. B., Bean, J., Briggle, T., and Sanchez-Ramos, J. R. (1994). Parkinson's disease and brain levels of organochlorine pesticides. *Ann. Neurol.* **36,** 100–103.

Gale, W. L., Patino, R., and Maule, A. G. (2004). Interaction of xenobiotics with estrogen receptors alpha and beta and a putative plasma sex hormone-binding globulin from channel catfish (Ictalurus punctatus). *Gen. Comp. Endocrinol.* **136,** 338–345.

Garcia-Segura, L. M., Azcoitia, I., and DonCarlos, L. L. (2001). Neuroprotection by estradiol. *Prog. Neurobiol.* **63,** 29–60.

Gonzalez, M., Miglioranza, K. S., Aizpun De Moreno, J. E., and Moreno, V. J. (2003). Occurrence and distribution of organochlorine pesticides (OCPs) in tomato (Lycopersicon esculentum) crops from organic production. *J. Agric. Food Chem.* **51,** 1353–1359.

Gottfried-Blackmore, A., Croft, G., Clark, J., McEwen, B. S., Jellinck, P. H., and Bulloch, K. (2007). Characterization of a cerebellar granule progenitor cell line, EtC.1, and its responsiveness to 17-beta-estradiol. *Brain Res.* **1186,** 29–40.

Grunfeld, H. T., and Bonefeld-Jorgensen, E. C. (2004). Effect of in vitro estrogenic pesticides on human oestrogen receptor alpha and beta mRNA levels. *Toxicol. Lett.* **151,** 467–480.

Hazell, G. G., Yao, S. T., Roper, J. A., Prossnitz, E. R., O'Carroll, A. M., and Lolait, S. J. (2009). Localisation of GPR30, a novel G protein-coupled oestrogen receptor, suggests multiple functions in rodent brain and peripheral tissues. *J. Endocrinol.* **202,** 223–236.

Hites, R. A., Foran, J. A., Carpenter, D. O., Hamilton, M. C., Knuth, B. A., and Schwager, S. J. (2004). Global assessment of organic contaminants in farmed salmon. *Science* **303,** 226–229.

Hojo, Y., Hattori, T. A., Enami, T., Furukawa, A., Suzuki, K., Ishii, H. T., Mukai, H., Morrison, J. H., Janssen, W. G., Kominami, S., *et al.* (2004). Adult male rat hippocampus synthesizes estradiol from pregnenolone by cytochromes P45017alpha and P450 aromatase localized in neurons. *Proc. Natl. Acad. Sci. U.S.A.* **101,** 865–870.

Killer, N., Hock, M., Gehlhaus, M., Capetian, P., Knoth, R., Pantazis, G., Volk, B., and Meyer, R. P. (2009). Modulation of androgen and estrogen receptor expression by antiepileptic drugs and steroids in hippocampus of patients with temporal lobe epilepsy. *Epilepsia* **50,** 1875–1890.

Lemaire, G., Mnif, W., Mauvais, P., Balaguer, P., and Rahmani, R. (2006). Activation of alpha- and beta-estrogen receptors by persistent pesticides in reporter cell lines. *Life Sci.* **79,** 1160–1169.

Li, J., Li, N., Ma, M., Giesy, J. P., and Wang, Z. (2008). In vitro profiling of the endocrine disrupting potency of organochlorine pesticides. *Toxicol. Lett.* **183,** 65–71.

Li, X., Zhang, S., and Safe, S. (2006). Activation of kinase pathways in MCF-7 cells by 17beta-estradiol and structurally diverse estrogenic compounds. *J. Steroid Biochem. Mol. Biol.* **98,** 122–132.

Liu, J., Morrow, A. L., Devaud, L., Grayson, D. R., and Lauder, J. M. (1997). GABAA receptors mediate trophic effects of GABA on embryonic brainstem monoamine neurons in vitro. *J. Neurosci.* **17,** 2420–2428.

Mannella, P., and Brinton, R. D. (2006). Estrogen receptor protein interaction with phosphatidylinositol 3-kinase leads to activation of phosphorylated Akt and extracellular signal-regulated kinase 1/2 in the same population of cortical neurons: a unified mechanism of estrogen action. *J. Neurosci.* **26,** 9439–9447.

Maranghi, F., Rescia, M., Macri, C., Di Consiglio, E., De Angelis, G., Testai, E., Farini, D., De Felici, M., Lorenzetti, S., and Mantovani, A. (2007). Lindane may modulate the female reproductive development through the interaction with ER-beta: an in vivo-in vitro approach. *Chem. Biol. Interact.* **169,** 1–14.

Mariscal-Arcas, M., Lopez-Martinez, C., Granada, A., Olea, N., Lorenzo-Tovar, M. L., and Olea-Serrano, F. (2010). Organochlorine pesticides in umbilical cord blood serum of women from Southern Spain and adherence to the Mediterranean diet. *Food Chem. Toxicol.* **48,** 1311–1315.

Mendez, P., Azcoitia, I., and Garcia-Segura, L. M. (2005). Interdependence of oestrogen and insulin-like growth factor-I in the brain: potential for analysing neuroprotective mechanisms. *J. Endocrinol.* **185,** 11–17.

Mendez, P., and Garcia-Segura, L. M. (2006). Phosphatidylinositol 3-kinase and glycogen synthase kinase 3 regulate estrogen receptor-mediated transcription in neuronal cells. *Endocrinology* **147,** 3027–3039.

Micevych, P., and Dominguez, R. (2009). Membrane estradiol signaling in the brain. *Front. Neuroendocrinol.* **30,** 315–327.

Minano, A., Cerbon, M. A., Xifro, X., Malagelada, C., Aguilera, J., and Rodriguez-Alvarez, J. (2007). 17Beta-estradiol does not protect cerebellar granule cells from excitotoxicity or apoptosis. *J. Neurochem.* **102,** 354–364.

Morissette, M., Le Saux, M., D'Astous, M., Jourdain, S., Al Sweidi, S., Morin, N., Estrada-Camarena, E., Mendez, P., Garcia-Segura, L. M., and Di Paolo, T. (2008). Contribution of estrogen receptors alpha and beta to the effects of estradiol in the brain. *J. Steroid Biochem. Mol. Biol.* **108,** 327–338.

Nativelle-Serpentini, C., Richard, S., Seralini, G. E., and Sourdaine, P. (2003). Aromatase activity modulation by lindane and bisphenol-A in human placental JEG-3 and transfected kidney E293 cells. *Toxicol. In Vitro* **17,** 413–422.

Ogiue-Ikeda, M., Tanabe, N., Mukai, H., Hojo, Y., Murakami, G., Tsurugizawa, T., Takata, N., Kimoto, T., and Kawato, S. (2008). Rapid modulation of synaptic plasticity by estrogens as well as endocrine disrupters in hippocampal neurons. *Brain Res. Rev.* **57,** 363–375.

Paul, V., Balasubramaniam, E., and Kazi, M. (1994). The neurobehavioural toxicity of endosulfan in rats: a serotonergic involvement in learning impairment. *Eur. J. Pharmacol.* **270,** 1–7.

Pomés, A., Rodriguez-Farré, E., and Suñol, C. (1994). Disruption of GABA-dependent chloride flux by cyclodienes and hexachlorocyclohexanes in primary cultures of cortical neurons. *J. Pharmacol. Exp. Ther.* **271,** 1616–1623.

Prewitt, A. K., and Wilson, M. E. (2007). Changes in estrogen receptor-alpha mRNA in the mouse cortex during development. *Brain Res.* **1134,** 62–69.

Roselli, C. E., Ellinwood, W. E., and Resko, J. A. (1984). Regulation of brain aromatase activity in rats. *Endocrinology* **114,** 192–200.

Sakamoto, H., Mezaki, Y., Shikimi, H., Ukena, K., and Tsutsui, K. (2003). Dendritic growth and spine formation in response to estrogen in the developing Purkinje cell. *Endocrinology* **144,** 4466–4477.

Schantz, S. L., and Widholm, J. J. (2001). Cognitive effects of endocrine-disrupting chemicals in animals. *Environ. Health Perspect.* **109,** 1197–1206.

Scippo, M. L., Argiris, C., Van De, W. C., Muller, M., Willemsen, P., Martial, J., and Maghuin-Rogister, G. (2004). Recombinant human estrogen, androgen and progesterone receptors for detection of potential endocrine disruptors. *Anal. Bioanal. Chem.* **378,** 664–669.

Silva, E., Kabil, A., and Kortenkamp, A. (2010). Cross-talk between non-genomic and genomic signalling pathways—distinct effect profiles of environmental estrogens. *Toxicol. Appl. Pharmacol.* **245,** 160–170.

Soto, A. M., Chung, K. L., and Sonnenschein, C. (1994). The pesticides endosulfan, toxaphene, and dieldrin have estrogenic effects on human estrogen-sensitive cells. *Environ. Health Perspect.* **102,** 380–383.

Soto, A. M., Sonnenschein, C., Chung, K. L., Fernandez, M. F., Olea, N., and Serrano, F. O. (1995). The E-SCREEN assay as a tool to identify estrogens: an update on estrogenic environmental pollutants. *Environ. Health Perspect.* **103**(Suppl. 7), 113–122.

Steinmetz, R., Young, P. C., Caperell-Grant, A., Gize, E. A., Madhukar, B. V., Ben Jonathan, N., and Bigsby, R. M. (1996). Novel estrogenic action of the pesticide residue beta-hexachlorocyclohexane in human breast cancer cells. *Cancer Res.* **56,** 5403–5409.

Sumbayev, V. V., Bonefeld-Jorgensen, E. C., Wind, T., and Andreasen, P. A. (2005). A novel pesticide-induced conformational state of the oestrogen receptor ligand-binding domain, detected by conformation-specific peptide binding. *FEBS Lett.* **579,** 541–548.

Thomas, P., and Dong, J. (2006). Binding and activation of the seven-transmembrane estrogen receptor GPR30 by environmental estrogens: a potential novel mechanism of endocrine disruption. *J. Steroid Biochem. Mol. Biol.* **102,** 175–179.

Tilson, H. A., Shaw, S., and McLamb, R. L. (1987). The effects of lindane, DDT, and chlordecone on avoidance responding and seizure activity. *Toxicol. Appl. Pharmacol.* **88,** 57–65.

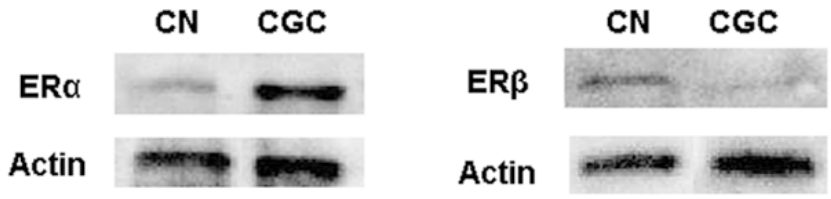
Tokuhara, D., Yokoi, T., Nakajima, R., Hattori, H., Matsuoka, O., and Yamano, T. (2005). Time course changes of estrogen receptor alpha expression in the adult rat hippocampus after kainic acid-induced status epilepticus. *Acta Neuropathol.* **110,** 411–416.

Topinka, M. A., Eisenstein, E. M., Siegel, D., Trofeli, R., and Barraco, D. (1984). The effects of dieldrin and chlordimeform on learning and memory in the cockroach, Periplaneta americana: a study in behavioral toxicology. *J. Toxicol. Environ. Health* **13,** 705–719.

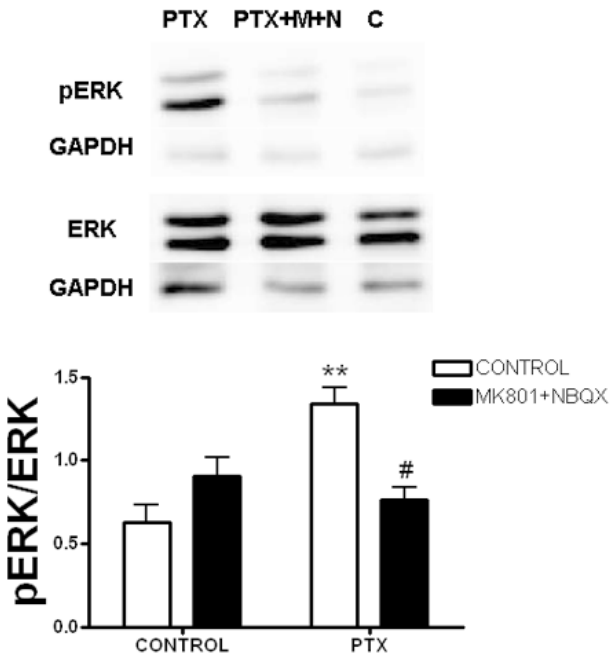
Vale, C., Fonfría, E., Bujons, J., Messeguer, A., Rodríguez-Farré, E., and Suñol, C. (2003). The organochlorine pesticides gamma-hexachlorocyclohexane (lindane), alpha-endosulfan and dieldrin differentially interact with GABA(A) and glycine-gated chloride channels in primary cultures of cerebellar granule cells. *Neuroscience* **117,** 397–403.

Zhao, L., and Brinton, R. D. (2007). Estrogen receptor alpha and beta differentially regulate intracellular Ca(2+) dynamics leading to ERK phosphorylation and estrogen neuroprotection in hippocampal neurons. *Brain Res.* **1172,** 48–59.

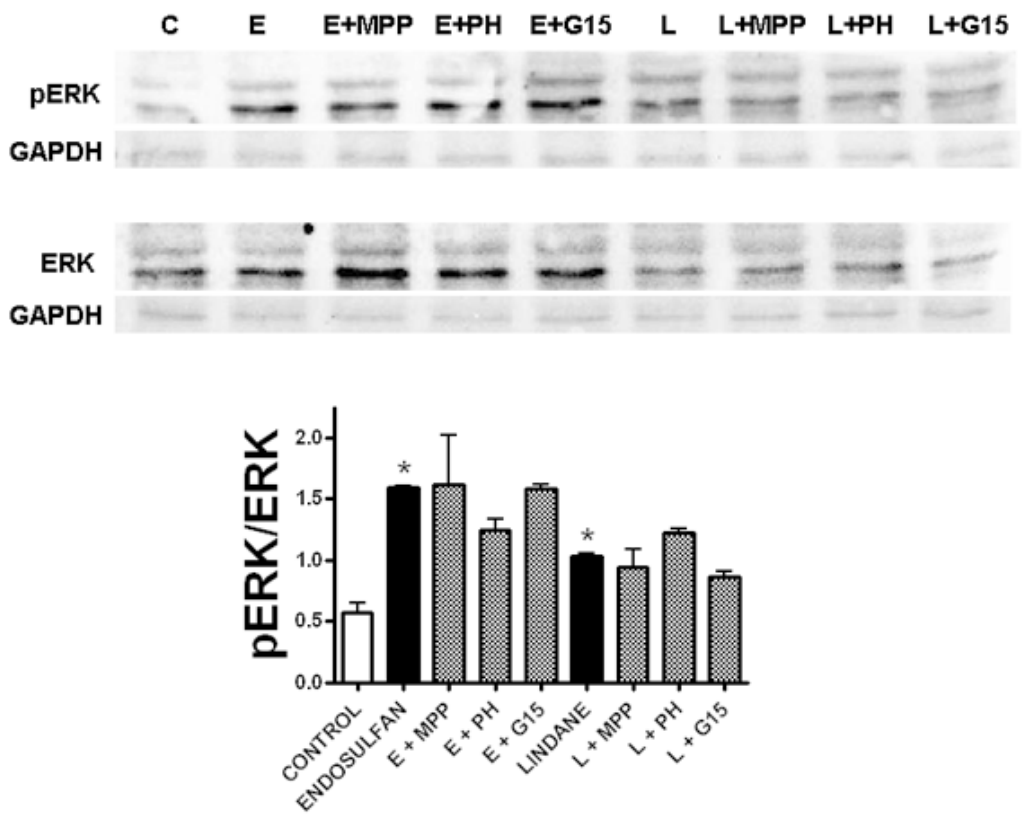
SUPPLEMENTARY DATA



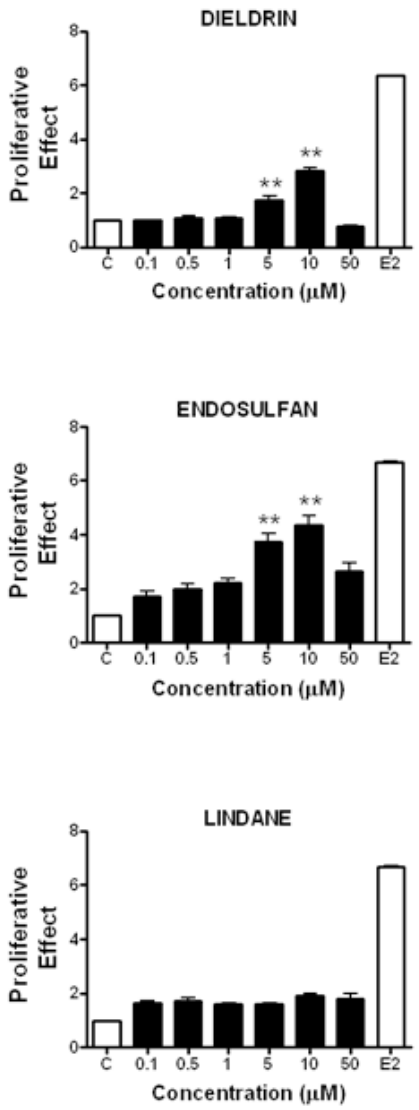
Supplementary Fig. 1. Differential expression of ERα and ERβ in primary cultures of cerebellar granule cells (CGC) and of cortical neurons (CN). After 7-8 DIV, neuronal cultures were subjected to Western Blot for the detection of ERα (66 KDa) and ERβ (56 KDa). Immunoblots for the indicated proteins are representative of 3 independent experiments.



Supplementary Fig. 2. Glutamate receptor antagonists inhibit picrotoxinin (PTX)-induced ERK1/2 phosphorylation in cortical neurons. Cells were treated with water (Control or C) or 100μM PTX for 5 h. both in the absence and presence of the glutamate receptor antagonists MK-801 and NBQX (M+N, both at 10μM). Densitometric quantification of the immunoblots is shown on the bottom and representative immunoblots for the indicated proteins are shown on the top of each panel. Data are mean ± SE of two independent experiments. Statistical comparisons were made by Two-way ANOVA: ** $p < 0.01$ versus control; # $p < 0.05$ PTX-treated cells.



Supplementary Fig. 3. ER antagonists failed to reverse endosulfan- and lindane-induced ERK1/2 activation in CN. Cells were treated with DMSO (Control or C), 10μM endosulfan (E) or 30μM lindane (L) for 5 h. both in the absence and presence of the ER antagonists MPP, PHTPP (PH) and G-15 (all at 1μM). Densitometric quantification of the immunoblots is shown on the bottom and representative immunoblots for the indicated proteins are shown on the top of each panel. Data are mean ± SE of two independent experiments. Statistical comparisons were made by One-way ANOVA: * $p < 0.05$ versus control.



Supplementary Fig. 4. Concentration-dependent proliferative effect of dieldrin, endosulfan and lindane in MCF-7 cells. Cells were incubated for 6 DIV at 37 °C in the presence of OCPs at the indicated concentrations. Results are expressed as proliferative effect (calculated as the ratio between the highest cell yield obtained with the chemical and the proliferation of hormone-free control cells). Data are mean ± SE of 3 independent experiments, each one performed in triplicates. Statistical comparisons were made by One-way ANOVA: ** $p < 0.01$ *vs*. control.

TRABAJO Nº 3

Allopregnanolone prevents dieldrin-induced NMDA receptor internalization and neurotoxicity by preserving $GABA_A$ receptor function

Víctor Briz[1], Jyoti Parkash[2], Sara Sánchez-Redondo[1], Vincent Prevot[2], Cristina Suñol [1]

[1]Departamento de Neuroquímica y Neurofarmacología, Instituto de Investigaciones Biomédicas de Barcelona, CSIC – IDIBAPS, Barcelona. [2] Centre de Recherche Jean-Pierre Aubert, Unité 837, Inserm, University of Lille Nord de France, Lille, France.

En este estudio hemos examinado los efectos del dieldrín sobre los receptores $GABA_A$ y NMDA y sobre la muerte celular en cultivos primarios de neuronas corticales en presencia de los neuroesteroides 17β-estradiol (E2) y alopregnanolona (AP). Hemos observado que el tratamiento con 1 nM E2 aumentó la expresión de los receptores NR1/NR2B y de la proteína PSD95 en la membrana celular, pero no indujo una asociación física entre ellos. Por el contrario, 10 nM E2 no tuvo ningún efecto sobre estas proteínas, pero redujo la expresión NR2A. También encontramos que la exposición a largo plazo a 60 nM de dieldrín causó la internalization de las subunidades NR1 y NR2B, pero no de NR2A. El tratamiento con 1 nM E2 o con AP previno la reducción de los receptores NR1/NR2B en la membrana inducida por el dieldrín. Además, la exposición prolongada a 200 nM dieldrin disminuyó la expresión génica de NR2A, efecto que fue revertido sólo por AP. En neuronas corticales maduras, el dieldrín causó el truncamiento de NR2B mediado por las calpaínas y redujo la interacción de ésta subunidad con SAP102, efecto de nuevo prevenido por AP. A pesar de que ambas hormonas restauraron la funcionalidad del receptor NMDA, AP (pero no E2) revirtió la inhibición del receptor $GABA_A$ y la muerte neuronal causadas por la exposición prolongada a dieldrín. Estos resultados indican que AP protege frente a la neurotoxicidad del dieldrín en neuronas corticales gracias a que es capaz de mantener la funcionalidad de los receptores $GABA_A$ y NMDA.

ABSTRACT

Dieldrin is an endocrine disruptor that accumulates in mammalian adipose tissue and brain. It induces convulsions due to its antagonism of the γ-aminobutyric acid A receptor ($GABA_AR$). We have previously reported that long-term exposure to dieldrin causes the internalization of the N-methyl-D-aspartate receptor (NMDAR) as a result of persistent $GABA_AR$ inhibition. As the neuroesteroids 17β-estradiol (E2) and allopregnanolone (AP) are known to modulate the trafficking of $GABA_AR$ and NMDAR at the cell membrane, we examined the effects of E2 and AP on dieldrin-induced $GABA_AR$ inhibition, NMDAR internalization and neuronal death in cortical neurons. We found that 1 nM E2 increased the membrane expression of NR1/NR2B receptors and postsynaptic density 95 (PSD95), but did not induce their physical association. In contrast, 10 nM E2 had no effect on these proteins but reduced NR2A membrane expression. We also found that long-term exposure to 60 nM dieldrin caused the internalization of the NMDAR subunits NR1 and NR2B, but not NR2A. Treatment with either 1 nM E2 or AP prevented the dieldrin-induced reduction in membrane levels of the NR1/NR2B receptors. Furthermore, prolonged exposure to 200 nM dieldrin down-regulated the expression of NR2A; this was inhibited only by AP. Although both hormones restored NMDAR function, as measured by the NMDA-induced rise in intracellular calcium, AP (but not E2) reversed the inhibition of $GABA_AR$ and neuronal death caused by prolonged exposure to dieldrin. Our results indicate that AP protects cortical neurons against the neurotoxicity caused by long-term exposure to dieldrin by maintaining $GABA_AR$ and NMDAR functionality.

Keywords: neuroprotection; neurosteroids; γ-aminobutyric acid A receptor ($GABA_A$); N-methyl-D-aspartate receptor (NMDAR); toxicology

INTRODUCTION

The neurosteroids 17β-estradiol (E2) and allopregnanolone (AP) are important for the development, maturation and function of the central nervous system (CNS). These hormones are involved in brain processes such as reproductive behavior, memory and neuronal survival. Moreover, these effects have been often related to their modulation of gamma-aminobutyric acid (GABA) and glutamate neurotransmission (1-4). For instance, E2 induces synaptogenesis and synaptic plasticity by increasing N-methyl-D-aspartate receptor (NMDAR) membrane expression (5,6) and regulates GABA synthesis (7). AP is a metabolite of progesterone that acts as a positive allosteric modulator of the $GABA_A$ receptor ($GABA_AR$) (8). Furthermore, both hormones are neuroprotective against different models of neurodegeneration, both *in vivo* and *in vitro* (1,9-15).

The NMDAR is responsible for many of the physiological and pathological actions of glutamate. This ionotropic glutamate receptor is a heterotetramer composed of two NR1 and two NR2 subunits. While the NR1 subunit is necessary for the cell membrane expression and functionality of the NMDAR, the NR2 (NR2A-D) subunit composition determines the calcium-permeable channel gating kinetics and the pharmacological properties of the receptor (16). The interaction of NR2A and NR2B with the scaffold proteins postsynaptic density 95 (PSD95) and synapse-associated protein 102 (SAP102) determines the synaptic localization and trafficking of the NMDAR (17). Moreover, the C-terminal tails of NR2A and NR2B are susceptible to cleavage by calpains (18) and also contain different endocytic motifs that regulate their trafficking to distinct intracellular pathways (19). Different roles in synaptic plasticity, memory, neuronal survival and excitotoxicity have also been attributed to NR2A- and NR2B-containing NMDARs (20-22).

The persistent organochlorine pesticide dieldrin is an endocrine disruptor due to its interaction with estrogen receptors (23), including those found in neurons (24). It also acts as a non-competitive antagonist at the $GABA_AR$, where it inhibits the binding of t-[^{35}S]butylbicyclophosphorothionate ([^{35}S]TBPS) and subsequently blocks the chloride flux through the channel pore (25). Dieldrin is also considered an environmental risk factor in the development of Parkinson's Disease (26,27). Whereas its neurotoxic action in mesencephalic dopaminergic neurons has been studied extensively, the effects of prolonged exposure to this pollutant in other neuronal systems, such as the GABAergic

and glutamatergic systems, are poorly understood. Dieldrin has been shown to disrupt neuronal development by altering the gene expression of $GABA_A$ receptor subunits (28). There is increasing evidence that long-term changes in glutamatergic and GABAergic neurotransmission eventually lead to regulation of NMDA receptor expression and trafficking, which restores the homeostatic balance between excitatory and inhibitory synaptic activity (29-31). We have previously reported that long-term exposure to dieldrin induces the internalization of the NMDAR in cerebellar granule cells and in cortical neurons as a consequence of permanent blockade of the $GABA_AR$ (32,33). These alterations may explain the cognitive and behavioral deficits observed in animals that are chronically exposed to dieldrin (34,35). However, the precise mechanisms underlying such impairments remain elusive.

The cerebral cortex is responsible for higher brain functions, such as learning and memory, and endogenous hormones play a crucial role in these processes through the modulation of the NMDAR and the $GABA_AR$. Here we examine the ability of the neurosteroids E2 and AP to prevent the reduction in GABA and glutamate neurotransmission observed after long-term exposure to dieldrin. Primary cultures of cortical neurons (mainly composed of GABAergic and glutamatergic neurons) were used to evaluate the effects of the hormones on $GABA_AR$ and NMDAR functionality after long-term exposure to dieldrin. We also measured the membrane expression of the NMDAR subunits NR1, NR2A and NR2B and the scaffold proteins PSD-95 and SAP102, under the above conditions. Finally, we tested the neuroprotective action of these hormones against the neuronal death caused by dieldrin. We found that AP, but not E2, prevented dieldrin-induced neurotoxicity in cortical neurons by maintaining $GABA_AR$ and NMDAR functionality.

RESULTS

AP and E2 prevented the dieldrin-induced reduction of NR1 and NR2B membrane expression and NMDAR functionality

We have previously reported that long-term exposure to dieldrin reduces the number of functional NMDARs and membrane NR1 immunostaining as a result of persistent $GABA_AR$ inhibition (32,33). Since NR2A and NR2B are the most expressed NR2 subunits in mature cortical neurons (16,36), we aimed to study the effects of long-term

exposure to dieldrin and/or to the neurosteroids AP and E2 on NR1, NR2A and NR2B protein levels in membrane extracts of cortical neurons. Exposure to 60 nM dieldrin for 6 DIV reduced the membrane expression of the NR1 (Fig. 1A) and NR2B (Fig. 2A) subunits of the NMDAR. No differences were observed in the total protein levels of NR2B from whole cell homogenates (Fig.2B), as we previously reported for NR1 (33). These observations suggest that dieldrin induces changes in the localization but not in the overall expression of these NMDAR subunits. Therefore, parallel experiments were conducted to analyze the cytosolic protein levels of the NMDAR subunits. Long-term exposure to dieldrin increased the expression of both NR1 and NR2B in the cytosolic fraction (Fig. 1B and 2C), indicating that these subunits are internalized after pesticide treatment. Treatment with 1 nM E2 but not with 10nM E2 increased the membrane expression of both NR1 and NR2B. Moreover, 1 nM E2 prevented the reduction in membrane expression of both subunits induced by dieldrin (Fig. 1A and Fig. 2A). However, E2 treatment did not prevent the increase in cytosolic NR1 (Fig. 1B) and NR2B (data not shown) levels induced by dieldrin. In contrast, treatment with 10 μM AP had no effect on the membrane expression of any of the NMDAR subunits studied, but it inhibited the dieldrin-induced reduction in membrane NR1 and NR2B levels (Fig. 1A and 2A). Furthermore, AP prevented the increase in the cytosolic levels of NR1 (Fig. 1B) and NR2B (Fig. 2C).

In order to confirm that the observed changes in NMDAR localization have functional relevance, we evaluated NMDAR function by measuring NMDA-induced increase in $[Ca^{2+}]_i$. We have previously reported that long-term exposure to 60nM dieldrin reduced the increase in $[Ca^{2+}]_i$ induced by NMDA (33). Figure 3 shows that this reduction was prevented by treatment with either E2 (at 1 nM but not at 10 nM) or 10 μM AP (Fig. 3). In addition, 1nM E2 enhanced the NMDA-induced increase in $[Ca^{2+}]_i$ with respect to control cells (Fig. 3A), unlike 10 nM E2 and AP (Fig. 3B).

AP but not E2 inhibited the down-regulation of NR2A expression caused by dieldrin

Prolonged exposure to dieldrin diminished NR2A membrane expression in cortical neurons in a concentration-dependent manner, achieving statistical significance at 200 nM (Fig. 4A). Addition of AP, but not E2, restored the basal levels of NR2A at the cell membrane (Fig. 4B). Unlike NR1 and NR2B, the membrane expression of NR2A was significantly reduced after treatment with 10 nM (but not 1 nM) E2 (Fig. 4A). However,

no concomitant increase of NR2A in the cytosolic fraction was observed after dieldrin or E2 exposure (Fig. 4C). This, prompted us to examine the effect of dieldrin on NR2A mRNA levels. Analysis by RT-PCR showed that long-term exposure to 200 nM dieldrin caused the down-regulation of NR2A, this effect being reverted by AP (Fig. 4D).

Estradiol promoted PSD95 expression but not its association with NMDARs

We next wanted to address the mechanisms underlying both the reduction and increase in NMDAR subunits in the cell membrane caused by dieldrin and E2, respectively. Since PSD95 and SAP102 are the two main scaffold proteins that hold the NMDAR on the cell membrane (17), we wondered whether the effects of these compounds on NMDAR subunits could be related to changes in the expression of these proteins. To test this hypothesis, we analyzed the protein levels of PSD95 and SAP102 in membrane extracts from cortical cultures after long-term exposure to dieldrin. Figure 5 shows that prolonged exposure to dieldrin (60–200 nM) did not alter the membrane protein levels of PSD95 nor SAP102. In contrast, treatment with 1 nM (but not 10 nM) E2 greatly increased the membrane expression of PSD95 (Fig. 5A). Consistent with the notion that PSD95 and SAP102 are mainly located on the cell membrane, no expression was detected in the cytosol (data not shown).

We then studied the association of PSD95 with the NMDAR subunits to determine whether the effects of E2 on PSD95 were accompanied by increased interaction with the NMDAR subunits. We also tested the possibility that dieldrin could disrupt the interaction between these proteins. Thus, we immunoprecipitated membrane extracts from our cultures with an antibody raised against PSD95 and subsequently analyzed the presence of NR2A and NR2B in the immunoprecipitates by immunoblotting. PSD95 was highly coupled to NR2A- but not to NR2B-containing NMDARs in our cultures (Fig. 5C–D). Moreover, long-term exposure to 200 nM dieldrin totally disrupted the physical association between PSD95 and NR2A (Fig. 5C). These observations confirm that the effects of dieldrin are specific to NMDARs and are not a consequence of the general reduction of the PSD, i.e. neurodegeneration. However, although 1 nM E2 promoted the membrane expression of PSD95 (Fig. 5A), this was not accompanied by increased interaction with NR2A (Fig. 5C). Likewise, E2 did not induce any physical association between PSD95 and NR2B (Fig. 5D). Consistent with this, immunoblot analysis of the supernatants after immunoprecipitation with PSD95 antibody showed increased expression of the obligatory NR1 subunit in E2-treated cultures, confirming

that E2-stimulated cell membrane insertion of those NMDARs not associated with PSD95 (Supplementary Fig. 1). Taken together, the results indicate that the positive effects of E2 on PSD95 and the NR1/NR2B receptors do not imply increased interaction between these proteins.

AP but not E2 reversed $GABA_AR$ inhibition and neuronal death induced by dieldrin

We also tested the possible neuroprotective effects of AP and E2 against dieldrin-induced neuronal death under different exposure paradigms, based on preliminary data from our laboratory. Cultured cortical neurons were exposed to 3–10 μM dieldrin for 4 DIV beginning on DIV 2 (immature neurons; Fig. 6A) or DIV 5 (mature neurons; Fig. 6B). Treatment with 10 μM but not with a lower concentration (1 μM) of AP significantly attenuated the cell death induced by dieldrin exposure, regardless of the maturity of the neurons in culture. In contrast, 1 nM E2 was unable to prevent the neuronal damage caused by dieldrin (Fig. 6).

In order to determine whether the neuroprotective effect of AP against dieldrin neurotoxicity was due to its positive modulation of the $GABA_AR$, we evaluated $GABA_AR$ function by measuring the ability of GABA to increase [^{3}H]-flunitrazepam binding (8,37). Cultured mature neurons were exposed to 10 μM dieldrin for 2 DIV, a condition that did not induce cell death (data not shown). Dieldrin did not modify basal [^{3}H]-flunitrazepam binding; however, it completely abolished the increase in [^{3}H]-flunitrazepam binding induced by 30 μM GABA. Treatment with 10 μM AP significantly reversed the effect of dieldrin on the $GABA_AR$. AP alone slightly reduced the effect of GABA on [^{3}H]-flunitrazepam binding (Fig. 7A). In contrast, 1 nM E2 did not affect the inhibition of $GABA_AR$ function caused by dieldrin (the GABA-induced increase in [^{3}H]-flunitrazepam binding amounted to 254±3%, 130±7% and 132±3% in control, dieldrin-treated and dieldrin+E2-treated cultures respectively, N=3). These results suggest that the protective effects of AP against dieldrin neurotoxicity are most likely mediated through modulation of the $GABA_AR$.

AP prevented dieldrin-induced NR2B cleavage and loss of association with SAP102 in mature neurons

Finally, we tested whether the effects of dieldrin on NMDAR subunits were also observed in mature neurons before the onset of the neuronal damage produced by this

pollutant. For this reason, we exposed the cells to 10 μM dieldrin for 2 DIV both in the absence and presence of AP. As previously observed at lower concentrations, dieldrin reduced both NR2A and NR2B membrane protein levels (Fig. 7B) and NR2A mRNA expression (Fig. 4D), these effects being prevented by AP (Fig. 4D; Fig. 7B). However, on this occasion there was no increase in cytosolic NR2B expression (data not shown). Conversely, a breakdown product of NR2B (~120 KDa) appeared in cells exposed to dieldrin. Again, this effect was reverted by treatment with AP. In contrast, no truncated NR2A fragments were observed after dieldrin exposure (Fig. 7B). Cleavage of NMDAR subunits is commonly triggered by calpains in neurons and heterologous cells (18,38). Both calpains and caspases degrade α-spectrin, a protein that links NMDARs to actin cytoskeleton (39). Therefore, we measured calpain activity by determining the proteolysis of α-spectrin under the above conditions. Dieldrin exposure induced the calpain- but not caspase-dependent cleavage of α-spectrin (fragments of 150 and 120 KDa, respectively), this effect being prevented by AP (Fig. 7C).

As SAP102 is the scaffold protein that usually holds NR1/NR2B receptors at the cell membrane in the adult cortex (17), we test whether this was also the case in mature cortical neurons in culture and moreover whether their interaction was affected by dieldrin-induced NR2B proteolysis. Thus, we performed immunoprecipitation of the NR2B subunit of NMDAR in membrane extracts followed by immunoblotting for SAP102. Figure 7D shows the physical association between NR2B and SAP102 in control neurons. Exposure to 10 μM dieldrin for 2 DIV reduced the levels of associated SAP102 in the immunoprecipitated fraction, this effect being prevented by AP treatment. In contrast, the membrane expression of SAP102 before immunoprecipitation remained unaffected in all conditions tested (Fig. 7D, bottom).

DISCUSSION

In the present study the neurosteroids AP and E2 prevented the reduction in NMDAR functionality produced by long-term exposure to the endocrine disruptor dieldrin in primary cultures of cortical neurons. Moreover, the results provide insights into the mechanisms of neurotoxicity of dieldrin since this persistent pesticide regulated NMDAR subunits in different ways, leading to the internalization of NR1/NR2B receptors and to down-regulation of NR2A. We also found that AP reverted the effects of dieldrin on all the NMDARs subunits studied, whereas E2 had a compensatory effect

only on NR1/NR2B receptors and did not prevent their internalization. In addition, we found that AP but not E2 is neuroprotective against the toxicity caused by dieldrin both in mature and immature cortical neurons through its positive modulation of the $GABA_AR$.

The concentrations of dieldrin used were similar to those found in the human brain (26), illustrating the toxicological relevance of the data presented. We have previously reported that prolonged exposure to low concentrations of this pollutant produces partial but permanent blockade of the $GABA_AR$, as measured by [^{35}S]TBPS binding (33). Disinhibition of synaptic activity induced by $GABA_AR$ antagonists initially causes hyperexcitability, which, if it persists, produces compensatory down-regulation of the post-synaptic response to glutamate over a 48-hour period (40). Furthermore, the homeostatic balance between GABA and glutamate neurotransmission, known as synaptic scaling, has been shown to involve NMDAR trafficking (29). Here, we found that long-term exposure to dieldrin reduced the membrane expression of all the NMDAR subunits tested. These results confirm our previous observations in which NR1 immunostaining was lower in the plasma membrane and higher in the cytosol after long-term exposure to dieldrin (33). Likewise, chronic treatment with other $GABA_AR$ antagonists has been shown to diminish NR1, NR2A and NR2B membrane levels (31). In our hands, the NR1 and NR2B subunits were those that were primarily affected by dieldrin exposure. In addition, unlike NR2A, which was regulated at mRNA level, these NMDAR subunits were internalized after dieldrin exposure. Overall, distinct mechanisms appear to be involved in the regulation of NMDAR subunits by this endocrine disruptor.

The alternative splicing of the C-terminal cassettes of NR1 determines its trafficking, and has been shown to be dependent on synaptic activity (36,41). Treatment with the $GABA_AR$ antagonist bicuculline increases the C2 splice variant with respect to C2' and as a result the rate of NR1 delivery to the cell membrane is retarded (41). Thus, the regulation of NR1 transcripts might underlie the reduced expression of NR1 on the membrane of cells exposed to dieldrin. On the other hand, NR2 trafficking is regulated by dual palmitoylation that can either cause retention in the Golgi apparatus or tyrosine phosphorylation-mediated membrane stabilization (42). Moreover, activity-dependent phosphorylation of NR2B at Ser-1480 disrupts its interaction with PSD-95 and SAP102, leading to reduced NMDAR membrane expression (43). Similarly, association with

PSD95 stops NR2A being internalized (19) and cleaved by calpains (18). Despite this, the NR2A and NR2B subunits have distinct endocytotic motifs in the C-terminal region and sort into different intracellular pathways after internalization (19). In agreement with previous studies (17), we found that PSD95 specifically interacts with NR2A- but not with NR2B-containing NMDARs in cortical neurons. In contrast, NR2B was physically associated with SAP102, which lacks palmitoylation motifs and so is unable to cluster and to prevent the calpain-dependent proteolysis of NR2 subunits (18). The subunit-specific association with these scaffold proteins most likely explains why NR2B but not NR2A was internalized together with NR1 after dieldrin exposure. Consistent with this, NR2B undergoes more robust constitutive internalization (especially when located extrasynaptically) than NR2A in mature neurons (19). Nevertheless, whether the phosphorylation and/or palmitoylation states of NR2B are altered before the receptor is internalized due to dieldrin exposure remains to be determined. The respective interactions with SAP102 and PSD95 may also explain why NR2B but not NR2A was degraded by calpains after exposure to dieldrin in mature neurons. Furthermore, the loss of association between SAP102 and NR2B observed after dieldrin treatment supports the idea that truncated NR2B subunits are no longer able to interact with scaffold proteins despite remaining on the cell membrane. The proteolysis of the NR2B but not the NR2A subunit by calpains in mature cortical neurons (38) and *in vivo* after ischemia or status epilepticus (44,45) has been reported previously. However, the physiological or pathological role of truncated NR2B subunits present in the membrane remains unknown.

While NR1 and NR2B are first expressed in cultured cortical neurons clustering at both synaptic and at extrasynaptic sites as early as 3 DIV, NR2A is almost undetectable up to 7 DIV, but becomes predominant at the synapsis in mature neurons (46). Therefore, the differential developmental expression of the NR2 subunits may also account for the preferential effects of dieldrin on NR2B-containing NMDARs, given NR2A-containing NMDARs were not functional for most of the time that the cells were exposed to the pesticide. In addition, it has been proposed that the developmental switch in synaptic NR2 subunit predominance is due to NR2A-enriched synaptogenesis rather than changes in the expression or lateral diffusion of NR2B (47). Our results indicate that dieldrin inhibits the developmental up-regulation of NR2A without affecting the expression of other proteins of the PSD. Consistent with this, NR2A but not NR2B

mRNA expression is dependent on synaptic activity mediated through NMDARs and L-type Ca^{2+} channels (36). Thus, dieldrin-induced internalization of NR2B-containing NMDARs precedes and maybe causes the disruption in the developmental up-regulation of NR2A observed at a higher concentration of pesticide. However, more studies are required to confirm this hypothesis. We recently reported that dieldrin mimics some of the effects of E2 by activating the ER in cortical neurons (24). Therefore, another possibility is that dieldrin could modulate NR2A expression through an ER-dependent mechanism. However, this is unlikely since the affinity of dieldrin for ER in cortical neurons is at least two orders of magnitude higher (~30 μM) than the concentrations used here for long-term experiments (60–200 nM). In contrast, we cannot rule out the involvement of ERs in NR2A down-regulation caused by 10μM dieldrin, given that this pesticide concentration has estrogenic effects (24).

E2 positively modulates glutamate neurotransmission in the hippocampus, enhancing synaptic plasticity and memory performance, these effects being associated with an increase in synaptic NR1, NR2B and PSD95 protein levels (2,5,6). In contrast, NMDAR expression in the cortex has been reported to either decrease, increase or remain unaffected after E2 treatment (14,48,49). In the present study, we found that treatment for 2 DIV with 1 nM but not 10 nM E2 increased the membrane expression of NR1, NR2B and PSD95. In contrast, 10 nM E2 reduced NR2A expression as previously described (14), whereas 1 nM E2 did not. Thus, the conflicting results obtained by different studies may be due to the different doses of E2 used and to the different NMDAR subunits analyzed. In support of this, it has been reported that the effects of E2 on neuronal outgrowth in cultured cortical neurons show an inverted U-shaped dose-response curve and moreover that they depend on the cortical region (1). The long-term effects of E2 on NMDAR expression involve *de novo* protein synthesis (48,49). Furthermore, E2 treatment did not prevent the internalization of NR1 and NR2B induced by dieldrin. All together, these findings indicate that the mechanisms by which these compounds modulate NR2B-containing NMDARs differ. In addition, we found that the increase in membrane PSD95 levels induced by 1nM E2 did not result in an increased association with NMDARs in cortical neurons. Similarly, we have recently reported that the association between PSD95 and NR2B in the preoptic area is not modified in proestrus *versus* diestrus or after E2 treatment of ovariectomized rats (50,51). Our results suggest different mechanisms for the regulation of PSD95 and

NMDARs expression by E2. Consistent with this, previous studies have shown that E2 promotes PSD95 expression via the PI3K/Akt pathway and ERβ activation in the hippocampus (2,52). On the other hand, activation of ERα is responsible for the E2-induced stimulation of NR1/NR2B receptor expression in hippocampal neurons, whereas both ERs appear to contribute in the cortex (5,49). Furthermore, inhibition of the MAPK pathway apparently mediates the NR2A down-regulation caused by E2 (14). Finally, PKC activation promotes the assembly of NR1 with NR2A and NR2B but not with PSD95 (53), and may explain the effects of E2 on NR2B-containing NMDARs.

Although E2 treatment counteracted the reduction of NR1 and NR2B membrane expression and NMDAR functionality induced by long-term exposure to dieldrin, it did not protect cortical neurons from its neurotoxic effects. The inability of E2 to prevent both $GABA_AR$ blockade and subsequent NR2A down-regulation induced by prolonged exposure to this pesticide could reflect its lack of neuroprotective effects against dieldrin-induced neuronal damage. In addition, it has been reported that E2 has negative effects on cortical GABA levels (7), which may also account for its lack of neuroprotection. In contrast, AP reversed the inhibitory effects of dieldrin at the $GABA_AR$ and restored the functionality and membrane expression of the NMDAR. These positive actions on both GABAergic and glutamatergic neurotransmissions may underlie the neuroprotective effect of AP against dieldrin-induced cell death. Consistent with this, neuroprotection by AP against both *in vitro* and *in vivo* models of ischemia has been related to $GABA_AR$ stabilization on the cell membrane (12). Although the EC_{50} of AP on [^{3}H]-flunitrazepam binding is around 1 μM, a full potentiation of $GABA_AR$ by 10 μM AP (8) was necessary to prevent the neuronal damage caused by this pesticide, as it was also the case for AMPA- and NMDA-mediated excitotoxicity (11,15). Both acute and prolonged exposure to dieldrin block the $GABA_AR$ through its interaction with the TBPS binding site (25,33). The neurosteroid AP has been shown to increase [^{3}H]-flunitrazepam binding and also to reduce [^{35}S]-TBPS binding (8). Its allosteric properties suggest that AP most likely interferes with dieldrin binding to $GABA_AR$ and as a result this neurosteroid can prevent the negative regulation of $GABA_ARs$ and NMDARs induced by long-term exposure to dieldrin. Consistent with this, AP was found to be anticonvulsant and neuroprotective against the noncompetitive antagonist picrotoxinin (9,54), which shares the same binding site with dieldrin at the $GABA_AR$ (25).

In contrast to the NMDAR, the number of functional $GABA_AR$s did not appear to be affected either by dieldrin or AP, since basal [^{3}H]-flunitrazepam binding was similar in all cases. Nevertheless, the expression of the different $GABA_AR$ subunits is developmentally regulated by $GABA_AR$ activity in cultured cortical neurons, being potentiated or inhibited by AP and TBPS, respectively (55). These changes in $GABA_AR$ subunit expression may explain the reduced effect of GABA on [^{3}H]-flunitrazepam binding in AP-treated cells. In addition, dieldrin has been shown to alter the expression of several $GABA_AR$ subunits in a similar way to TBPS (28). Thus, long-term exposure to dieldrin disrupts the developmental switch in $GABA_AR$ subunit composition and most likely interferes with the positive effects of endogenously synthesized AP. Furthermore, these changes certainly modify the functional properties of $GABA_AR$ and may also affect neurosteroid sensitivity (3).

The neurotoxicity of dieldrin in dopaminergic neurons involves the production of reactive oxygen species and caspase-dependent apoptosis (27). We have recently observed that the dieldrin-induced apoptosis of cerebellar granule cells is also caspase-mediated (manuscript in preparation). However, these proteases do not appear to be activated in cortical neurons after dieldrin exposure, as indicated by the absence of caspase-dependent spectrin degradation. In contrast, the 150- kDa fragment in the α-spectrin proteolytic pattern suggests that calpains and/or cathepsins were active before neuronal death was observed in dieldrin-treated cortical neurons. Nevertheless, the results do not rule out the possible involvement of caspases in late stages of the neuronal damage caused by dieldrin, since the caspase-specific fragments of α-spectrin have been shown to appear later than calpain-dependent fragments after prolonged seizures (56). Furthermore, excessive NMDA-mediated calpain activation can trigger pro-apoptotic pathways through the Bcl-2 family of proteins as well as cathepsin-mediated necrosis, as has been reported to occur after status epilepticus and oxygen-glucose deprivation (57,58). In this regard, AP has been reported to protect against different toxic insults through the modulation of the Bcl-2 proteins (10,15). Overall, the involvement of cysteine proteases and the Bcl-2 family of proteins in both the toxicity of dieldrin and the neuroprotective action of AP in cortical neurons deserves further investigation. Suppression of NMDAR activity (as observed in long-term dieldrin-treated cells) can also trigger apoptosis in cortical neurons, which can be reversed by restoring synaptic activity (59). In addition, NR2A-containing NMDARs appear to

mediate the pro-survival effects of NMDA whereas activation of NR2B-containing NMDARs results in excitotoxicity (21). This might explain why prolonged exposure to dieldrin initially prevents glutamate excitotoxicity (33) but eventually leads to neurodegeneration at higher concentrations (this study). All together, the results suggest that the regulation of NR2A expression plays an important role in the neurotoxic effects of dieldrin as well as in neuroprotection by AP.

In summary, the present study shows that prolonged $GABA_AR$ blockade by the pesticide dieldrin triggers the internalization of NR2B-containing NMDARs and also disrupts NR2A subunit developmental up-regulation, which may be responsible for the neuronal death observed at higher concentrations. Both NR2A- and NR2B-containing NMDARs have a crucial role in cortical synaptic plasticity and memory (20,22). Therefore, the results presented here may contribute to our understanding of the molecular mechanisms underlying the cognitive and behavioral deficits found in animals after chronic exposure to this endocrine disruptor (34,35). In addition, the observed neuroprotective actions of AP support its use as a potential therapeutic hormone against insults or neurodegenerative disorders in which GABAergic and/or glutamatergic systems are compromised.

MATERIALS AND METHODS

Materials

Pregnant NMRI mice (16^{th} gestational day) were obtained from Charles River, Iffa Credo (Saint Germain-sur-l'Arbreste, France). Plastic multiwell plates were from Nunc™ (Rockilde, Denmark). Fetal calf serum was from Gibco (Invitrogen, Carlsbad, CA). Dulbecco's modified Eagle's minimum essential medium (DMEM) was from Biochrom (Berlin, Germany). Isofluorane (FORANE®) was from Abbot Laboratories (Madrid, Spain). Trypsin, soybean trypsin inhibitor, DNAse, L-pyruvic acid, reduced β-nicotineamide adenine dinucleotide (NADH) disodium salt, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT), diazepam, dimethyl sulfoxide (DMSO), dieldrin, 17β-estradiol (E2), γ-amino-n-butyric acid (GABA), L-glycine, N-methyl-D-aspartate (NMDA), β-glycerophosphate, sodium pyrophosphate and sodium orthovanadate were from Sigma (St. Louis, MO, USA). 3α-hydroxy-5α-pregnan-20-one or allopregnanolone (AP) was from the Institute of Industrial Organic Chemistry

(Prague, Czech Republic) (8). Fluo-3 AM was from Molecular Probes (Leiden, The Netherlands). [^{3}H]-Flunitrazepam was from PerkinElmer (Boston, MA). Optiphase 'Hisafe'2 liquid scintillation cocktail was from Wallac Oy (Turku, Finland).

Neuronal cultures

Primary cultures of cortical neurons (CN) were prepared from the cerebral cortices of embryonic day 16 mouse fetuses as described previously (33). Briefly, pregnant animals were anesthetized with isofluorane and killed by cervical dislocation and the fetuses were extracted. Cortices were dissected using forceps and mechanically minced, and cells were then dissociated by mild trypsinization (0.02% [wt/vol]) at 37 ºC for 10 min followed by trituration in a DNAse solution (0.004% [wt/vol]) containing soybean trypsin inhibitor (0.05% [wt/vol]). The cells were then suspended in DMEM containing 5 mM KCl, 31 mM glucose and 0.2 mM glutamine supplemented with p-aminobenzoate, insulin, penicillin and 10% fetal calf serum. The cell suspension (1.5 x 10^6 cells/ml) was seeded in 6-, 24- or 96-well plates pre-coated with poly-D-lysine and incubated for at least 7 days in a humidified 5% CO_2/95% air atmosphere at 36.8 ºC. A mixture of 5 μM 5-fluoro-2'-deoxyuridine and 20 μM uridine was added after 1–2 days in vitro (DIV) to prevent glial proliferation. Animals were handled in compliance with protocol DMA1852 of the University of Barcelona, approved by the Generalitat de Cataluña, Spain, following European Union guidelines.

Chemical treatments

Stock solutions of each compound were prepared in DMSO and frozen in aliquots of 100 μl. The final concentration of DMSO in the culture medium was <0.5%. To avoid cross-contamination between wells in the same plate, DMSO or dieldrin treatments were applied to separate plates. Cultured neurons were treated with dieldrin at 2 DIV or 6DIV by adding the stock solution to the culture medium. The medium remained unchanged until the experiments were performed at 8 DIV (exposure for 6 and 2 DIV respectively). In some experiments neurons were treated with dieldrin at 1–2 DIV and the experiments were performed at 4–5 DIV. Treatments with the neurosteroids E2 and AP were generally applied from 6 to 8 DIV, unless otherwise stated. Preliminary experiments showed that 10 μM but not 1 μM AP was effective against dieldrin-induced cell death (see Fig. 6). Therefore, 10 μM AP was used for the remaining experiments. The concentrations of dieldrin used for long-term experiments (60 and 200 nM, 6DIV) were devoid of cytotoxicity (33). With the exception of the cell viability assay,

experiments with mature neurons in which a higher concentration of dieldrin (10 μM) was used, were performed 2 DIV before neuronal damage was detected.

[^{3}H]-Flunitrazepam binding

The binding assay was performed on cells grown in 24-well plates as previously described (37), with a few modifications. After long-term treatment with AP and/or dieldrin, cultures were washed three times in HBSS buffer solution (136 mM NaCl, 5.4 mM KCl, 1.2 mM $CaCl_2$, 1.4 mM $MgCl_2$, 1.0 mM NaH_2P0_4, 10 mM HEPES, and 9 mM glucose, adjusted to pH 7.3). Cells were then exposed to 1.3–2.0 nM [^{3}H]-Flunitrazepam in HBSS in the absence or presence of 30 μM GABA. After 30 min of incubation at 25 °C with gentle shaking, the solutions were removed and the cells were rinsed three times in ice-cold HBSS. Thereafter, cultures were disaggregated in 0.2 N NaOH overnight, and their radioactivity was determined by liquid scintillation counting (Optiphase 'Hisafe'2; PerkinElmer, Waltham, MA). Nonspecific binding was measured in the presence of 20 μM diazepam.

Determination of intracellular calcium concentration

The intracellular calcium concentration ($[Ca^{2+}]_i$) was determined by measuring Fluo-3 fluorescence, as previously described (33). This fluorescent probe is specific for the evaluation of glutamate receptor function (32,33).

Cell viability

Cell viability was assessed by measuring the reduction of the colored formazan salt MTT by mitochondrial activity. After dieldrin and/or neurosteroid treatment, cells grown in 96-well plates were incubated with MTT (500 μg/ml) dissolved in Hank's solution (1.3 mM CaCl, 5.4 mM KCl, 0.4 mM KH_2PO_4, 0.5 mM $MgCl_2$, 0.4 mM $MgSO_4$, 137 mM NaCl, 4.2 mM $NaHCO_3$, 0.3 mM Na_2HPO_4, 8 mM HEPES, and 5.5 mM glucose, adjusted to pH 7.4) at 37 ºC for 1 h, while protected from light. After washing off the excess MTT, the cells were disaggregated with 5% sodium dodecyl sulfate (SDS) and the amount of colored formazan salt formed was measured at 560 nm in a spectrophotometer plate reader (iEMS Reader MF, Labsystems, Helsinki, Finland).

Reverse transcription-polymerase chain reaction (RT-PCR)

Total cell RNA was purified following the method of Chomczynski and Sacchi (60). RNA integrity and concentration were assessed by spectral analysis using a NanoDrop

Spectrophotometer (Thermo Scientific). Equal amounts of RNA (~1 µg) were retro-transcribed with Omniscript reverse transcriptase (Qiagen) following the manufacturer's guidelines. To ensure that cDNA samples were not contaminated with genomic DNA, all samples were also incubated with an RT reaction mix lacking Omniscript reverse transcriptase. Amplification of each transcript was performed in 20 µl of a PCR cocktail containing cDNA derived from 50 ng of mRNA, 8 pmols of each primer and 0.6 U of KAPA Taq DNA polymerase (Kapa Biosystems). After incubation at 95 °C for 5 min, 32 cycles of amplification (15 s at 95 °C, 30 s at 62 °C and 40 s at 72 °C) or 30 cycles of amplification (15 s at 95 °C, 30 s at 56 °C and 15 s at 72 °C) were performed for NR2A and GAPDH, respectively. The number of amplification cycles for each primer pair and cDNA preparation was set to ensure that no saturation occurred. Sense and antisense PCR primers were as follows: 5'-CCCACCTACTCAGGCCACTT-3' and 5'-CCGACTGTCCCTGGAGCAAT-3' (nt 3664–3683 and 4251–4232 in databank accession NM_008170.2) for NR2A; 5'- AACGACCCCTTCATTGAC-3' and 5'-TCCACGACATACTCAGCAC-3' (nt 144–161 and 334–316 in databank accession NM_008084.2) for GAPDH.

Antibodies

The following antibodies were used for co-immunoprecipitation: mouse monoclonal anti-PSD95 (MA1-045 and MA1-046, both used at 2 µg/750 µl) was purchased from Affinity BioReagents (Golden, CO), and rabbit polyclonal anti-NR2B (71-8600, 2µg/750µl) from Zymed (San Francisco, CA). The specificity of both anti-PSD-95 antibodies was described previously (61). The antibodies used for immunodetection were: mouse monoclonal anti-NMDAR1 (1:3000, Upstate, Cell Signaling, MA), anti-PSD95 (MA1-046, 1:500) and anti-α-spectrin (1:1000, Sigma); and rabbit polyclonal anti-NR2A (1:500, Millipore corp., MA), anti-NR2B (1:500, Invitrogen) and anti-SAP102 (1:1000, Cell Signaling). A goat polyclonal anti-actin (sc-1616, 1:1000, Santa Cruz Biotechnology, Santa Cruz, CA) was always used as a control of the amount of protein loaded. Secondary antibodies used for Western blot detection were: anti-mouse (1:8000), anti-rabbit (1:10,000) and anti-goat/sheep (1:10,000), all horseradish peroxidase (HRP)-conjugated (Sigma).

Membrane extraction and co-immunoprecipitation

Membrane extraction was performed as previously described (62), with a few modifications. In brief, cells grown on 6-well plates were washed three times with ice-

cold PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 2 mM KH_2PO_4). Protein extracts from three wells were prepared in 750 µl of lysis buffer (pH 7.4, 25 mM Tris, 50 mM β-glycerophosphate, 1.5 mM EGTA, 0.5 mM EDTA, 1 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 10 µg/ml leupeptin and pepstatin, 10 µg/ml aprotinin, 100 µg/ml PMSF) by trituration through 26-gauge needles. The cell lysates were cleared by centrifugation at 4600 x *g* for 10 min. The supernatant was then collected and ultracentrifuged at 59,000 x g in Beckmann tubes for 1 h at 4 °C. After ultracentrifugation, the pellet was resuspended in 50 µl of lysis buffer containing 1% triton X-100 (lysis buffer X-1) and briefly sonicated. Protein content was determined using the Bradford method (Bio-Rad, Hercules, CA). The membrane samples were mixed with the same volume of 2X sample buffer (Invitrogen) and boiled for 5 min. Supernatants from ultracentrifugation (cytosolic fraction) were diluted in 4X sample buffer (Invitrogen) and boiled for 5 min.

Co-immunoprecipitation was performed as described previously (50,51,62). Briefly, similar amounts of protein (~0.5 mg) from membrane extracts were prepared in a total volume of 750 µl of lysis buffer X-1, and then incubated overnight with gentle rocking at 4°C with 2 µg of both anti-PSD95 antibodies (MA1-045 and MA1-046) or 2 µg of anti-NR2B. Thereafter, 60 µl of protein A–Sepharose beads (1:1 slurry in lysis buffer X-1; P3391, Sigma) was added to each sample and incubated for 4 h with gentle rocking at 4° C. The Sepharose beads were pelleted by brief centrifugation, the supernatant was collected, and 4X sample buffer was added for analysis of nonimmunoprecipitated proteins. The beads were washed three times in ice-cold lysis buffer X-1 and boiled for 5 min in 2X sample buffer. When necessary, the samples were stored at -80 °C until use.

Western blot

Before loading, the samples were boiled and 50 µg of protein from membrane fraction samples or 25 µg from cytosolic fraction samples were subjected to electrophoresis at 150 mV for 75 min in 3–8% Tris-acetate precast SDS-polyacrylamide gels according to the protocol supplied with the NuPAGE system (Invitrogen). After size fractionation, the proteins were transferred onto nitrocellulose membrane (0.2 µm pore-size membranes; LC2002; Invitrogen) in the blot module of the NuPAGE system at 30V for 90 min at 4ºC. Blots membranes were incubated with 5% nonfat dry milk in Tris-buffered Saline Tween-20 (TBST, 20mM Tris-HCl at pH 7.6, 140mM NaCl, 0.1%

Tween-20) for 45–60 min at room temperature and then incubated overnight at 4 ºC with primary antibodies, all prepared in TBST containing 5% nonfat dry milk. The membranes were then washed in TBST and incubated for 1.5 h with the respective secondary antibodies diluted in 5% nonfat dry milk in TBST. The membranes were washed and immunoreactions were detected via enhanced chemiluminescence (NEL101; PerkinElmer, Boston, MA). Densitometric quantification of the immunoblots was performed using J image software and is expressed as the ratio of specific protein *vs*. actin.

Data analysis

Unless otherwise stated, at least three experiments from independent culture batches were performed, each one in triplicate. Statistical comparisons were made by one-way ANOVA followed by Dunnett's post-comparison test when comparing more than two groups, and two-way ANOVA followed by Bonferroni *post-hoc* test when comparing two factors. The level of significance was set at $p < 0.05$. Graphics were created and statistical analysis performed using the software package Graph-Pad Prism (version 4.0, 2003, Graph-Pad Software Inc., San Diego, CA, USA).

ACKNOWLEDGEMENTS

We thank Dr Alexander Kasal (Inst. Org. Chem. Biochem., Prague) for supplying allopregnanolone. This work was supported by Ministry of Science and Innovation (FIS 10/0453); Generalitat de Catalunya (2009/SGR/214); Inserm, University of Lille 2 and the Agence Nationale de la Recherche (ANR).

Disclosure Summary: The authors have nothing to disclose.

REFERENCES

1. **Brinton RD, Tran J, Proffitt P, Montoya M** 1997 17 beta-Estradiol enhances the outgrowth and survival of neocortical neurons in culture. Neurochem Res 22:1339-1351
2. **Liu F, Day M, Muniz LC, Bitran D, Arias R, Revilla-Sanchez R, Grauer S, Zhang G, Kelley C, Pulito V, Sung A, Mervis RF, Navarra R, Hirst WD, Reinhart PH, Marquis KL, Moss SJ, Pangalos MN, Brandon NJ** 2008 Activation of estrogen receptor-beta regulates hippocampal synaptic plasticity and improves memory. Nat Neurosci 11:334-343

3. **Mellon SH** 2007 Neurosteroid regulation of central nervous system development. Pharmacol Ther 116:107-124
4. **Schwarz JM, McCarthy MM** 2008 Steroid-induced sexual differentiation of the developing brain: multiple pathways, one goal. J Neurochem 105:1561-1572
5. **Jelks KB, Wylie R, Floyd CL, McAllister AK, Wise P** 2007 Estradiol targets synaptic proteins to induce glutamatergic synapse formation in cultured hippocampal neurons: critical role of estrogen receptor-alpha. J Neurosci 27:6903-6913
6. **Smith CC, McMahon LL** 2006 Estradiol-induced increase in the magnitude of long-term potentiation is prevented by blocking NR2B-containing receptors. J Neurosci 26:8517-8522
7. **Blurton-Jones M, Tuszynski MH** 2006 Estradiol-induced modulation of estrogen receptor-beta and GABA within the adult neocortex: a potential transsynaptic mechanism for estrogen modulation of BDNF. J Comp Neurol 499:603-612
8. **Suñol C, Garcia DA, Bujons J, Kristofikova Z, Matyas L, Babot Z, Kasal A** 2006 Activity of B-nor analogues of neurosteroids on the GABA(A) receptor in primary neuronal cultures. J Med Chem 49:3225-3234
9. **Brinton RD** 1994 The neurosteroid 3 alpha-hydroxy-5 alpha-pregnan-20-one induces cytoarchitectural regression in cultured fetal hippocampal neurons. J Neurosci 14:2763-2774
10. **Charalampopoulos I, Tsatsanis C, Dermitzaki E, Alexaki VI, Castanas E, Margioris AN, Gravanis A** 2004 Dehydroepiandrosterone and allopregnanolone protect sympathoadrenal medulla cells against apoptosis via antiapoptotic Bcl-2 proteins. Proc Natl Acad Sci U S A 101:8209-8214
11. **Ciriza I, Azcoitia I, Garcia-Segura LM** 2004 Reduced progesterone metabolites protect rat hippocampal neurones from kainic acid excitotoxicity in vivo. J Neuroendocrinol 16:58-63
12. **Kelley MH, Taguchi N, Ardeshiri A, Kuroiwa M, Hurn PD, Traystman RJ, Herson PS** 2008 Ischemic insult to cerebellar Purkinje cells causes diminished GABAA receptor function and allopregnanolone neuroprotection is associated with GABAA receptor stabilization. J Neurochem 107:668-678
13. **Mendez P, Azcoitia I, Garcia-Segura LM** 2005 Interdependence of oestrogen and insulin-like growth factor-I in the brain: potential for analysing neuroprotective mechanisms. J Endocrinol 185:11-17
14. **Numakawa Y, Matsumoto T, Yokomaku D, Taguchi T, Niki E, Hatanaka H, Kunugi H, Numakawa T** 2007 17beta-estradiol protects cortical neurons against oxidative stress-induced cell death through reduction in the activity of mitogen-activated protein kinase and in the accumulation of intracellular calcium. Endocrinology 148:627-637
15. **Xilouri M, Papazafiri P** 2006 Anti-apoptotic effects of allopregnanolone on P19 neurons. Eur J Neurosci 23:43-54
16. **Monyer H, Burnashev N, Laurie DJ, Sakmann B, Seeburg PH** 1994 Developmental and regional expression in the rat brain and functional properties of four NMDA receptors. Neuron 12:529-540

17. **van Zundert B, Yoshii A, Constantine-Paton M** 2004 Receptor compartmentalization and trafficking at glutamate synapses: a developmental proposal. Trends Neurosci 27:428-437
18. **Dong YN, Waxman EA, Lynch DR** 2004 Interactions of postsynaptic density-95 and the NMDA receptor 2 subunit control calpain-mediated cleavage of the NMDA receptor. J Neurosci 24:11035-11045
19. **Lavezzari G, McCallum J, Dewey CM, Roche KW** 2004 Subunit-specific regulation of NMDA receptor endocytosis. J Neurosci 24:6383-6391
20. **Barker GR, Warburton EC, Koder T, Dolman NP, More JC, Aggleton JP, Bashir ZI, Auberson YP, Jane DE, Brown MW** 2006 The different effects on recognition memory of perirhinal kainate and NMDA glutamate receptor antagonism: implications for underlying plasticity mechanisms. J Neurosci 26:3561-3566
21. **Liu Y, Wong TP, Aarts M, Rooyakkers A, Liu L, Lai TW, Wu DC, Lu J, Tymianski M, Craig AM, Wang YT** 2007 NMDA receptor subunits have differential roles in mediating excitotoxic neuronal death both in vitro and in vivo. J Neurosci 27:2846-2857
22. **Massey PV, Johnson BE, Moult PR, Auberson YP, Brown MW, Molnar E, Collingridge GL, Bashir ZI** 2004 Differential roles of NR2A and NR2B-containing NMDA receptors in cortical long-term potentiation and long-term depression. J Neurosci 24:7821-7828
23. **Soto AM, Chung KL, Sonnenschein C** 1994 The pesticides endosulfan, toxaphene, and dieldrin have estrogenic effects on human estrogen-sensitive cells. Environ Health Perspect 102:380-383
24. **Briz V, Molina-Molina JM, Sánchez-Redondo S, Fernández MF, Grimalt JO, Olea N, Rodríguez-Farré E, Suñol C** 2011 Differential estrogenic effects of the persistent organochlorine pesticides dieldrin, endosulfan, and lindane in primary neuronal cultures. Toxicol Sci 120:413-427
25. **Pomés A, Rodríguez-Farré E, Suñol C** 1994 Disruption of GABA-dependent chloride flux by cyclodienes and hexachlorocyclohexanes in primary cultures of cortical neurons. J Pharmacol Exp Ther 271:1616-1623
26. **Corrigan FM, Wienburg CL, Shore RF, Daniel SE, Mann D** 2000 Organochlorine insecticides in substantia nigra in Parkinson's disease. J Toxicol Environ Health A 59:229-234
27. **Kitazawa M, Anantharam V, Kanthasamy AG** 2003 Dieldrin induces apoptosis by promoting caspase-3-dependent proteolytic cleavage of protein kinase Cdelta in dopaminergic cells: relevance to oxidative stress and dopaminergic degeneration. Neuroscience 119:945-964
28. **Liu J, Morrow AL, Devaud LL, Grayson DR, Lauder JM** 1997 Regulation of GABA(A) receptor subunit mRNA expression by the pesticide dieldrin in embryonic brainstem cultures: a quantitative, competitive reverse transcription-polymerase chain reaction study. J Neurosci Res 49:645-653
29. **Perez-Otaño I, Ehlers MD** 2005 Homeostatic plasticity and NMDA receptor trafficking. Trends Neurosci 28:229-238
30. **Rao A, Craig AM** 1997 Activity regulates the synaptic localization of the NMDA receptor in hippocampal neurons. Neuron 19:801-812

31. **Swann JW, Le JT, Lam TT, Owens J, Mayer AT** 2007 The impact of chronic network hyperexcitability on developing glutamatergic synapses. Eur J Neurosci 26:975-991
32. **Babot Z, Vilaró MT, Suñol C** 2007 Long-term exposure to dieldrin reduces gamma-aminobutyric acid type A and N-methyl-D-aspartate receptor function in primary cultures of mouse cerebellar granule cells. J Neurosci Res 85:3687-3695
33. **Briz V, Galofré M, Suñol C** 2010 Reduction of glutamatergic neurotransmission by prolonged exposure to dieldrin involves NMDA receptor internalization and metabotropic glutamate receptor 5 downregulation. Toxicol Sci 113:138-149
34. **Carlson JN, Rosellini RA** 1987 Exposure to low doses of the environmental chemical dieldrin causes behavioral deficits in animals prevented from coping with stress. Psychopharmacology (Berl) 91:122-126
35. **Topinka MA, Eisenstein EM, Siegel D, Trofeli R, Barraco D** 1984 The effects of dieldrin and chlordimeform on learning and memory in the cockroach, Periplaneta americana: a study in behavioral toxicology. J Toxicol Environ Health 13:705-719
36. **Hoffmann H, Gremme T, Hatt H, Gottmann K** 2000 Synaptic activity-dependent developmental regulation of NMDA receptor subunit expression in cultured neocortical neurons. J Neurochem 75:1590-1599
37. **Galofré M, Babot Z, García DA, Iraola S, Rodríguez-Farré E, Forsby A, Suñol C** 2010 GABAA receptor and cell membrane potential as functional endpoints in cultured neurons to evaluate chemicals for human acute toxicity. Neurotoxicol Teratol 32:52-61
38. **Dong YN, Wu HY, Hsu FC, Coulter DA, Lynch DR** 2006 Developmental and cell-selective variations in N-methyl-D-aspartate receptor degradation by calpain. J Neurochem 99:206-217
39. **Rajgopal Y, Vemuri MC** 2002 Calpain activation and alpha-spectrin cleavage in rat brain by ethanol. Neurosci Lett 321:187-191
40. **Turrigiano GG, Leslie KR, Desai NS, Rutherford LC, Nelson SB** 1998 Activity-dependent scaling of quantal amplitude in neocortical neurons. Nature 391:892-896
41. **Mu Y, Otsuka T, Horton AC, Scott DB, Ehlers MD** 2003 Activity-dependent mRNA splicing controls ER export and synaptic delivery of NMDA receptors. Neuron 40:581-594
42. **Hayashi T, Thomas GM, Huganir RL** 2009 Dual palmitoylation of NR2 subunits regulates NMDA receptor trafficking. Neuron 64:213-226
43. **Chung HJ, Huang YH, Lau LF, Huganir RL** 2004 Regulation of the NMDA receptor complex and trafficking by activity-dependent phosphorylation of the NR2B subunit PDZ ligand. J Neurosci 24:10248-10259
44. **Araujo IM, Xapelli S, Gil JM, Mohapel P, Petersen A, Pinheiro PS, Malva JO, Bahr BA, Brundin P, Carvalho CM** 2005 Proteolysis of NR2B by calpain in the hippocampus of epileptic rats. Neuroreport 16:393-396
45. **Simpkins KL, Guttmann RP, Dong Y, Chen Z, Sokol S, Neumar RW, Lynch DR** 2003 Selective activation induced cleavage of the NR2B subunit by calpain. J Neurosci 23:11322-11331

46. **Zhong J, Russell SL, Pritchett DB, Molinoff PB, Williams K** 1994 Expression of mRNAs encoding subunits of the N-methyl-D-aspartate receptor in cultured cortical neurons. Mol Pharmacol 45:846-853
47. **Liu XB, Murray KD, Jones EG** 2004 Switching of NMDA receptor 2A and 2B subunits at thalamic and cortical synapses during early postnatal development. J Neurosci 24:8885-8895
48. **Brann DW, Zamorano PL, Chorich LP, Mahesh VB** 1993 Steroid hormone effects on NMDA receptor binding and NMDA receptor mRNA levels in the hypothalamus and cerebral cortex of the adult rat. Neuroendocrinology 58:666-672
49. **Morissette M, Le Saux M, Di Paolo T** 2008 Effect of oestrogen receptor alpha and beta agonists on brain N-methyl-D-aspartate receptors. J Neuroendocrinol 20:1006-1014
50. **d'Anglemont dT, X, Campagne C, Dehouck B, Leroy D, Holstein GR, Beauvillain JC, Buee-Scherrer V, Prevot V** 2007 Coupling of neuronal nitric oxide synthase to NMDA receptors via postsynaptic density-95 depends on estrogen and contributes to the central control of adult female reproduction. J Neurosci 27:6103-6114
51. **d'Anglemont dT, X, Campagne C, Steculorum S, Prevot V** 2009 Estradiol induces physical association of neuronal nitric oxide synthase with NMDA receptor and promotes nitric oxide formation via estrogen receptor activation in primary neuronal cultures. J Neurochem 109:214-224
52. **Akama KT, McEwen BS** 2003 Estrogen stimulates postsynaptic density-95 rapid protein synthesis via the Akt/protein kinase B pathway. J Neurosci 23:2333-2339
53. **Lin Y, Skeberdis VA, Francesconi A, Bennett MV, Zukin RS** 2004 Postsynaptic density protein-95 regulates NMDA channel gating and surface expression. J Neurosci 24:10138-10148
54. **Belelli D, Bolger MB, Gee KW** 1989 Anticonvulsant profile of the progesterone metabolite 5 alpha-pregnan-3 alpha-ol-20-one. Eur J Pharmacol 166:325-329
55. **Poulter MO, Ohannesian L, Larmet Y, Feltz P** 1997 Evidence that GABAA receptor subunit mRNA expression during development is regulated by GABAA receptor stimulation. J Neurochem 68:631-639
56. **Wang S, Wang S, Shan P, Song Z, Dai T, Wang R, Chi Z** 2008 Mu-calpain mediates hippocampal neuron death in rats after lithium-pilocarpine-induced status epilepticus. Brain Res Bull 76:90-96
57. **Fujikawa DG** 2005 Prolonged seizures and cellular injury: understanding the connection. Epilepsy Behav 7 Suppl 3:S3-11
58. **Yamashima T** 2000 Implication of cysteine proteases calpain, cathepsin and caspase in ischemic neuronal death of primates. Prog Neurobiol 62:273-295
59. **Leveille F, Papadia S, Fricker M, Bell KF, Soriano FX, Martel MA, Puddifoot C, Habel M, Wyllie DJ, Ikonomidou C, Tolkovsky AM, Hardingham GE** 2010 Suppression of the intrinsic apoptosis pathway by synaptic activity. J Neurosci 30:2623-2635
60. **Chomczynski P, Sacchi N** 1987 Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 162:156-159

61. **Kornau HC, Schenker LT, Kennedy MB, Seeburg PH** 1995 Domain interaction between NMDA receptor subunits and the postsynaptic density protein PSD-95. Science 269:1737-1740

62. **Parkash J, d'Anglemont dT, X, Bellefontaine N, Campagne C, Mazure D, Buee-Scherrer V, Prevot V** 2010 Phosphorylation of N-methyl-D-aspartic acid receptor-associated neuronal nitric oxide synthase depends on estrogens and modulates hypothalamic nitric oxide production during the ovarian cycle. Endocrinology 151:2723-2735

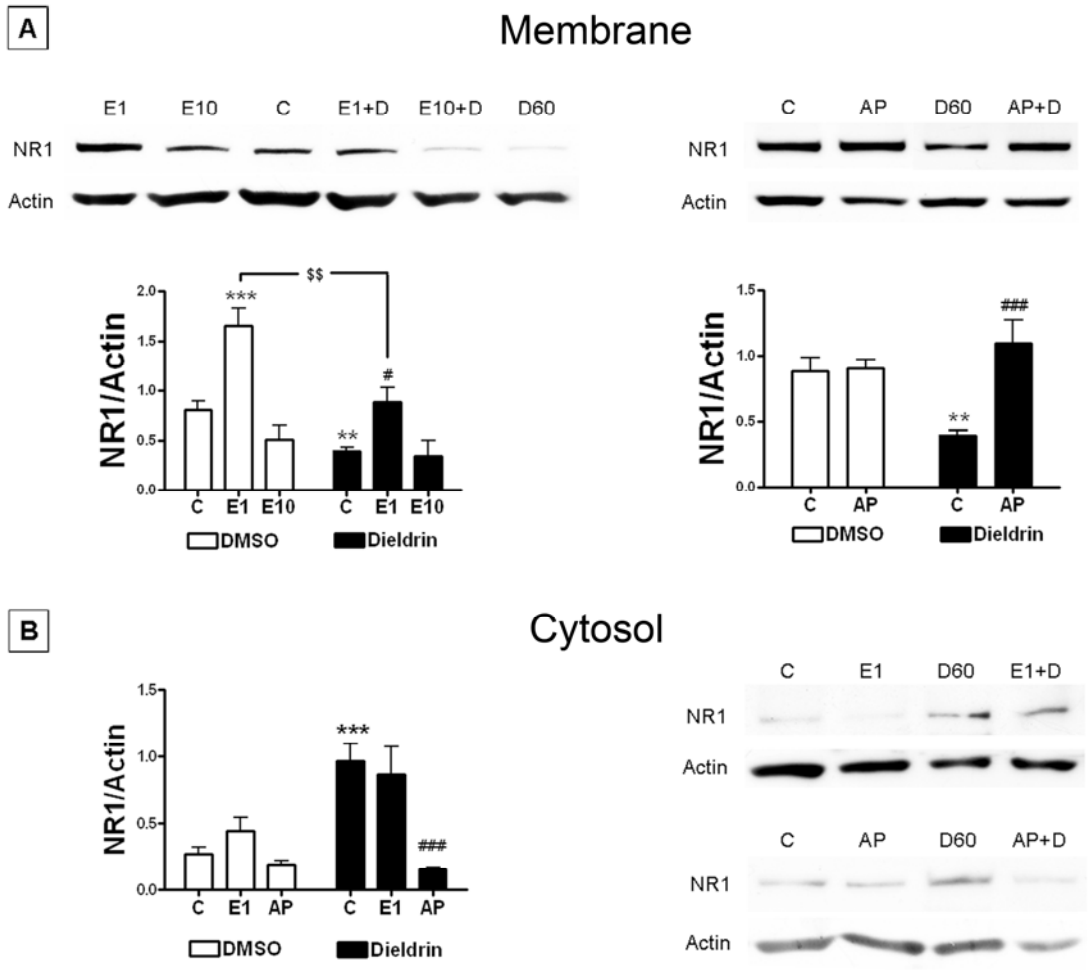


Fig. 1. Effects of neurosteroids on dieldrin-induced internalization of NR1 subunit of NMDAR. Cortical neurons were exposed to DMSO (C) or 60nM dieldrin (D60) for 6DIV and to 10µM AP, 1nM E2 (E1) or 10nM E2 (E10) for 2 DIV. The protein levels of NR1 and actin were analyzed on membrane (panel A) and cytosolic fractions (panel B) from the cultures by Western Blot. Representative immunoblots are shown on the top (A) or on the right (B) and densitometric quantification of the immunoblots is shown on the bottom (A) or on the left (B) of each panel. Data are mean ± SE of 3-7 independent experiments. Statistical comparisons were made by Two-way ANOVA: **, $P < 0.01$; ***, $P < 0.001$ *vs*. control; $^{\#}$, $P < 0.05$; $^{\#\#\#}$, $P < 0.001$ *vs*. dieldrin-treated cells; $^{\$\$}$, $P < 0.01$ *vs*. E1-treated cells.

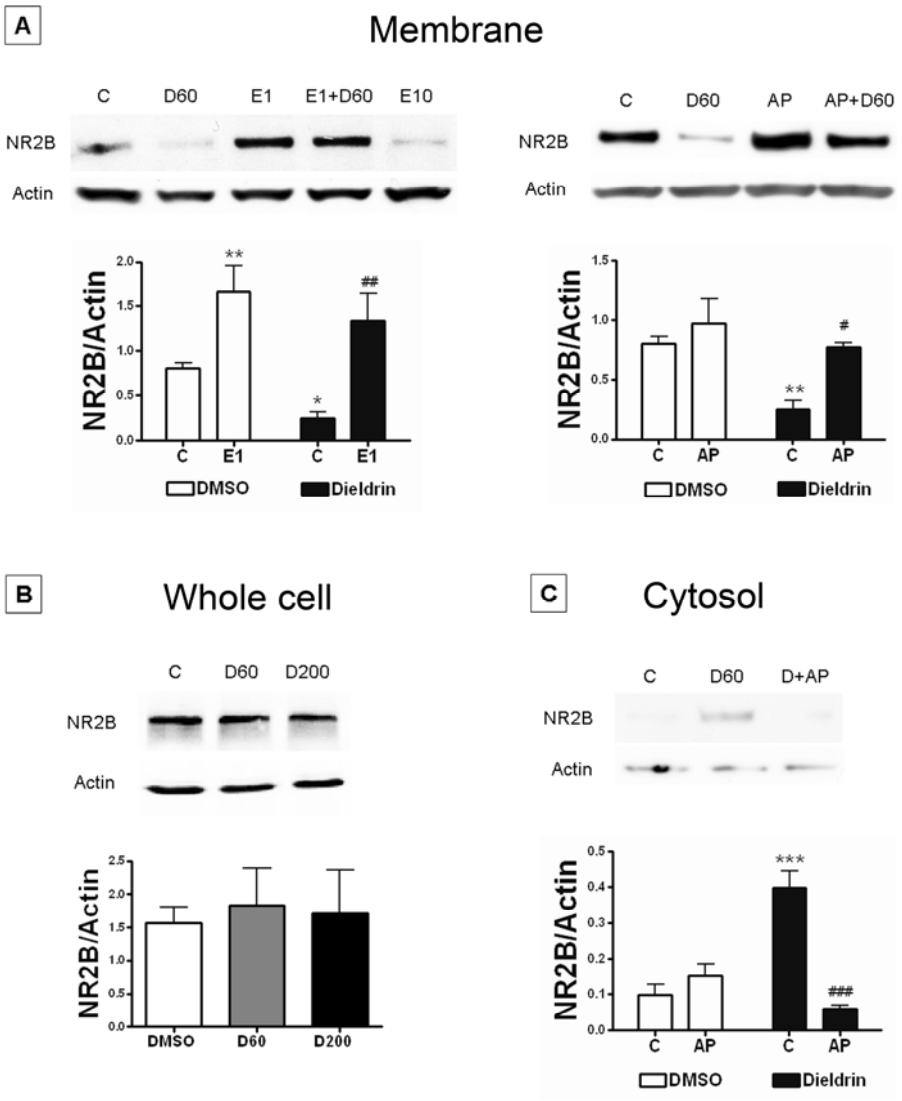


Fig. 2. Effects of neurosteroids on dieldrin-induced internalization of NR2B subunit of NMDAR. Cortical neurons were exposed to DMSO (control or C), 60nM dieldrin (D60) or 200nM dieldrin (D200) for 6DIV and to 10μM AP or 1nM E (E1) for 2 DIV (and combinations) and subsequently the protein levels of NR2B and actin were analyzed on membrane fractions (panel A), whole cell lysates (panel B) and cytosolic fractions (panel C) from the cultures by Western Blot. Representative immunoblots are shown on the top and densitometric quantification of the immunoblots is shown on the bottom of each panel. Data are mean ± SE of 3-6 independent experiments. Statistical comparisons were made by Two-way ANOVA: *, $P < 0.05$ vs. control; **, $P < 0.01$ *vs*. control; ***, $P < 0.001$ *vs*. control; $^{\#}$, $P < 0.05$ *vs*. dieldrin-treated cells; $^{\#\#}$, $P < 0.01$ *vs*. dieldrin-treated cells; $^{\#\#\#}$, $P < 0.001$ *vs*. dieldrin-treated cells.

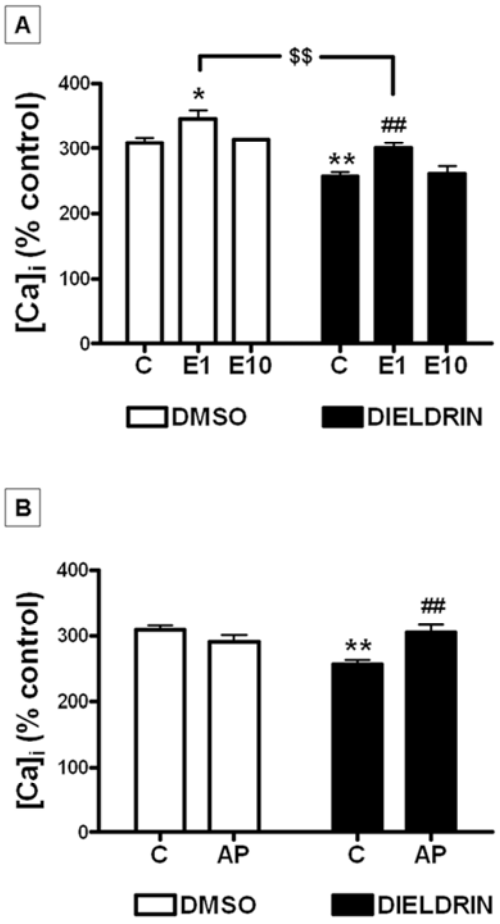


Fig. 3. Effects of the neurosteroids estradiol (panel A) and allopregnanolone (panel B) on dieldrin-induced reduction of NMDAR functionality. Fluo-3 fluorescence was measured immediately after treatment with 100μM NMDA in neurons exposed to DMSO or dieldrin for 6 DIV and to 10μM AP, 1nM E (E1) or 10nM E (E10) for 2 DIV (and combinations). $[Ca^{2+}]_i$ (calculated from fluorescence values) is expressed as % with respect to cells incubated with Hank's solution without NMDA and are mean ± SE of 3-5 independent experiments, each performed in triplicate. Statistical comparisons were made by Two-way ANOVA: *, $P < 0.05$ vs. control; **, $P < 0.01$ *vs*. control; $^{\#\#}$, $P < 0.01$ *vs*. dieldrin-treated cells; $^{\$\$}$, $P < 0.01$ *vs*. E1-treated cells.

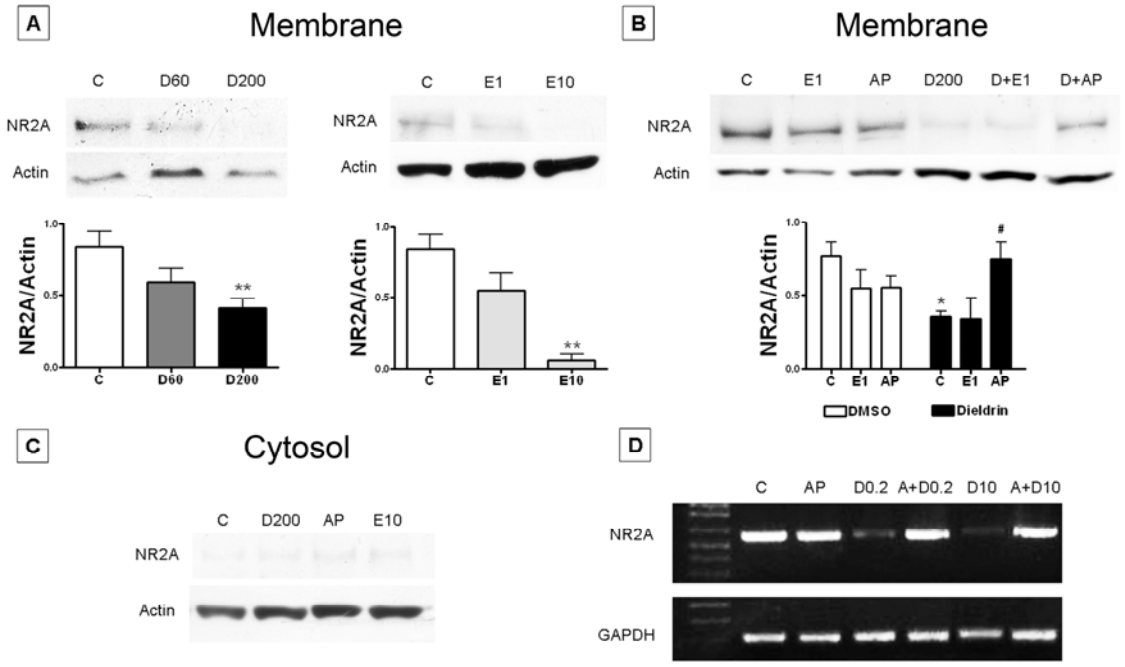


Fig. 4. Effects of neurosteroids on dieldrin-induced down-regulation of NR2A subunit of NMDAR. Cortical neurons were exposed to DMSO (control or C), 60nM dieldrin (D60) or 200nM dieldrin (D200) for 6DIV and to 10μM AP, 1nM E (E1) or 10nM E (E10) for 2 DIV (and combinations) and subsequently the protein levels of NR2A and actin were analyzed on membrane (panels A and B) and cytosolic fractions (panel C) from the cultures by Western Blot. Representative immunoblots are shown on the top and densitometric quantification of the immunoblots is shown on the bottom of each panel. (A and B). Data are mean ± SE of 3-5 independent experiments. Statistical comparisons were made by One-way ANOVA (panel A) or Two-way ANOVA (panel B): *, $P < 0.05$ *vs*. control; **, $P < 0.01$ *vs*. control; $^{\#}$, $P < 0.05$ *vs*. dieldrin-treated cells. D) Cortical neurons were exposed to DMSO (C) or 0.2μM dieldrin (D0.2) for 6DIV and to 10μM AP or 10 μM dieldrin (D10) for 2 DIV (and combinations) and subsequently the mRNA levels of NR2A and GAPDH were analyzed by RT-PCR.

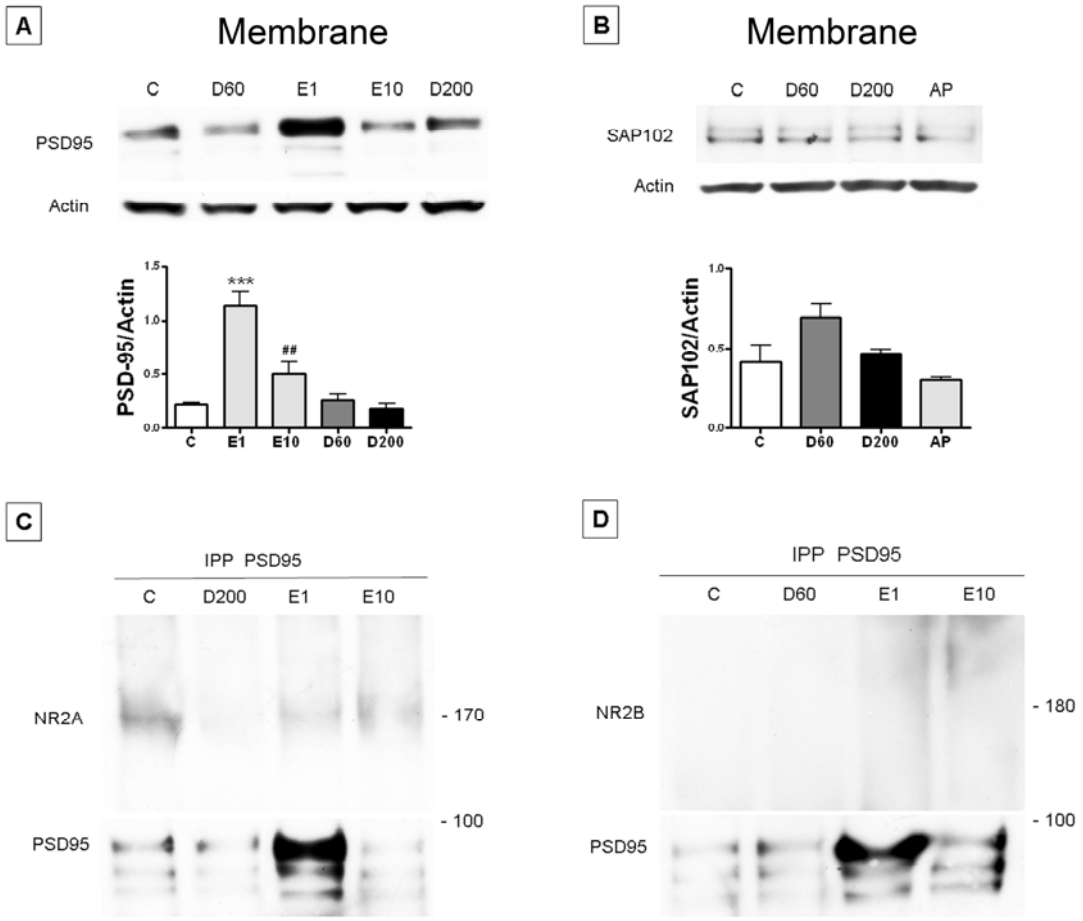


Fig. 5. Estradiol increases PSD95 membrane expression but not its association with NR2 subunits of NMDAR. Cortical neurons were exposed to DMSO (control or C), 60nM dieldrin (D60) or 200nM dieldrin (D200) for 6DIV and to 10µM AP, 1nM E (E1) or 10nM E (E10) for 2 DIV and subsequently the protein levels of PSD95 (panel A) and SAP102 (panel B) were analyzed on membrane fractions from the cultures by Western Blot. Representative immunoblots are shown on the top and densitometric quantification of the immunoblots is shown on the bottom of each panel. (A and B). Data are mean ± SE of 2-6 independent experiments. Statistical comparisons were made by One-way ANOVA: ***, $P < 0.001$ *vs*. control; $^{\#\#}$, $P < 0.01$ *vs*. E1-treated cells. Panels C and D) After the treatments, membrane extracts from the cultures were immunoprecipitated with an antibody raised against PSD95 and then the proteins levels of NR2A (panel C), NR2B (panel D) and PSD95 were analyzed by Western Blot. Molecular weights are indicated on the right of the immunoblots.

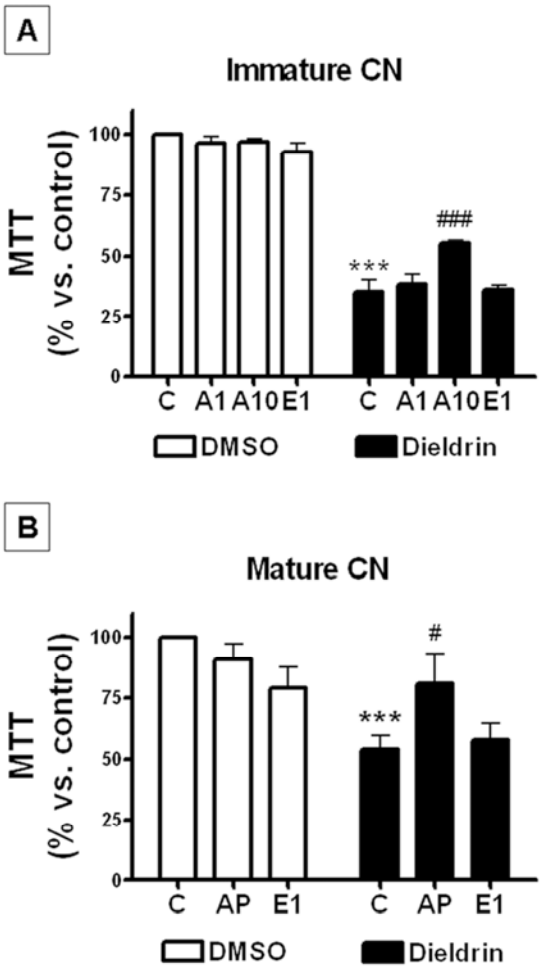


Fig. 6. Effects of neurosteroids on dieldrin-induced reduction of cell viability in immature (panel A) and mature (panel B) cortical neurons. A) Immature cortical neurons were exposed from 2 to 5 DIV to DMSO or 3μM dieldrin both in the absence (C) and presence of 1μM AP (A1), 10μM AP (A10) or 1nM E (E1). B) Mature cortical neurons were exposed from 5 to 9 DIV to DMSO or 10μM dieldrin both in the absence (C) and presence of 10μM AP (A10) or 1nM E (E1). MTT values are expressed as % with respect to control (DMSO-treated cells) and are mean ± SE of 4-5 independent experiments, each in triplicate. Statistical comparisons were made by Two-way ANOVA: ***, $P < 0.001$ *vs*. control; $^{\#}$, $P < 0.05$ *vs*. dieldrin-treated cells; $^{\#\#\#}$, $P < 0.001$ *vs*. dieldrin-treated cells.

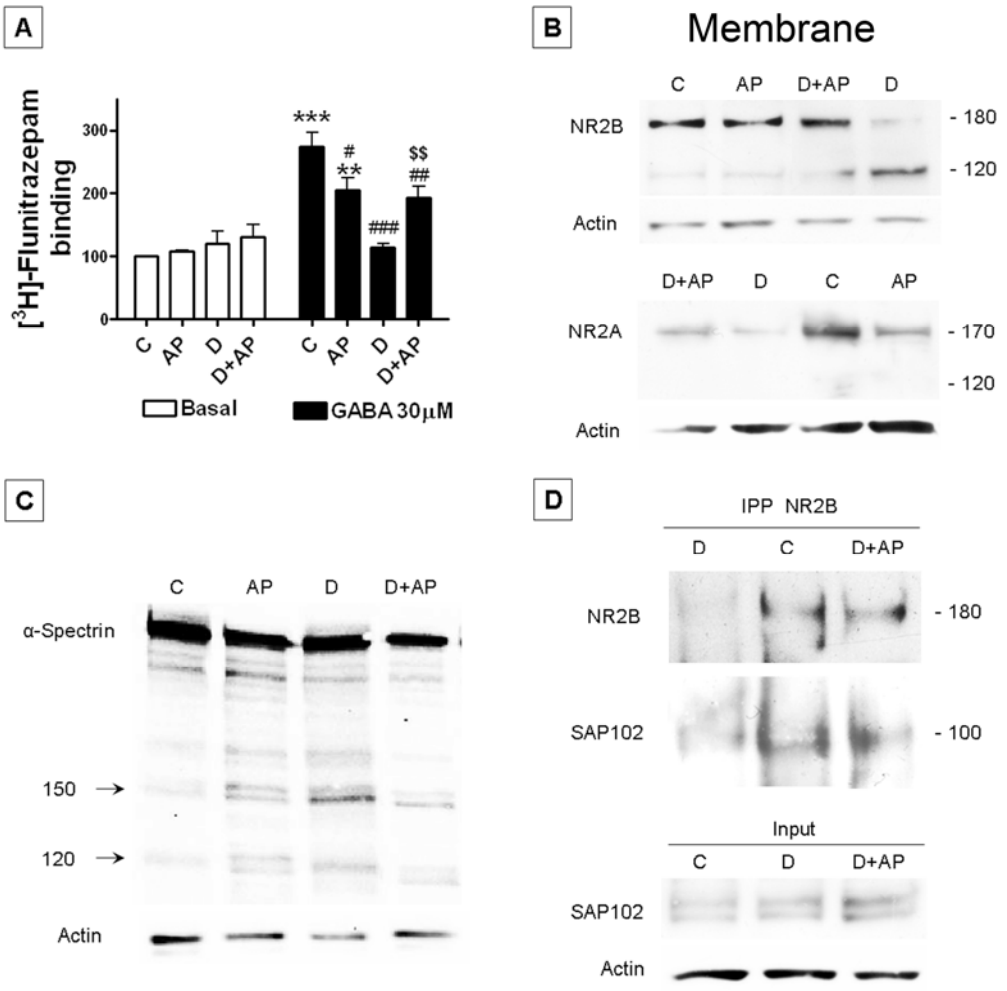
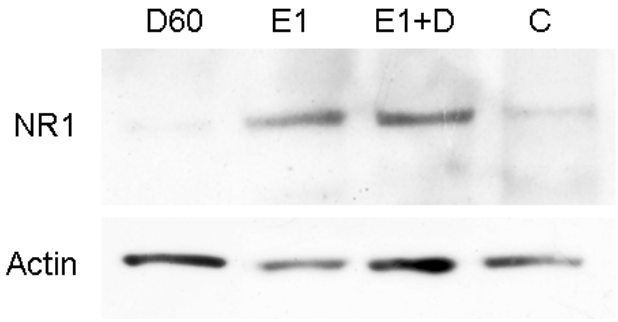


Fig. 7. Effects of AP and dieldrin on $GABA_A$ functionality, calpain activity and calpain-mediated cleavage of NR2 subunits of NMDAR in mature cortical neurons. Cells were exposed to DMSO (C) or 10µM dieldrin (D) for 2DIV both in the absence and presence of 10µM AP. Panel A) After the treatments, the cells were exposed to 1.3-2.0nM [^{3}H]-Flunitrazepam in HBSS in the absence (basal) or presence of 30µM GABA. Values are expressed as % of basal binding and are mean ± SE of 3 independent experiments, each in triplicate. Statistical comparisons were made by Two-way ANOVA: ***, $P < 0.001$ *vs*. basal; $^{\#}$, $P < 0.05$ *vs*. DMSO-treated cells; $^{\#\#\#}$, $P < 0.001$ vs. DMSO-treated cells; $^{\$\$}$, $P < 0.01$ *vs*. dieldrin-treated cells. Panel B) After the treatments, the protein levels of NR2A (bottom), NR2B (top) and actin were analyzed on membrane fractions from the cultures by Western Blot. C) Calpain and caspase activities were determined by Western Blot analysis of α-spectrin in whole cell lysates from cortical neurons previously exposed to 10µM dieldrin and/or 10µM AP for 2DIV. D) After the treatments, membrane extracts from the cultures were straightly subjected to Western Blot analysis of SAP102 and actin (bottom) or immunoprecipitated with an antibody raised against NR2B and subsequently analyzed by Western Blot for NR2B and SAP102 (top). Molecular weights are indicated on the right (panels B and D) or on the left (panel C) of the immunoblots.

SUPPLEMENTARY DATA



Supplementary Fig. 1. E2 increases and dieldrin decreases NMDARs not associated with PSD95. Cortical neurons were exposed to DMSO (control or C) or 60nM dieldrin (D60) for 6DIV and to 1nM E (E1) for 2 DIV. Membrane extracts from the cultures were immunoprecipitated with an antibody raised against PSD95 and then the supernatants after immunoprecipitation were used to determine the proteins levels of NR1 by Western Blot.

TRABAJO Nº 4

Glutamatergic neurons are highly sensitive to organochlorine pesticide long-term toxicity

Víctor Briz, Sara Sánchez-Redondo, Cristina Suñol

Departamento de Neuroquímica y Neurofarmacología, Instituto de Investigaciones Biomédicas de Barcelona, CSIC – IDIBAPS, Barcelona.

En el presente estudio hemos comparado la neurotoxicidad de los pesticidas organoclorados (OCPs) en dos cultivos neuronales diferentes: las células granulares de cerebelo (CGC) y las neuronas corticales (CN). El dieldrín y el endosulfán fueron más tóxicos que el lindano en ambos cultivos. Se observó también que las CGC maduras son más sensibles que las neuronas corticales a la neurotoxicidad inducida por los OCPs. Por el contrario, las neuronas corticales inmaduras fueron más susceptibles a la exposición prolongada a concentraciones bajas de estos pesticidas. El marcaje inmuncitoquímico con vGLUT1 y GAD67 reveló que las neuronas glutamatérgicas corticles constituyen un tipo neuronal más sensible que las GABAérgicas frente a la toxicidad del dieldrín. Además, la fosforilación de Akt y ERK1/2 se vio disminuida tras 24 horas de exposición a dieldrín o endosulfan en CGC. Por otra parte, el tratamiento con estradiol e insulina previno la muerte apoptótica inducida por estos pesticidas en CGC a través de la activación conjunta de ERK1/2 y de Akt. Estos resultados muestran por primera vez la susceptibilidad específica de las neuronas glutamatérgicas frente a la exposición prolongada a estos contaminantes y destacan la participación de las vías PI3K/Akt y MAPK en la neurotoxicidad de los OCPs.

ABSTRACT

BACKGROUND: The organochlorine pesticides (OCPs) endosulfan, dieldrin, and lindane are highly persistent in the environment and they accumulate in plants and animals, causing escalating exposure. These three compounds are convulsants due to their antagonism of the γ-aminobutyric acid-A ($GABA_A$) receptor. They are also endocrine disruptors because of their interaction with the estrogen receptor (ER). However, little is known about the effects of prolonged exposure to OCPs in neurons. OBJECTIVES: The aim of this study is to compare the neurotoxicity of OCPs in two different neuronal cultures: cerebellar granule cells (CGC) and cortical neurons (CN). We also tested different neuroprotective agents in order to address the mechanisms of OCP-induced neurotoxicity. RESULTS: Dieldrin and endosulfan were more toxic than lindane after long-term exposure in the two cultures studied. In addition, we observed that mature CGC are more sensitive than mature CN to OCP-induced neurotoxicity. In contrast, immature CN are primarily affected by prolonged exposure to low concentrations of OCPs. Furthermore, our results revealed that glutamatergic neurons constituted a more sensitive neuronal type than GABAergic neurons to dieldrin-induced toxicity. We also observed that 24 h of exposure to OCPs in CGC reduced the phosphorylation state of both Akt and ERK1/2. Moreover, treatment with estradiol and insulin prevented OCP-induced apoptosis in CGC in an ERK- and Akt-dependent mechanism. CONCLUSIONS: The present study highlights the susceptibility of glutamatergic neurons against prolonged exposure to OCPs. The results also point out the involvement of Akt and ERK1/2 pathways in OCP's neurotoxicity.

Keywords: Pesticides; neurotoxicity; protein kinase B (Akt); extracellular-regulated kinase (ERK); hormones.

INTRODUCTION

Dieldrin, endosulfan and lindane are organochlorine pesticides (OCPs) that have been extensively used in agriculture for decades. Because of their high persistence in the environment, they contaminate the water and food, leading to escalating exposure (Chopra *et al.*, 2011; Schafer and Kegley, 2002). For instance, OCPs were detected in 95% of umbilical cord blood samples from a Spanish mother-child cohort and their presence was associated with consumption of vegetables and fruits (Mariscal-Arcas *et al.*, 2010). Dieldrin and lindane have been recently listed in the Stockholm Convention on Persistent Organic Pollutants (http://www.pops.int) due to their toxicity and environmental health risk; while the status of endosulfan is currently under review. Despite the use and production of dieldrin and lindane are banned in most developed countries since several years ago, the general population is still exposed to OCPs (Bouvier *et al.*, 2006).

Acute exposure to these OCPs induce a hyperexcitability syndrome accompanied by convulsions in mammals, due to their antagonism of the gamma-aminobutyric acid-A ($GABA_A$) receptor (Lawrence and Casida, 1984; Pomes *et al.*, 1993; Pomes *et al.*, 1994b). Furthermore, the cyclodienes dieldrin and endosulfan interact at the $GABA_A$ receptor with higher affinity than lindane as a result of their more hydrophilic chemical structure, whereas all three compounds show the same inhibitory potency at the glycine receptor (Vale *et al.*, 2003). However, little is known about the effects of chronic exposure to low concentrations of these chemicals. These pesticides have deleterious effects on the development of GABAergic, glutamatergic and serotonergic neurotransmissions (Briz *et al.*, 2010; Cabaleiro *et al.*, 2008; Liu *et al.*, 1997; Slotkin and Seidler, 2008; Slotkin and Seidler, 2009). In addition, dieldrin and lindane have been proposed as environmental risk factors in the development of Parkinson's disease (Corrigan *et al.*, 2000; Kanthasamy *et al.*, 2003). Furthermore, early studies reported behavioural alterations in animals after chronic exposure to OCPs (Mariussen and Fonnum, 2006; Paul *et al.*, 1994; Schantz and Widholm, 2001; Tilson *et al.*, 1987; Topinka *et al.*, 1984). However, the molecular mechanisms responsible of such cognitive deficits are poorly understood.

OCPs are also considered endocrine disruptors because they interact with several nuclear receptors, including the estrogen receptor (ER). Dieldrin, endosulfan and lindane have demonstrated *in vitro* estrogenic activity in MCF-7 human breast cancer-

derived cells (Soto *et al.*, 1994) and recombinant cell lines (Lemaire *et al.*, 2006; Maranghi *et al.*, 2007). In addition, OCPs are able to rapidly activate ER-associated signaling pathways in cell lines such as mitogen-activated protein kinase (MAPK) and phosphoinositide 3-Kinase (PI3K)/protein kinase B (Akt) cascades (Bulayeva and Watson, 2004). We have recently reported that dieldrin, endosulfan and lindane cause a sustained activation of extracellular-regulated kinase 1 and 2 (ERK1/2) and Akt as a result of their interaction with ER and $GABA_A$ receptor in primary neuronal cultures (Briz *et al.*, 2011). However, whether these estrogenic effects are involved in the neurotoxic actions of OCPs remain to be determined.

Estrogens and insulin are hormones that have an important role in the development, maturation and function of the central nervous system (CNS). 17β-estradiol (E_2) is known to influence different functions both in the developing and adult brain through the activation of neuronal ERs, such as neuroendocrine events, reproductive behavior synaptic plasticity and neuronal survival (Garcia-Segura *et al.*, 2001; McEwen *et al.*, 1995; Micevych and Dominguez, 2009). In these regards, MAPK and PI3K/Akt pathways have been implicated in ER-mediated synaptogenesis (Akama and McEwen, 2003; Ogiue-Ikeda *et al.*, 2008; Schwarz and McCarthy, 2008) as well as in neuroprotection against different models of neurodegeneration (Bourque *et al.*, 2009; Mendez *et al.*, 2005; Numakawa *et al.*, 2007; Zhao and Brinton, 2007). Similarly, activation of insulin-like growing factor-I receptor (IGF-IR) can modulate these signaling pathways to induce neuroprotection in different neuronal populations (Subramaniam *et al.*, 2005; Willaime-Morawek *et al.*, 2005). Furthermore, a functional association between ER and IGF-IR has been shown to cooperatively regulate several functions in the CNS, including neuroprotection. Indeed, PI3K/Akt and MAPK pathways represent a point of convergence by which E_2 and IGF-I synergistically promote neuronal survival (Mendez *et al.*, 2005).

In the present work, primary cultures of cerebellar granule cells (CGC) and cortical neurons (CN) were used to compare the long-term neurotoxic effects of dieldrin, endosulfan and lindane along the maturation process of the neurons *in vitro*. We have previously observed that OCPs have differential effects in these neuronal cultures through different modulation of ERs, GABA and glycine receptors (Briz *et al.*, 2011; Vale *et al.*, 1998a; Vale *et al.*, 1998b; Vale *et al.*, 2003). Moreover, these neuronal cultures have been extensively used to predict human toxicity of OCPs and other

environmental pollutants (Galofre *et al.*, 2010; Sunol *et al.*, 2008). Whereas CGC are a quite homogenous neuronal population containing predominantly glutamatergic neurons (> 90%), CN have more GABAergic neurons but also contain glutamatergic and cholinergic neurons (Kinney *et al.*, 2006; Sonnewald *et al.*, 2004; Thomas, 1985). Further, the use of cell-specific immunolabeling allowed us to compare the selective susceptibility to OCP-induced cell death of glutamatergic versus GABAergic neurons. Finally, we evaluated the possible therapeutic effects of several neuroprotective agents, including the hormones E_2 and insulin, against OCP-induced neurotoxicity. The results obtained from the present work point out the susceptibility of two important brain regions such as the cortex and cerebellum against OCPs exposure, especially during the development of the CNS.

RESULTS

Differential toxicity of OCPs after long-term exposure in primary neuronal cultures

We first characterized the toxicological profile of dieldrin, endosulfan and lindane after long-term exposure in primary cultures of CN and CGC. Two different times of exposure (2 and 6 DIV) were chosen in order to study the time-dependent susceptibility to OCPs of the neurons in culture. Exposure to all OCPs for 2-6 DIV caused a concentration-dependent reduction of cell viability in the cultures studied (Fig. 1). After 2 DIV of exposure, CGC were more sensitive than CN to OCP-induced neuronal death for all the compounds tested except for lindane (Fig.1A-C). In addition, dieldrin and endosulfan were significantly more toxic than lindane both in CN and CGC. Table 1 and 2 summarize the half lethal concentration (LC_{50}) values for each chemical in MTT assay after 2 and 6 DIV of exposure, respectively. Similar results were obtained with LDH assay (data not shown). Unlike that observed with the shorter exposure time, the toxicity of dieldrin after 6 DIV of exposure was higher in CN than in CGC. Again, dieldrin and endosulfan were significantly more toxic than lindane in both CGC and CN. In addition, dieldrin was more toxic than endosulfan in CN (Fig. 1D-F; Table 2). Surprisingly, low concentrations of dieldrin (200-600 nM) significantly increase rather than decrease cell viability in CGC (Fig. 1D). Finally, comparison of LC50 values from tables 1 and 2 show that the neurotoxicity of all OCPs increased over time in culture

both in CGC ($p<0.01$ for dieldrin; $p<0.05$ for endosulfan and lindane) and in CN ($p<0.001$ for dieldrin and endosulfan; $p<0.05$ for lindane).

Nevertheless, the higher susceptibility of the cultured neurons against OCP-induced neurotoxicity after 6 DIV of exposure with respect to 2 DIV can be either due to the duration of the treatment or to the immaturity of the neurons when first exposed to the pesticides. In order to discriminate between these two possibilities, we treated CN with 3 μM dieldrin at DIV 2 and we determined cell viability 3 or 6 DIV later. Interestingly, when exposed to immature CN, dieldrin (3 μM) had a similar toxicity despite of the duration of the treatment (MTT values with respect to control were 35.3% ± 4.7 and 23.4% ± 3.1 for 3 and 6 DIV of exposure, respectively, N=4). In contrast, mature CN were resistant to the neuronal death induced by dieldrin (up to 10 μM) (Fig. 1A). Likewise, immature CGC were more susceptible than mature ones to the neuronal death induced by 10 μM dieldrin (MTT values with respect to control were 41.3% ± 1.8 and 60.7% ± 3.1 for exposures from DIV 2 to DIV 4 and from DIV 6 to DIV 8, respectively, $P<0.01$, N=3). As found in CN, the toxicity of dieldrin in CGC was not further increased when the duration of the exposure was prolonged up to 6 DIV (Fig 1D). These results indicate that the maturity of the cells rather than the duration of the treatment determines the toxicity of dieldrin in these cultures.

Glutamatergic neurons are more sensitive than GABAergic neurons against dieldrin toxicity

We next wanted to investigate whether the observed differences in OCP-neurotoxicity between the cultures studied might be due to different susceptibility of the neuronal types present in the cultures. Because GABAergic neurons have been described as less susceptible against dieldrin-induced cell death than dopaminergic neurons in primary cultures of mesencephalic neurons (Sanchez-Ramos *et al.*, 1998), we wondered whether glutamatergic neurons were more sensitive than GABAergic neurons to the neurotoxicity caused by dieldrin. To address this, we performed immunocitochemistry against vGLUT1 and GAD67 in order to detect glutamatergic and GABAergic neurons, respectively, present in CN. Around 50% of neurons were positive for vGLUT1 immunostaining and almost 20% were GAD67-positive neurons (Fig. 2A). Exposure to 3μM dieldrin from 2 to 5 DIV killed about 65% of the neurons, as measured by counting DAPI-positive neurons. Interestingly, 78% of the vGLUT1-positive neurons

died, while more than 60% of the GABAergic and around 45% of non stained neurons were still alive after dieldrin exposure (Fig. 2A).

OCP-induced neuronal death is prevented by co-treatment with E_2 and insulin in an Akt and ERK-dependent mechanism

Because mature CGC are more sensitive than mature CN to OCP-induced neurotoxicity, we aimed to study the mechanisms by which OCPs produce neuronal loss in these cells. Exposure for 48 h (from DIV 6 to DIV 8) to 10 μM dieldrin reduced cell viability of CGC by 40% while CN remained unaffected under the same conditions (Fig. 1A and 2B). Since the production of reactive oxygen species (ROS) has been involved in dieldrin neurotoxicity of dopaminergic neurons (Chun *et al.*, 2001; Kitazawa *et al.*, 2001; Sharma *et al.*, 2010), we tested whether dieldrin increase the levels of ROS by monitoring the fluorescence of dichlorofluoresceine (DCF) at different times (from 1 h up to 24h after dieldrin exposure) in CGC. However, dieldrin did not increase the levels of ROS with respect to DMSO-treated cells at any time of exposure tested. In addition, the antioxidant Trolox (10 μM) failed to protect CGC from dieldrin-induced neuronal death (see Supplemental Material, Figure 1). We have previously shown that lindane toxicity could be ameliorated by glutamate receptor antagonists both in CGC and CN (Vale *et al.*, 1998a; Vale *et al.*, 1998b). However, treatment with a mixture of glutamate receptor antagonists (MK-801 plus NBQX) did not prevent dieldrin-induced toxicity (see Supplemental Material, Figure 1).

E_2 and insulin have been found to be neuroprotective against several experimental paradigms and neurotoxins (Bourque *et al.*, 2009; Lee *et al.*, 2009; Mendez *et al.*, 2005; Mielke and Wang, 2005; Willaime-Morawek *et al.*, 2005). Neither 1nM E_2 nor 500 nM insulin were able to prevent dieldrin neurotoxicity when treated separately. However, co-treatment with both hormones exerted a significant neuroprotection against 10μM dieldrin-induced death in CGC (Figure 2B). Likewise, E_2 and insulin prevented the reduction of CGC viability caused by endosulfan (see Supplemental Material, Figure 1). We have recently reported that exposure to dieldrin for 1-5 h increase the phosphorylation of both Akt and ERK1/2 in CGC (Briz et al., 2011). We further studied the time dependence of kinase phosphorylation induced by dieldrin in these neurons. Dieldrin caused a sustained activation of both Akt and ERK1/2 lasting 5 h, followed by suppression 24 h after treatment (see Supplemental Material, Figure 2). Thus, we next wondered whether the activation of ER or the MAPK and PI3K/Akt pathways caused

by dieldrin could be related to its neurotoxic effects. In order to test this hypothesis, we pretreated CGC with the respective inhibitors of ER (ICI 182780, 1 μM), MAPK (U0126, 20 μM) or PI3K (LY294002, 20 μM) for 30 min and subsequently we exposed CGC to dieldrin for 48 h together with the inhibitors either in the absence or presence of E_2 and insulin. None of the three inhibitors significantly affected the viability of control (data not shown) or dieldrin-treated cells (Figure 2C). However, the neuroprotection afforded by the simultaneous treatment with E_2 and insulin was prevented by U0126 and LY294002, suggesting that activation of both MAPK and PI3K/Akt pathways are required for the cooperative neuroprotection of the two hormones. Pretreatment with the ER antagonist ICI 182780 reduced the neuroprotective effects of E_2 and insulin, but the effect did not reach statistical significance (Figure 2C).

Subsequently, we determined the phosphorylation state of ERK1/2 and Akt 24 h after dieldrin exposure in the presence of both E_2 and insulin in order to confirm the involvement of these signalling pathways in hormone mediated-neuroprotection. Exposure to 10 μM dieldrin for 24 h reduced the levels of phospho-Akt (Fig 3A) and phospho-ERK1/2 (Fig. 4A). Treatment with either E_2 or insulin alone did not reverse this effect, but when combined they significantly restored the basal levels of kinase activation (Fig 3A and 4A). Similar effects were observed for endosulfan (see Supplemental Material, Figure 2). Consistent with this, immunostaining of pAkt and pERK was reduced after dieldrin or endosulfan exposure and hormone treatment prevented this effect (Fig. 3B and 4B). These results confirm that the combined activation of Akt and MAPK pathways by E_2 and insulin is necessary to prevent dieldrin-induced cell death.

E_2 and insulin inhibited OCP-induced caspase-3 activation and apoptosis.

Because alterations of Akt and MAPK pathways may lead to apoptosis in CGC and other cells (La Sala *et al.*, 2009; Subramaniam *et al.*, 2005; Willaime-Morawek *et al.*, 2005), we wanted to address whether OCP-induced neuronal death of CGC was apoptotic and caspase-dependent or not. We found that dieldrin and endosulfan increased the number of condensed nuclei after 24 h of exposure, indicating that the neuronal death induced by these OCPs had apoptotic features. Treatment with E_2 and insulin reduced the number of apoptotic nuclei after OCP exposure (Fig. 5A). Also, we measured caspase and calpain activities by immunoblot against α-spectrin. This protein can be differently degraded by caspases and calpains/cathepsins and as a result we

would obtain proteolytic fragments of ~120 KDa or ~150 KDa, respectively. Exposure to dieldrin and endosulfan for 24 h strongly increased caspase-dependent α-spectrin proteolysis. In contrast, calpain/cathepsin activity remains unaffected by OCPs (Fig. 5B). In addition, we found that both dieldrin and endosulfan increased the cleavage of caspase-3, which was prevented by E_2 and insulin treatment (Fig. 5C).

DISCUSSION

The present study shows that primary cultures of CN and CGC represent two highly sensitive neuronal populations against the toxicity induced by long-term exposure to OCPs. To the best of our knowledge, this is the first study demonstrating the selective toxicity of OCPs for glutamatergic neurons. We have previously reported that long-term exposure to dieldrin disrupts the development of glutamatergic neurotransmission as a consequence of permanent $GABA_A$ receptor inhibition both in CN and CGC. However, the effects of this pesticide in CN were observed at concentration 50 times lower than in CGC (Babot *et al.*, 2007; Briz *et al.*, 2010). Thus, the higher susceptibility of CN vs. CGC against dieldrin-induced neurotoxicity after 6 DIV of exposure is likely related to its different efficiency in affecting GABAergic and glutamatergic neurotransmission in these cultures. Supporting this, recent findings from our laboratory indicate that the neurotoxic effects of dieldrin in CN are due to its $GABA_A$ receptor-dependent negative modulation of NMDA receptors (manuscript in preparation). Conversely, exposure to low concentrations of dieldrin for 6 DIV slightly increased immature CGC viability rather than reduced it. This might be explained by the fact that, unlike CN, CGC are grown in high potassium medium to maintain a mild but continue depolarization, necessary for their survival. In this sense, partial blockade of the $GABA_A$ receptor by 200-600 nM dieldrin (Vale *et al.*, 2003) would initially produce a slight hyperactivation of the glutamatergic system that may contribute to the activity-dependent survival of CGC. On the other hand, chronic exposure to E2 in immature CGC has been shown to increase their survival through ER-dependent transcriptional activity, whereas pulse treatment with E2 is toxic in developing CGC (Wong *et al.*, 2003). Since dieldrin is able to activate the ER in these cells at lower concentrations than in CN (Briz *et al.*, 2011), another possibility is that long-term exposure to low concentrations of dieldrin could mimics the neuroprotective effects of E2 under these conditions.

Several studies have shown that monoaminergic (dopaminergic and serotonergic) neurones are selectively sensitive to dieldrin-induced cytotoxicity after acute and prolonged exposure (Kitazawa *et al.*, 2001; Liu *et al.*, 1997; Sanchez-Ramos *et al.*, 1998). Here, we show that mature CGC, which are predominantly glutamatergic neurons (Sonnewald *et al.*, 2004), constituted a highly sensitive neuronal population against OCP toxicity, with LC_{50} values (~10 μM) similar to those previously observed in monoaminergic neurons (Chan *et al.*, 2006; Liu *et al.*, 1997; Sanchez-Ramos *et al.*, 1998). Instead, mature CN were more resistant to OCP exposure, probably because they are enriched in GABAergic neurons, which have been described as less sensitive to dieldrin- and NMDA-mediated cell death (Kitazawa *et al.*, 2001; Liu *et al.*, 1997; Sanchez-Ramos *et al.*, 1998; Tecoma and Choi, 1989). Consistent with this, we now show that cortical glutamatergic neurons were more susceptible than GABAergic neurons to dieldrin toxicity. It is worth noting that the susceptibility of both cortical and cerebellar glutamatergic neurons to dieldrin-induced cell death is strongly dependent on the day of treatment, being much more sensitive when treated within the firsts DIV. Synaptic activity has a crucial role in the survival and differentiation of developing neurons and determine the formation and strengthening of neuronal networks. Alterations of $GABA_A$ and glutamate receptor expression and function as that produced by long-term exposure to dieldrin in our cultures (Babot *et al.*, 2007; Briz *et al.*, 2010), may underlie the higher susceptibility of immature CGC and CN against OCP neurotoxicity. Likewise, dieldrin has been shown to disrupt the GABAergic-dependent development of monoamine neurons by modulating the expression of monoamine transporters and receptors (Liu *et al.*, 1997; Slotkin and Seidler, 2008). Since GABA and glutamate neurotransmission are the main inhibitory and excitatory neuronal systems, respectively, prenatal and/or early postnatal exposure to OCPs may have a strong impact in the development of the CNS.

As we previously described for shorter exposure times, the neurotoxic potency of lindane was similar between CGC and CN, although it is apparently related to different modulation of $GABA_A$ and $GABA_B$ receptors (Vale *et al.*, 1998a; Vale *et al.*, 1998b). Also, we have demonstrated that lindane toxicity of CGC is Ca-dependent and it can be prevented by glutamate receptor antagonists, whereas that of CN is Ca-independent (Pomes *et al.*, 1994a; Rosa *et al.*, 1997). Instead, the mechanisms of neurotoxicity for dieldrin and endosulfan in our cultures are not yet fully understood. Because both

dieldrin and endosulfan are more potent inhibitors than lindane at the $GABA_A$ receptor (Vale *et al.*, 2003), their higher toxicity with respect to lindane observed in the present work (Table 1 and 2) might be indirectly a consequence of excitotoxicity as a result of $GABA_A$ receptor blockade. However, a cocktail of glutamate receptor antagonists failed to prevent dieldrin-induced neuronal death in CGC. On the contrary, our previous observations indicate that $GABA_A$ receptor antagonists (including dieldrin) prevent rather than accentuated glutamate-mediated excitotoxicity (Babot *et al.*, 2005; Babot *et al.*, 2007; Briz *et al.*, 2010).

The generation of ROS is well known to be involved in OCP toxicity of dopaminergic neurons (Chun *et al.*, 2001; Jia and Misra, 2007; Kitazawa *et al.*, 2001; Sharma *et al.*, 2010). However, in our system dieldrin did not cause oxidative stress, at least within the first 24 h of exposure. Moreover, the antioxidant trolox was unable to protect CGC from dieldrin toxicity. Similarly, (Slotkin and Seidler, 2010) recently found that antioxidants can prevent the oxidative stress but failed to prevent cell loss caused by dieldrin in PC12 cells, suggesting that additional mechanisms should be implicated in dieldrin neurotoxicity. We have recently reported that OCPs have different effects on Akt and ERK1/2 phosphorylation within short exposure times (< 6 h) through their interaction with ERs in CGC. Moreover, dieldrin has higher affinity for ER and it is more effective in triggering Akt and ERK1/2 activation than endosulfan and lindane in these cells (Briz *et al.*, 2011). Here, the time-dependence of kinase activation followed an inverted U-shape curve within the first hours and felt below the basal levels 24 h after pesticide treatment. It is well known that physiological activation of these signaling pathways plays an important role in neuronal survival. Instead, their disruption has been associated with the neuronal damage observed under pathological conditions. For instance, low-potassium containing-medium is a commonly used model to trigger apoptosis in CGC, and it has been associated with reduction of Akt phosphorylation and with delayed ERK1/2 phosphorylation (Subramaniam *et al.*, 2005). Moreover, a sustained inhibition or persistent activation of these kinases has been related to the cell death produced by several environmental pollutants, including pesticides (Bourque *et al.*, 2009; Du *et al.*, 2002; Kang *et al.*, 2001; La Sala *et al.*, 2009; Tan *et al.*, 2009). Prolonged activation of PI3K, ERK or ER appear not to be involved in dieldrin neurotoxicity since the specific inhibitors of these proteins did not rescue CGC from dieldrin-mediated cell death. In contrast, the results presented here suggest that the

reduction of Akt and ERK1/2 phosphorylation observed 24 h after dieldrin and endosulfan exposure could be responsible, at least in part, for the neuronal death observed 24 h later (2 DIV after OCP exposure). According to that previously described in cell lines (Jia and Misra, 2007; Kanthasamy *et al.*, 2003), dieldrin and endosulfan caused caspase-3 activation and apoptosis in our neuronal cultures. In contrast, lysosomal-dependent necrosis or autophagy seem not to be involved in OCP-induced cell death since calpain/cathepsin activity was unaffected by either dieldrin or endosulfan treatment. In dopaminergic neurons, dieldrin-induced mithocondrial failure and DNA damage involves activation of protein kinase C-δ and can be prevented by overexpression of Bcl-2 (Kanthasamy *et al.*, 2003). Similarly, the negative modulation of Akt and ERK1/2 activity may lead to apoptosis through different regulation of pro- and anti-apoptotic mithocondrial proteins in CGC (Barneda-Zahonero *et al.*, 2009; Linseman *et al.*, 2002). Thus, although the signalling pathways associated with OCP-induce apoptosis are apparently different between CGC and dopaminergic neurons, they converge to activate caspase-3-dependent neuronal death.

The present study shows that E_2 and insulin cooperatively protect CGC from dieldrin-induced apoptotic cell death and this effect is PI3K- and ERK-dependent but ER-independent. Likewise, E_2 and tamoxifen neuroprotection against manganese toxicity has been reported to be ER-independent but associated with the activation of PI3K/Akt and MAPK pathways (Lee *et al.*, 2009). In addition, several studies have shown that the PI3K/Akt and MAPK pathways mediate the pro-survival effects of E_2 and/or insulin/IGF-I against different neurotoxic insults both *in vivo* and *in vitro* (Bourque *et al.*, 2009; Numakawa *et al.*, 2007; Subramaniam *et al.*, 2005; Willaime-Morawek *et al.*, 2005; Zhao and Brinton, 2007). Furthermore, a cross talk between E_2 and IGF-I signaling through these pathways has emerged as a crucial point of convergence by which the two hormones modulate different functions in the brain, including neuronal survival (Mendez *et al.*, 2005). In addition, E_2 and insulin have been shown to increase GABA levels and $GABA_A$ receptor cell membrane expression in hippocampal neurons, respectively (Mielke and Wang, 2005; Shen *et al.*, 2005; Zhou *et al.*, 2007). The positive effect of insulin on $GABA_A$ receptors is apparently related to Akt activation (Fujii et al., 2010) and is responsible for its neuroprotective action against oxygen-glucose deprivation in vitro (Mielke and Wang, 2005), whereas E2 prevent bicuculline-induced spine loss by restoring GABA synthesis (Fujii *et al.*, 2010; Zhou *et al.*, 2007).

Thus, it is possible that insulin and E_2 cooperatively increase GABAergic neurotransmission and this in turn may counteract the permanent inhibition of $GABA_A$ receptor and probably the neuronal death caused by prolonged exposure to dieldrin. Overall, the alterations in GABA and glutamate neuronal systems described in the present study may contribute to the cognitive and behavioural deficits reported in animals chronically exposed to OCPs (Mariussen and Fonnum, 2006; Paul *et al.*, 1994; Schantz and Widholm, 2001; Tilson *et al.*, 1987; Topinka *et al.*, 1984). Further, the beneficial effects of E_2 and insulin against OCP neurotoxicity support their potential therapeutic use against other neurotoxins and neurodegenerative diseases in which the PI3K/Akt and MAPK pathways are involved.

MATERIALS AND METHODS

Materials

Pregnant NMRI mice (16^{th} gestational day) and mice pups (7^{th} postnatal day) were obtained from Charles River, Iffa Credo (Saint Germain-sur-l'Arbreste, France). Plastic multiwell plates were from Nunc™ (Rockilde, Denmark). Fetal calf serum was obtained from Gibco (Invitrogen, Barcelona, Spain). Dulbecco's modified Eagle's minimum essential medium (DMEM) was from Biochrom (Berlin, Germany). Isofluorane (FORANE®) was from Abbot Laboratories (Madrid, Spain). Trypsin, soybean trypsin inhibitor, DNAse, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), dieldrin, α-endosulfan, 17β-estradiol (E_2), insulin, picrotoxinin, (+)-MK-801 hydrogen maleate (MK-801), LY294002 and sulphorhodamine-B (SRB) were from Sigma (St. Louis, MO, USA). β-HCH was from LGC (Teddington Middlesex, U.K.). Lindane was from the Institute of Industrial Organic Chemistry (Warsaw, Poland). ICI 182780 (ICI) and 2,3-dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[f]quinoxaline-7-sulfonamide (NBQX) were from Tocris Cookson (Bristol, U.K.). U0126 was from Calbiochem (EMD Bioscience Inc., La Jolla, CA, USA).

Neuronal Cultures

Primary cultures of cortical neurons (CN) and cerebellar granule cells (CGC) were prepared from cerebral cortices of embryonic day 18 mice fetuses and from cerebellum of 7-day-old mice pups respectively as we previously described (Briz *et al.*, 2011).

Briefly, pregnant animals were anesthetized with isofluorane, killed by cervical dislocation and fetuses extracted. Cortices were dissected with forceps, mechanically minced and cells were then dissociated by mild trypsinization [0.02% (w/v)] at 37ºC for 10 min followed by trituration in a DNAse solution [0.004% (w/v)] containing soybean trypsin inhibitor (0.05% [w/v)]. The cells were then suspended in DMEM containing 5 mM KCl, 31 mM glucose and 0.2 mM glutamine supplemented with p-aminobenzoate, insulin, penicillin and 10% foetal calf serum. The cell suspension (1.5 x 10^6 cells/ml) was seeded in 6-, 24- or 96-well plates precoated with poly-D-lysine and incubated for at least 8 days in a humidified 5% CO_2/95% air atmosphere at 36.8ºC. A mixture of 5 µM 5-fluoro-2'-deoxyuridine and 20 µM uridine was added after 1-2 days *in vitro* (DIV) to prevent glial proliferation. In the case of CGC, cerebellum was removed after decapitation and cells were dissociated by mild trypsinization. From this step the procedure is the same as for CN, but DMEM contains 25 mM KCl. Animals were handled in compliance with protocol DMA1852 of the University of Barcelona, approved by the Generalitat de Cataluña, following the EU guidelines.

Chemical Treatments

Stock solutions for each compound were prepared in DMSO and frozen in aliquots of 100 µl. The final concentration of DMSO in the culture medium was <0.5%. To avoid cross-contamination between different wells in the same plate, DMSO or pesticide treatments were applied on separate plates. Cultured neurons were treated with OCPs by adding the stock pesticide solution to the culture medium at day *in vitro* (DIV) 1-2 (when cultured neurons are still immature) or DIV 6 (when cultured neurons are considered mature) (Frandsen and Schousboe, 1990). The medium was not change until the experiments were performed at 7-8 DIV (exposure times referred as exposure for 6 and 2 DIV, respectively). In some experiments neurons were treated at DIV 1-2 and the experiments were performed at 4-5 DIV. Pre-treatments with ICI and the inhibitors of kinases (U0126 and LY294002) were performed in Hank's solution (1.3mM CaCl, 5.4mM KCl, 0.4mM KH_2PO_4, 0.5mM MgCl, 0.4mM $MgSO_4$, 137mM NaCl, 4.2mM $NaHCO_3$, 0.3mM Na_2HPO_4, 8mM HEPES, 5.5mM glucose, adjusted to pH 7.4) and after washing them out, the culture medium containing the indicated treatments was replaced.

Cytotoxicity

Cell viability was assessed by measuring the reduction of the colored formazan salt MTT by mithocondrial activity, whereas cytotoxicity was assessed by quantifying the release of the cytosolic enzyme lactate dehydrogenase (LDH) as described by (Briz *et al.*, 2010). The methods were used simultaneously for every experiment. In some experiments, cell counting was also used to determine the number of live/death neurons, e.g. DAPI-positive neurons or ToPro-3-positive neurons (see below).

Immunocytochemistry

vGLUT1 and GAD67 immunostaining. Cells were grown in 24-well plates and immunostaining was performed as previously described by (Briz *et al.*, 2010), with modifications. Cultures were rinsed with PBS, fixed in 4% PFA for 15 min and after washing out they were incubated with methanol at -20ºC for 10 min. Cells were blocked in 10% FBS in PBS for 30 min at room temperature. Subsequently, cultures were incubated overnight at 4ºC with primary guinea pig anti-vGLUT1 (1:2000) and mouse anti-GAD67 (1:500) antibodies in a solution containing 5% BSA in PBS (PBS-BSA) and rinsed three times with PBS for 5 min. Cells were first incubated with PBS-BSA containing biotinylated protein A (1µg/ml) for 2 h at room temperature and then incubated with PBS-BSA containing secondary streptavidine-conjugated Alexa 594 (1:1000; Molecular Probes) antibody for another 2 h at room temperature. After three rinses with PBS, cells were incubated with secondary donkey anti-mouse Alexa 488 (1:1000; Molecular Probes). After washing out with PBS, cells were incubated with DAPI (1:10000) for 15 min at room temperature. The immunostained cells were examined in a fluorescence microscope (Nikon). Cell counting was performed in five different fields (using an objective 20X) per well, and 4-5 different wells for each treatment were used for quantification. Results were expressed as percentage of stained neurons with respect to total number of neurons (DAPI-positive neurons).

phospho-Akt and phospho-ERK1/2 immunostaining. Cells were grown in Permanox chamber slides (Nunc) treated with poly-D-lysine and immunostaining was performed as described above, with little modifications. Cells were blocked in 10% FBS in PBS for 1 h at room temperature and then incubated overnight at 4ºC with primary rabbit anti-phospho-Akt (Ser473) or anti-phospho-p44/42 MAPK (Thr202/Tyr204) antibodies (both 1:200, Cell Signaling, Danvers, MA) prepared in PBS-BSA. Cells were incubated with secondary goat anti-rabbit Alexa 488 (1:1000; Molecular Probes) antibody prepared in PBS-BSA for 2 h and then with ToPro-3 (1:10000, Invitrogen) for 20 min at

room temperature. After rinsing with PBS, the slides were coverslipped with Mowiol. The immunostained cells were examined in a confocal fluorescence microscope (Leica microsystems) using the same excitation laser intensity for control, dieldrin- and hormone-treated cells. Apoptotic nuclei counting was performed in four different fields (using an objective 20X) per well and 2-3 different wells for each treatment were used for quantification. Results were expressed as percentage of condensed nuclei with respect to total number of nuclei (ToPro-3-positive neurons).

Western Blot

Neuronal cultures grown in 6-well plates were washed twice with cold Hank's solution and cells were harvested with 0.2 ml of loading buffer [62.5mM Tris-HCl (pH 6.8), 10% glycerol, 2% SDS and 50mM dithiothreitol] and briefly sonicated. After boiling for 5 min and being centrifuged at 16100 x g for 5 min , 25 µl of the homogenate was subjected to SDS-PAGE using 10-12% polyacrilamide resolving gel at 60 mA for 1.5-2 h. Proteins were transferred into a nitrocellulose membrane and incubated with 5% nonfat dry milk in TBS-T (20mM Tris-HCl (pH 7.6), 140mM NaCl, 0.1% Tween-20). Membranes were incubated overnight at 4ºC with the following primary antibodies: rabbit polyclonal anti-Akt, rabbit monoclonal anti-p44/42 MAPK, anti-phospho-Akt (Ser473), anti-phospho-p44/42 MAPK (Thr202/Tyr204) (All 1:2000, Cell Signaling). All primary antibodies were diluted in TBS-T containing 5% bovine serum albumin (BSA), except anti-ERβ which was diluted in TBS-T containing 5% nonfat dry milk. After the membranes were washed, they were incubated for 1 h with anti-rabbit horseradish peroxidase-conjugated (HRP) secondary antibody (1:4000, Jackson InmunoResearch, West Grove, PA). On all the membranes, anti-GAPDH (1:4000, Assay Designs, Ann Arbor, MI) and a secondary HRP-linked anti-mouse (1:8000, Jackson InmunoResearch) antibodies were used as a control of the amount of protein loaded. The membranes were washed and incubated for 4 min in a chemiluminescent solution (Immun-Star HRP Kit, Bio-Rad, Hercules, CA). Luminescence was quantified with a Versadoc Imagine System (Bio-Rad). Digital images were then quantified in the Quantity One software (Bio-Rad).

Data analysis

Data are shown as mean ± SE. Unless otherwise stated, at least three experiments from independent culture batches were performed, each one in triplicate. Individual dose-response curves were fitted using the sigmoid dose-response function of a graphics and

statistics software package (Graph-Pad Prism, version 4.0, 2003, Graph-Pad Software Inc., San Diego, CA, USA). Statistical comparisons were made by *t*-test when comparing two groups, one-way ANOVA followed by Dunnett's post-comparison test when comparing more than two groups, and two-way ANOVA followed by Bonferroni post-test when comparing two factors. Differences were considered statistically significant when $P < 0.05$ (95% confidence).

REFERENCES

Akama KT and McEwen BS. 2003. Estrogen stimulates postsynaptic density-95 rapid protein synthesis via the Akt/protein kinase B pathway. J Neurosci 23:2333-2339.

Babot Z, Cristofol R, Sunol C. 2005. Excitotoxic death induced by released glutamate in depolarized primary cultures of mouse cerebellar granule cells is dependent on GABAA receptors and niflumic acid-sensitive chloride channels. Eur J Neurosci 21:103-112.

Babot Z, Vilaro MT, Sunol C. 2007. Long-term exposure to dieldrin reduces gamma-aminobutyric acid type A and N-methyl-D-aspartate receptor function in primary cultures of mouse cerebellar granule cells. J Neurosci Res 85:3687-3695.

Barneda-Zahonero B, Minano-Molina A, Badiola N, Fado R, Xifro X, Saura CA et al. 2009. Bone morphogenetic protein-6 promotes cerebellar granule neurons survival by activation of the MEK/ERK/CREB pathway. Mol Biol Cell 20:5051-5063.

Bourque M, Dluzen DE, Di Paolo T. 2009. Neuroprotective actions of sex steroids in Parkinson's disease. Front Neuroendocrinol 30:142-157.

Bouvier G, Blanchard O, Momas I, Seta N. 2006. Pesticide exposure of non-occupationally exposed subjects compared to some occupational exposure: a French pilot study. Sci Total Environ 366:74-91.

Briz V, Galofre M, Sunol C. 2010. Reduction of glutamatergic neurotransmission by prolonged exposure to dieldrin involves NMDA receptor internalization and metabotropic glutamate receptor 5 downregulation. Toxicol Sci 113:138-149.

Briz V, Molina-Molina JM, Sanchez-Redondo S, Fernandez MF, Grimalt JO, Olea N et al. 2011. Differential estrogenic effects of the persistent organochlorine pesticides dieldrin, endosulfan, and lindane in primary neuronal cultures. Toxicol Sci 120:413-427.

Bulayeva NN and Watson CS. 2004. Xenoestrogen-induced ERK-1 and ERK-2 activation via multiple membrane-initiated signaling pathways. Environ Health Perspect 112:1481-1487.

Cabaleiro T, Caride A, Romero A, Lafuente A. 2008. Effects of in utero and lactational exposure to endosulfan in prefrontal cortex of male rats. Toxicol Lett 176:58-67.

Chan MP, Morisawa S, Nakayama A, Kawamoto Y, Yoneda M. 2006. Development of an in vitro blood-brain barrier model to study the effects of endosulfan on the permeability of tight junctions and a

comparative study of the cytotoxic effects of endosulfan on rat and human glial and neuronal cell cultures. Environ Toxicol 21:223-235.

Chopra AK, Sharma MK, Chamoli S. 2011. Bioaccumulation of organochlorine pesticides in aquatic system--an overview. Environ Monit Assess 173:905-916.

Chun HS, Gibson GE, DeGiorgio LA, Zhang H, Kidd VJ, Son JH. 2001. Dopaminergic cell death induced by MPP(+), oxidant and specific neurotoxicants shares the common molecular mechanism. J Neurochem 76:1010-1021.

Corrigan FM, Wienburg CL, Shore RF, Daniel SE, Mann D. 2000. Organochlorine insecticides in substantia nigra in Parkinson's disease. J Toxicol Environ Health A 59:229-234.

Du S, McLaughlin B, Pal S, Aizenman E. 2002. In vitro neurotoxicity of methylisothiazolinone, a commonly used industrial and household biocide, proceeds via a zinc and extracellular signal-regulated kinase mitogen-activated protein kinase-dependent pathway. J Neurosci 22:7408-7416.

Frandsen A and Schousboe A. 1990. Development of excitatory amino acid induced cytotoxicity in cultured neurons. Int J Dev Neurosci 8:209-216.

Fujii M, Kanematsu T, Ishibashi H, Fukami K, Takenawa T, Nakayama KI et al. 2010. Phospholipase C-related but catalytically inactive protein is required for insulin-induced cell surface expression of gamma-aminobutyric acid type A receptors. J Biol Chem 285:4837-4846.

Galofre M, Babot Z, Garcia DA, Iraola S, Rodriguez-Farre E, Forsby A et al. 2010. GABAA receptor and cell membrane potential as functional endpoints in cultured neurons to evaluate chemicals for human acute toxicity. Neurotoxicol Teratol 32:52-61.

Garcia-Segura LM, Azcoitia I, DonCarlos LL. 2001. Neuroprotection by estradiol. Prog Neurobiol 63:29-60.

Jia Z and Misra HP. 2007. Reactive oxygen species in in vitro pesticide-induced neuronal cell (SH-SY5Y) cytotoxicity: role of NFkappaB and caspase-3. Free Radic Biol Med 42:288-298.

Kang KS, Park JE, Ryu DY, Lee YS. 2001. Effects and neuro-toxic mechanisms of 2, 2', 4, 4', 5, 5'-hexachlorobiphenyl and endosulfan in neuronal stem cells. J Vet Med Sci 63:1183-1190.

Kanthasamy AG, Kitazawa M, Kaul S, Yang Y, Lahiri DK, Anantharam V et al. 2003. Proteolytic activation of proapoptotic kinase PKCdelta is regulated by overexpression of Bcl-2: implications for oxidative stress and environmental factors in Parkinson's disease. Ann N Y Acad Sci 1010:683-686.

Kinney JW, Davis CN, Tabarean I, Conti B, Bartfai T, Behrens MM. 2006. A specific role for NR2A-containing NMDA receptors in the maintenance of parvalbumin and GAD67 immunoreactivity in cultured interneurons. J Neurosci 26:1604-1615.

Kitazawa M, Anantharam V, Kanthasamy AG. 2001. Dieldrin-induced oxidative stress and neurochemical changes contribute to apoptopic cell death in dopaminergic cells. Free Radic Biol Med 31:1473-1485.

La Sala G, Farini D, De Felici M. 2009. Proapoptotic effects of lindane on mouse primordial germ cells. Toxicol Sci 108:445-451.

Lawrence LJ and Casida JE. 1984. Interactions of lindane, toxaphene and cyclodienes with brain-specific t-butylbicyclophosphorothionate receptor. Life Sci 35:171-178.

Lee ES, Yin Z, Milatovic D, Jiang H, Aschner M. 2009. Estrogen and tamoxifen protect against Mn-induced toxicity in rat cortical primary cultures of neurons and astrocytes. Toxicol Sci 110:156-167.

Lemaire G, Mnif W, Mauvais P, Balaguer P, Rahmani R. 2006. Activation of alpha- and beta-estrogen receptors by persistent pesticides in reporter cell lines. Life Sci 79:1160-1169.

Linseman DA, Phelps RA, Bouchard RJ, Le SS, Laessig TA, McClure ML et al. 2002. Insulin-like growth factor-I blocks Bcl-2 interacting mediator of cell death (Bim) induction and intrinsic death signaling in cerebellar granule neurons. J Neurosci 22:9287-9297.

Liu J, Morrow AL, Devaud L, Grayson DR, Lauder JM. 1997. GABAA receptors mediate trophic effects of GABA on embryonic brainstem monoamine neurons in vitro. J Neurosci 17:2420-2428.

Maranghi F, Rescia M, Macri C, Di Consiglio E, De Angelis G, Testai E et al. 2007. Lindane may modulate the female reproductive development through the interaction with ER-beta: an in vivo-in vitro approach. Chem Biol Interact 169:1-14.

Mariscal-Arcas M, Lopez-Martinez C, Granada A, Olea N, Lorenzo-Tovar ML, Olea-Serrano F. 2010. Organochlorine pesticides in umbilical cord blood serum of women from Southern Spain and adherence to the Mediterranean diet. Food Chem Toxicol 48:1311-1315.

Mariussen E and Fonnum F. 2006. Neurochemical targets and behavioral effects of organohalogen compounds: an update. Crit Rev Toxicol 36:253-289.

McEwen BS, Gould E, Orchinik M, Weiland NG, Woolley CS. 1995. Oestrogens and the structural and functional plasticity of neurons: implications for memory, ageing and neurodegenerative processes. Ciba Found Symp 191:52-66.

Mendez P, Azcoitia I, Garcia-Segura LM. 2005. Interdependence of oestrogen and insulin-like growth factor-I in the brain: potential for analysing neuroprotective mechanisms. J Endocrinol 185:11-17.

Micevych P and Dominguez R. 2009. Membrane estradiol signaling in the brain. Front Neuroendocrinol 30:315-327.

Mielke JG and Wang YT. 2005. Insulin exerts neuroprotection by counteracting the decrease in cell-surface GABA receptors following oxygen-glucose deprivation in cultured cortical neurons. J Neurochem 92:103-113.

Numakawa Y, Matsumoto T, Yokomaku D, Taguchi T, Niki E, Hatanaka H et al. 2007. 17beta-estradiol protects cortical neurons against oxidative stress-induced cell death through reduction in the activity of mitogen-activated protein kinase and in the accumulation of intracellular calcium. Endocrinology 148:627-637.

Ogiue-Ikeda M, Tanabe N, Mukai H, Hojo Y, Murakami G, Tsurugizawa T et al. 2008. Rapid modulation of synaptic plasticity by estrogens as well as endocrine disrupters in hippocampal neurons. Brain Res Rev 57:363-375.

Paul V, Balasubramaniam E, Kazi M. 1994. The neurobehavioural toxicity of endosulfan in rats: a serotonergic involvement in learning impairment. Eur J Pharmacol 270:1-7.

Pomes A, Frandsen A, Sunol C, Sanfeliu C, Rodriguez-Farre E, Schousboe A. 1994a. Lindane cytotoxicity in cultured neocortical neurons is ameliorated by GABA and flunitrazepam. J Neurosci Res 39:663-668.

Pomes A, Rodriguez-Farre E, Sunol C. 1993. Inhibition of t-[35S]butylbicyclophosphorothionate binding by convulsant agents in primary cultures of cerebellar neurons. Brain Res Dev Brain Res 73:85-90.

Pomes A, Rodriguez-Farre E, Sunol C. 1994b. Disruption of GABA-dependent chloride flux by cyclodienes and hexachlorocyclohexanes in primary cultures of cortical neurons. J Pharmacol Exp Ther 271:1616-1623.

Rosa R, Sanfeliu C, Sunol C, Pomes A, Rodriguez-Farre E, Schousboe A et al. 1997. The mechanism for hexachlorocyclohexane-induced cytotoxicity and changes in intracellular Ca2+ homeostasis in cultured cerebellar granule neurons is different for the gamma- and delta-isomers. Toxicol Appl Pharmacol 142:31-39.

Sanchez-Ramos J, Facca A, Basit A, Song S. 1998. Toxicity of dieldrin for dopaminergic neurons in mesencephalic cultures. Exp Neurol 150:263-271.

Schafer KS and Kegley SE. 2002. Persistent toxic chemicals in the US food supply. J Epidemiol Community Health 56:813-817.

Schantz SL and Widholm JJ. 2001. Cognitive effects of endocrine-disrupting chemicals in animals. Environ Health Perspect 109:1197-1206.

Schwarz JM and McCarthy MM. 2008. Steroid-induced sexual differentiation of the developing brain: multiple pathways, one goal. J Neurochem 105:1561-1572.

Sharma H, Zhang P, Barber DS, Liu B. 2010. Organochlorine pesticides dieldrin and lindane induce cooperative toxicity in dopaminergic neurons: role of oxidative stress. Neurotoxicology 31:215-222.

Shen H, Gong QH, Yuan M, Smith SS. 2005. Short-term steroid treatment increases delta GABAA receptor subunit expression in rat CA1 hippocampus: pharmacological and behavioral effects. Neuropharmacology 49:573-586.

Slotkin TA and Seidler FJ. 2008. Developmental neurotoxicants target neurodifferentiation into the serotonin phenotype: Chlorpyrifos, diazinon, dieldrin and divalent nickel. Toxicol Appl Pharmacol 233:211-219.

Slotkin TA and Seidler FJ. 2009. Oxidative and excitatory mechanisms of developmental neurotoxicity: transcriptional profiles for chlorpyrifos, diazinon, dieldrin, and divalent nickel in PC12 cells. Environ Health Perspect 117:587-596.

Slotkin TA and Seidler FJ. 2010. Oxidative stress from diverse developmental neurotoxicants: antioxidants protect against lipid peroxidation without preventing cell loss. Neurotoxicol Teratol 32:124-131.

Sonnewald U, Olstad E, Qu H, Babot Z, Cristofol R, Sunol C et al. 2004. First direct demonstration of extensive GABA synthesis in mouse cerebellar neuronal cultures. J Neurochem 91:796-803.

Soto AM, Chung KL, Sonnenschein C. 1994. The pesticides endosulfan, toxaphene, and dieldrin have estrogenic effects on human estrogen-sensitive cells. Environ Health Perspect 102:380-383.

Subramaniam S, Shahani N, Strelau J, Laliberte C, Brandt R, Kaplan D et al. 2005. Insulin-like growth factor 1 inhibits extracellular signal-regulated kinase to promote neuronal survival via the phosphatidylinositol 3-kinase/protein kinase A/c-Raf pathway. J Neurosci 25:2838-2852.

Sunol C, Babot Z, Fonfria E, Galofre M, Garcia D, Herrera N et al. 2008. Studies with neuronal cells: From basic studies of mechanisms of neurotoxicity to the prediction of chemical toxicity. Toxicol In Vitro 22:1350-1355.

Tan DH, Peng SQ, Wu YL, Wang YM, Lu CF, Ding W et al. 2009. Chlorpyrifos induces delayed cytotoxicity after withdrawal in primary hippocampal neurons through extracellular signal-regulated kinase inhibition. Biol Pharm Bull 32:1649-1655.

Tecoma ES and Choi DW. 1989. GABAergic neocortical neurons are resistant to NMDA receptor-mediated injury. Neurology 39:676-682.

Thomas WE. 1985. Morphology of acetylcholinesterase-containing neurons in primary cultures of dissociated rat cerebral cortex. Brain Res 361:392-395.

Tilson HA, Shaw S, McLamb RL. 1987. The effects of lindane, DDT, and chlordecone on avoidance responding and seizure activity. Toxicol Appl Pharmacol 88:57-65.

Topinka MA, Eisenstein EM, Siegel D, Trofeli R, Barraco D. 1984. The effects of dieldrin and chlordimeform on learning and memory in the cockroach, Periplaneta americana: a study in behavioral toxicology. J Toxicol Environ Health 13:705-719.

Vale C, Damgaard I, Sunol C, Rodriguez-Farre E, Schousboe A. 1998a. Cytotoxic action of lindane in cerebellar granule neurons is mediated by interaction with inducible GABA(B) receptors. J Neurosci Res 52:286-294.

Vale C, Damgaard I, Sunol C, Rodriguez-Farre E, Schousboe A. 1998b. Cytotoxic action of lindane in neocortical GABAergic neurons is primarily mediated by interaction with flunitrazepam-sensitive GABA(A) receptors. J Neurosci Res 52:276-285.

Vale C, Fonfria E, Bujons J, Messeguer A, Rodriguez-Farre E, Sunol C. 2003. The organochlorine pesticides gamma-hexachlorocyclohexane (lindane), alpha-endosulfan and dieldrin differentially interact with GABA(A) and glycine-gated chloride channels in primary cultures of cerebellar granule cells. Neuroscience 117:397-403.

Willaime-Morawek S, Arbez N, Mariani J, Brugg B. 2005. IGF-I protects cortical neurons against ceramide-induced apoptosis via activation of the PI-3K/Akt and ERK pathways; is this protection independent of CREB and Bcl-2? Brain Res Mol Brain Res 142:97-106.

Wong JK, Le HH, Zsarnovszky A, Belcher SM. 2003. Estrogens and ICI182,780 (Faslodex) modulate mitosis and cell death in immature cerebellar neurons via rapid activation of p44/p42 mitogen-activated protein kinase. J Neurosci 23:4984-4995.

Zhao L and Brinton RD. 2007. Estrogen receptor alpha and beta differentially regulate intracellular Ca(2+) dynamics leading to ERK phosphorylation and estrogen neuroprotection in hippocampal neurons. Brain Res 1172:48-59.

Zhou L, Lehan N, Wehrenberg U, Disteldorf E, von Lossow R, Mares U et al. 2007. Neuroprotection by estradiol: a role of aromatase against spine synapse loss after blockade of GABA(A) receptors. Exp Neurol 203:72-81.

TABLE 1

Toxicity of OCPs after exposure for 2 DIV in primary neuronal cultures

LC50 (µM)	Dieldrin	Endosulfan	Lindane
CGC	14.7 ± 0.6**$^{,\#\#\#}$	14.5 ± 1.7**$^{,\#}$	72.7 ± 14.9
CN	33.2 ± 0.2*	27.2 ± 3.3*	80.1 ± 18.6

Primary cultures of cortical neurons (CN) and cerebellar granule cells (CGC) were treated with OCPs and 2 DIV later cell viability was determined by the MTT assay. LC50 values were calculated by adjusting the curves from Fig.1A-C into a non-linear fit regression (mean ± SE of 3-4 independent experiments). Statistical differences by one-way ANOVA were obtained for dieldrin and endosulfan in CGC (**P<0.01) and CTX (*P<0.05) with respect to lindane; and comparing CGC and CN for endosulfan and dieldrin ($^{\#}$P<0.05, $^{\#\#\#}$P<0.001, two-tailed *t*-test).

TABLE 2

Toxicity of OCPs after exposure for 6 DIV in primary neuronal cultures

LC50 (µM)	Dieldrin	Endosulfan	Lindane
CGC	8.7± 0.7***	6.2± 1.8***	28.3± 0.7
CN	1.9 ± 0.3***$^{,\$\$,\#\#\#}$	10.5 ± 2.5***	39.5 ± 2.0$^{\#}$

Primary cultures of cortical neurons (CN) and cerebellar granule cells (CGC) were treated with OCPs and 6 DIV later cell viability was determine by the MTT assay. LC50 values were calculated by adjusting the curves from Fig.1D-F into a non-linear fit regression (mean ± SE of 3-4 independent experiments) Statistical differences by one-way ANOVA were obtained for dieldrin and endosulfan with respect to lindane in CN and CGC (***P<0.01); for dieldrin with respect to endosulfan in CN ($^{\$\$}$P<0.01); and for dieldrin and lindane comparing CGC and CN ($^{\#}$P<0.05; $^{\#\#\#}$P<0.001, two-tailed *t*-test).

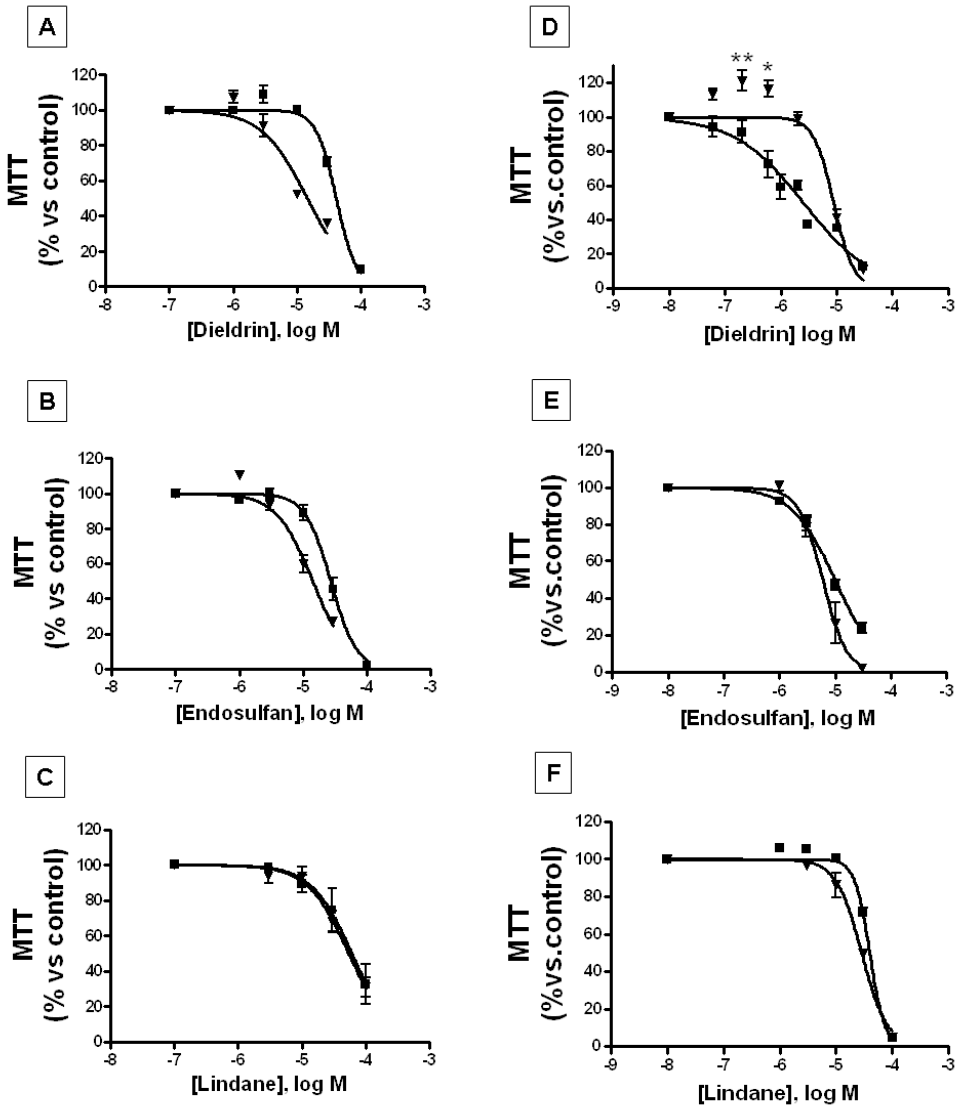


Figure 1. Concentration-dependent reduction of cell viability after long-term exposure to OCPs. Primary cultures of cortical neurons (■) and cerebellar granule cells (▼) were exposed to different concentrations (1-100 μM) of dieldrin, endosulfan and lindane for 2 (panels A-C) or 6 DIV (panels D-F). MTT values are expressed as % with respect to cells exposed to DMSO (mean ± SE, N = 3-4).

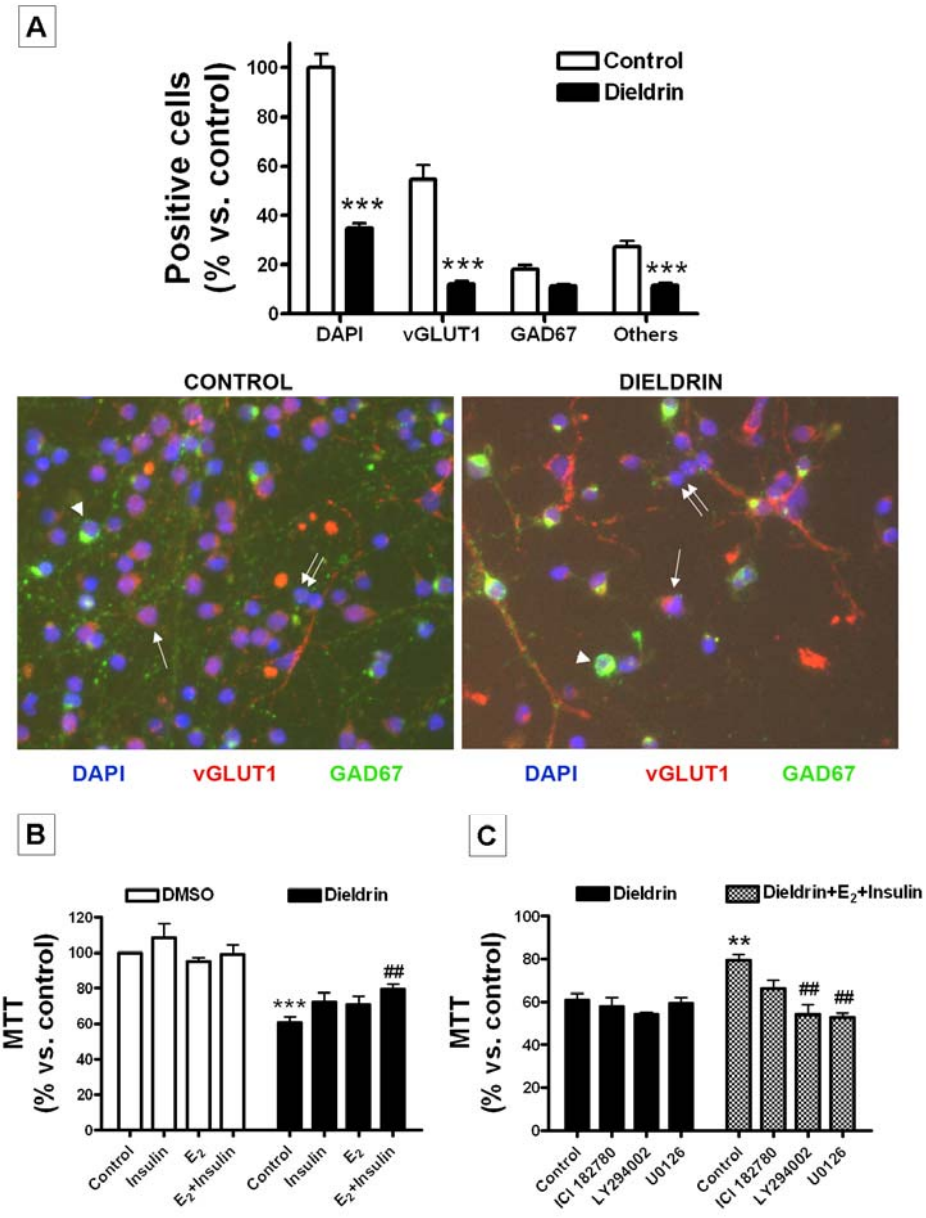


Figure 2. Selective toxicity of dieldrin in glutamatergic neurons. Neuroprotection by E2 and insulin against dieldrin neurotoxicity is Akt and ERK-dependent. A) Immunocytochemistry for vGLUT1 (red) and GAD67 (green) was performed to specifically label glutamatergic (arrows) and GABAergic (arrowheads) neurons, respectively, present in immature cortical neurons. Non-stained neurons for neither vGLUT1 (red) nor GAD67 were designate as others (double arrows). DAPI (blue) immunostaining was performed to detect the total number of nuclei. Statistical differences in cell counting (top of the panel) were obtained by two-way ANOVA: *** p <0.001 vs. DMSO-treated cells. B) Mature cerebellar granule cells were exposed to DMSO or 10 μM dieldrin for 2 DIV both in the absence and presence of E_2 (1nM), insulin (500nM) and a mixture of both hormones. MTT values are expressed as % with respect to cells exposed to DMSO (mean ± SE, N = 3-7). Statistical comparisons were made by two-way ANOVA: *** p <0.001 vs. DMSO-treated cells; $^{\#\#}$ p < 0.01 vs. dieldrin-treated cells. C) Mature cerebellar granule cells

were pre-treated with ICI 182780 (1 μM), U0126 (20 μM) or LY294002 (20 μM) for 30 min and then exposed to 10 μM dieldrin for 2 DIV either in the absence or presence of E_2 (1nM) and insulin (500nM). MTT values are expressed as % with respect to cells exposed to DMSO (mean ± SE, N = 2-7). Statistical comparisons were made by two-way ANOVA: ** p <0.01 vs. dieldrin-treated cells; $^{\#\#}$ p < 0.01 vs. E2+Insulin-treated cells.

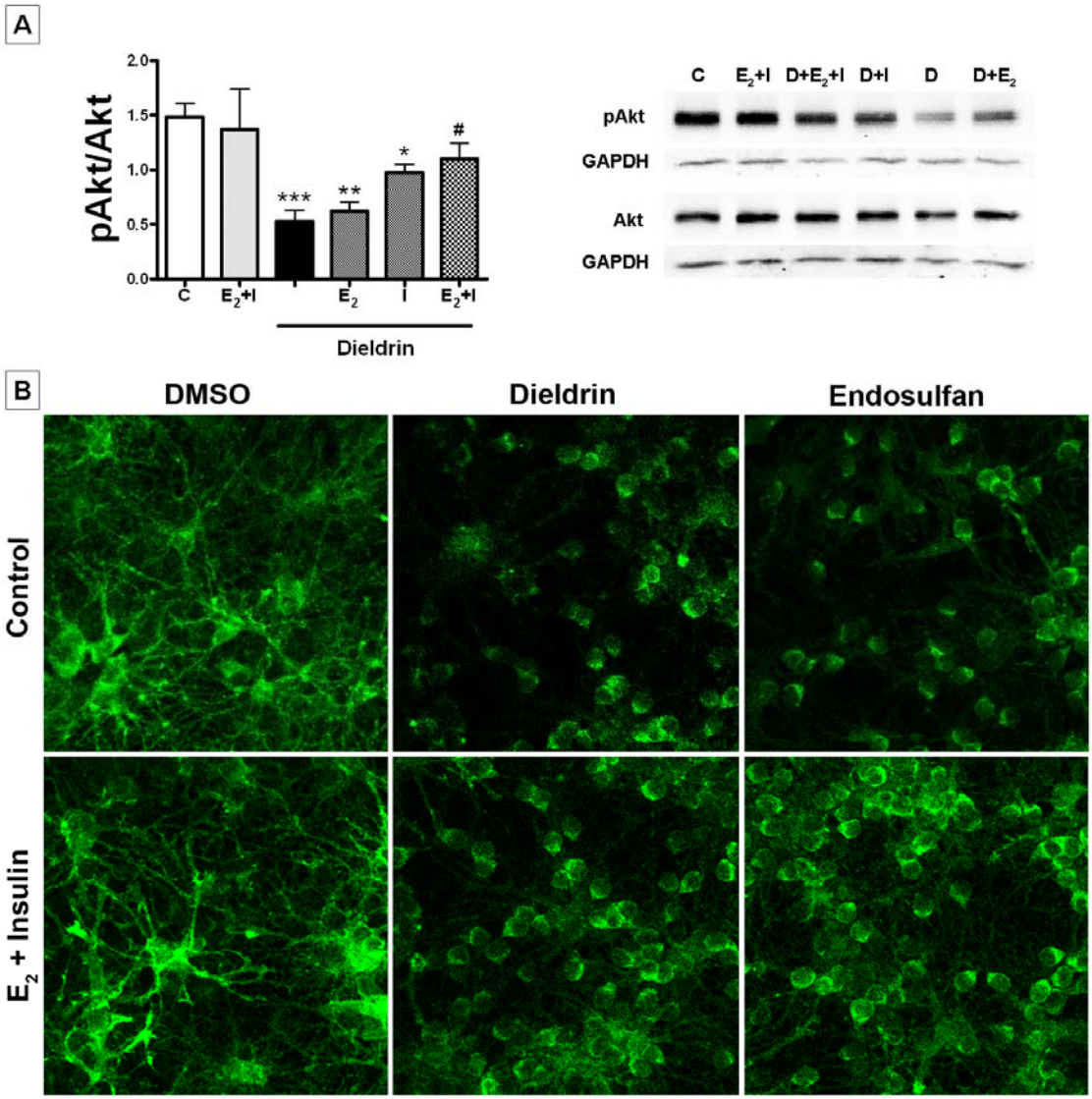


Figure 3. E_2 and insulin prevented dieldrin- and endosulfan-induced reduction of Akt phosphorylation. A) Cerebellar granule cells were exposed to DMSO (C) or 10 μM dieldrin (D) for 24 h both in the absence and presence of E_2 (1nM), insulin (500nM) and a mixture of both hormones (E_2+I) and then the protein levels of GAPDH, Akt and phospho-Akt were determined by *Western Blot*. Densitometric quantification of the immunoblots is shown on the left and a representative immunoblot for the indicated proteins is shown on the right of the panel. Data are mean ± SE of three independent experiments. Statistical comparisons were made by one-way ANOVA: * p < 0.05, ** p <0.01, *** p <0.001 vs. DMSO-treated cells; $^{\#}$ p < 0.05 vs. dieldrin-treated cells. B) Cerebellar granule cells were

exposed to DMSO, 10 μM dieldrin or 10 μM endosulfan for 24 h both in the absence and presence of E_2 (1nM) and insulin (500nM) and then neurons were immnunostained for phospho-Akt.

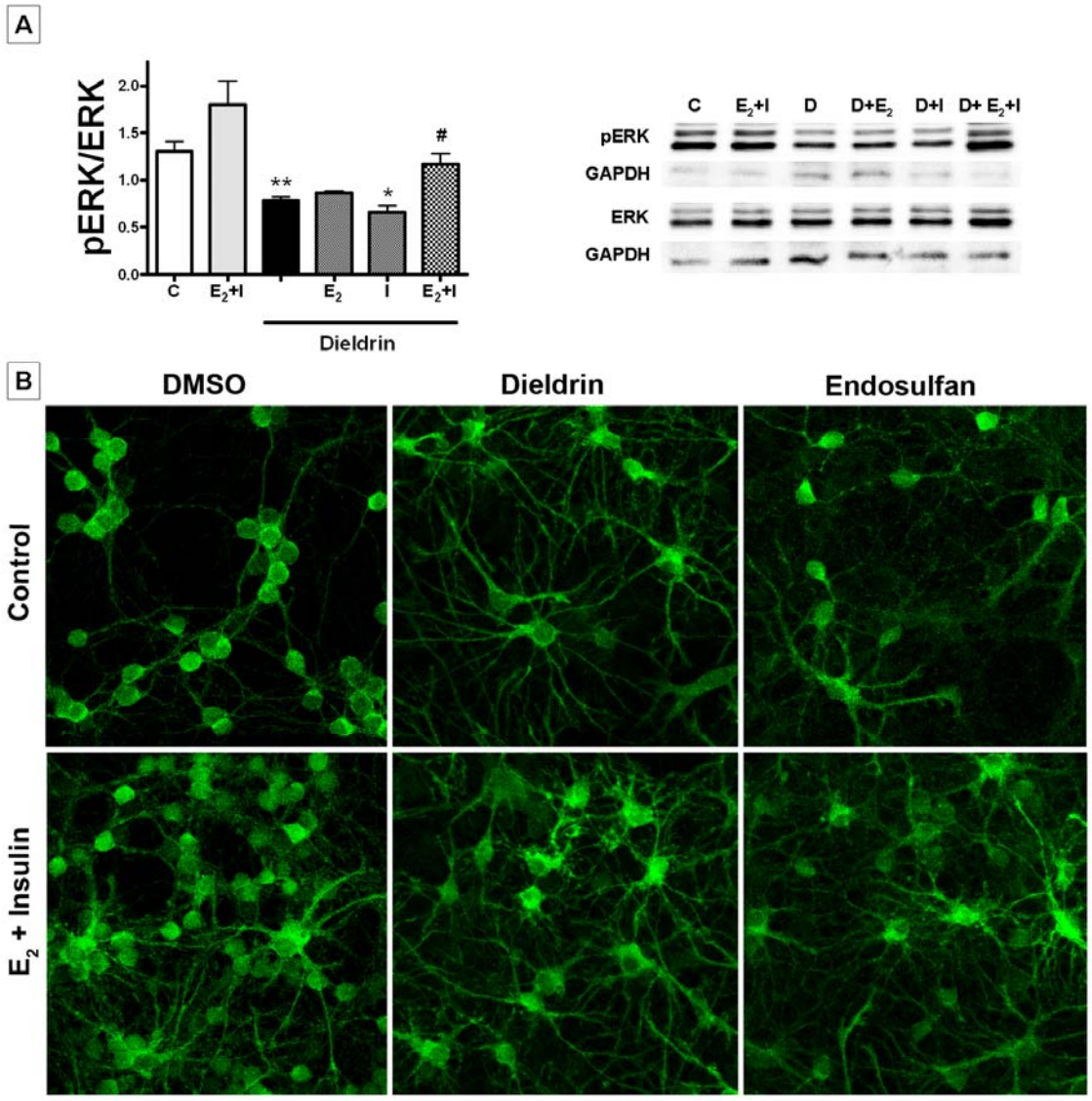


Figure 4. E_2 and insulin prevented dieldrin- and endosulfan-induced reduction of ERK1/2 phosphorylation. A) Cerebellar granule cells were exposed to DMSO (C) or 10 μM dieldrin (D) for 24 h both in the absence and presence of E_2 (1nM), insulin (500nM) and a mixture of both hormones (E_2+I) and then the protein levels of GAPDH, ERK1/2 and phospho-ERK1/2 were determined by *Western Blot*. Densitometric quantification of the immunoblots is shown on the left and a representative immunoblot for the indicated proteins is shown on the right of the panel. Data are mean ± SE of 2-6 independent experiments. Statistical comparisons were made by one-way ANOVA: * $p < 0.05$, ** $p < 0.01$ vs. DMSO-treated cells; $^{\#}p < 0.05$ vs. dieldrin-treated cells. B) Cerebellar granule cells were exposed to DMSO, 10 μM dieldrin or 10 μM endosulfan for 24 h both in the absence and presence of E_2 (1nM) and insulin (500nM) and then neurons were immnunostained for phospho-ERK1/2.

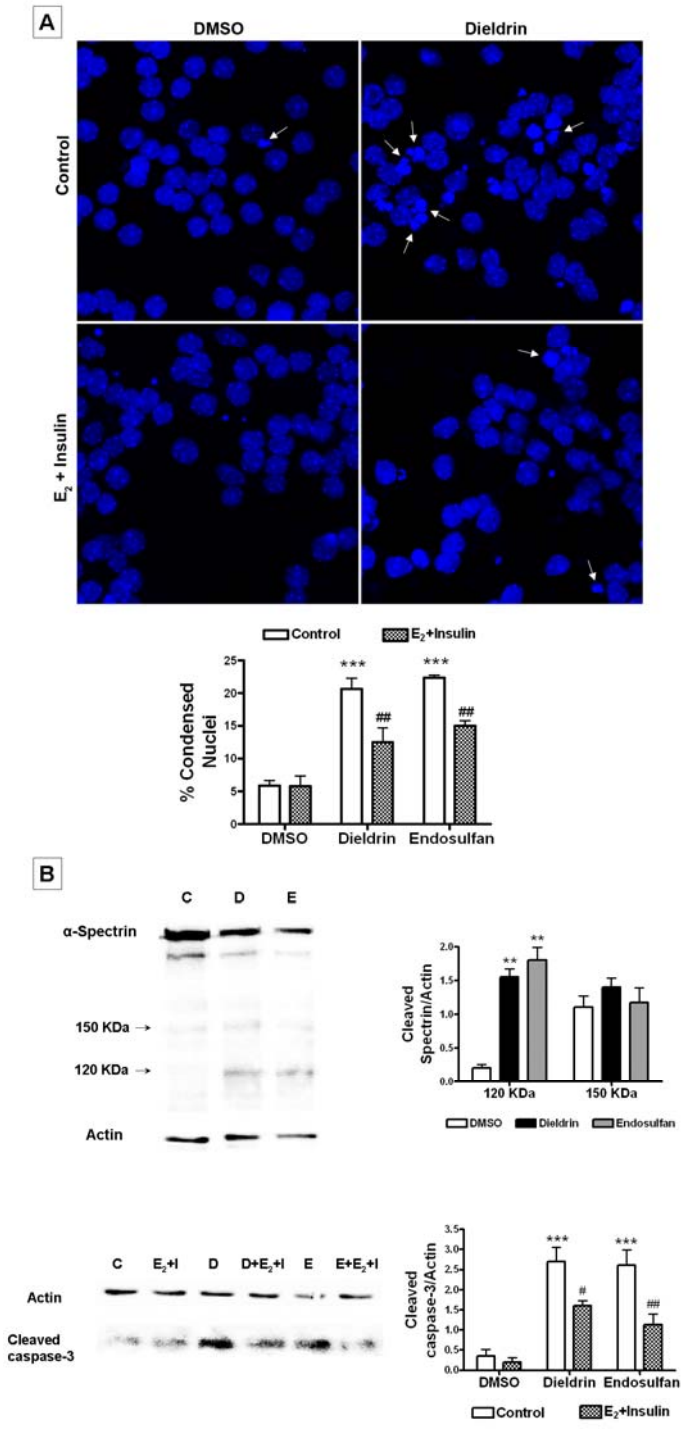
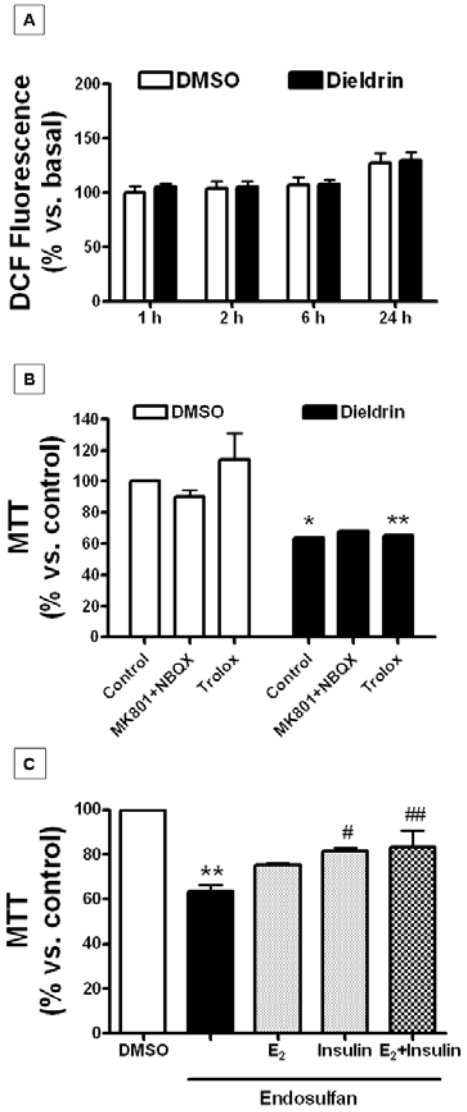


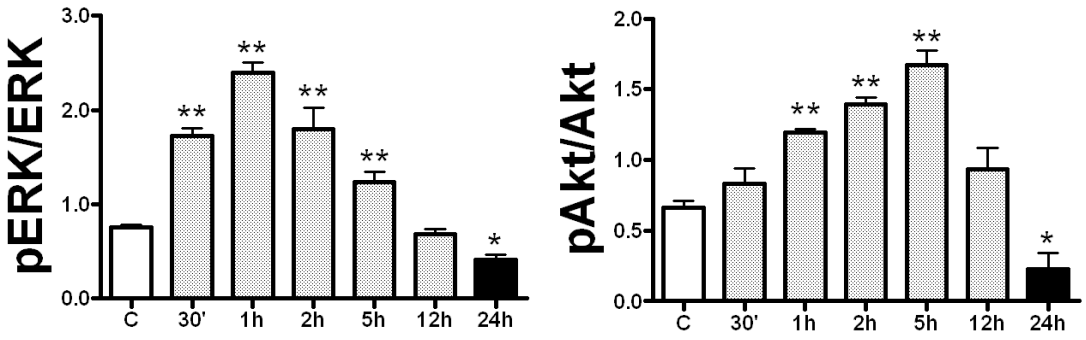
Figure 5. Dieldrin- and endosulfan-induced apoptosis is dependent of caspase-3. A) Cerebellar granule cells were exposed to DMSO, 10 μM dieldrin or 10 μM endosulfan for 24 h both in the absence and presence of E_2 (1nM) and insulin (500nM) and then neurons were labeled with the nuclear dye To-Pro-3. The number of apoptotic cells was determined by counting neurons with marked condensed nuclei (arrows) and was expressed as % compared with total cells (bottom of the panel). Statistical differences were obtained by two-way ANOVA: *** p <0.001 versus DMSO-treated cells; $^{\#\#}$ p < 0.01 vs. OCP-treated cells. B) Calpain/cathepsin and caspase activities were determined by Western Blot analysis of α-

spectrin in cerebellar granule cells previously exposed to DMSO (C), 10 μM dieldrin (D) or 10 μM endosulfan (E) for 24 h. Data are mean ± SE of three independent experiments. Statistical comparisons were made by one-way ANOVA (** p <0.01 vs. DMSO-treated cells). C) Caspase-3 activity was determined by Western Blot analysis of cleaved caspase-3 in cerebellar granule cells previously exposed to DMSO (C), 10 μM dieldrin (D) or 10 μM endosulfan (E) for 24 h both in the absence and presence of E_2 (1nM) and insulin (500nM) (E_2+I). Data are mean ± SE of three independent experiments. Statistical comparisons were made by two-way ANOVA: *** p <0.001 vs. DMSO-treated cells; $^{\#}p < 0.05$, $^{\#\#}p <$ 0.01 vs. OCP-treated cells.

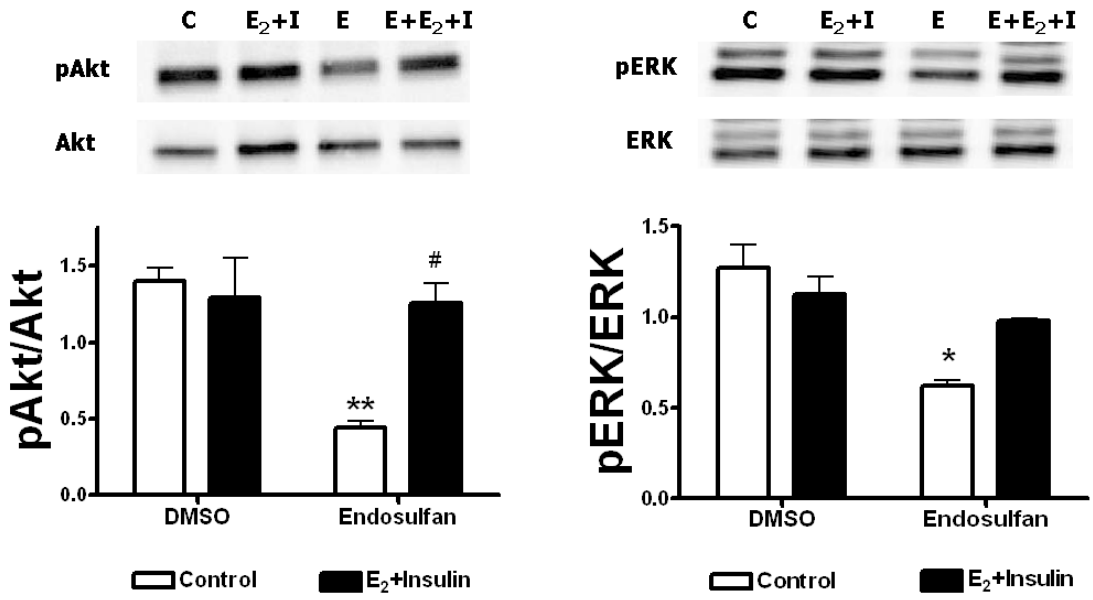
SUPPLEMENTARY DATA



Supplementary Fig. 1A. Exposure to dieldrin did not produce oxidative stress in cerebellar granule cells. Fluorescence of DCF was determined at 1, 2, 6 and 24 h after exposure to DMSO (control) or 10μM dieldrin. Results are expressed as % with respect to basal fluorescence measure before treatments (mean ± SE, N =3-4). **1B. Effect of antioxidants and glutamate receptor antagonists on dieldrin-induced reduction of cell viability in cerebellar granule cells.** MTT values were obtained from neurons exposed to DMSO (white bars) or 10μM dieldrin (black bars) for 2 DIV either in the absence or presence of the antioxidant Trolox (10μM) and the glutamate receptor antagonists MK801 and NBQX (both at 10μM). Results are expressed as % with respect to cells incubated with Hank's solution (mean ± SE, N =2). Statistical analysis was obtained by two-way ANOVA: * $p < 0.05$, ** $p < 0.01$ compared with DMSO-treated cells. **1C. Effect of E_2 and insulin on endosulfan-induced reduction of cell viability in cerebellar granule cells.** MTT values were obtained from neurons exposed to DMSO or 10μM endosulfan for 2 DIV alone or in combination with E_2 (1nM), insulin (500nM) and a mixture of both hormones. Results are expressed as % with respect to cells incubated with Hank's solution (mean ± SE, N =2-5). Statistical analysis was obtained by one-way ANOVA: ** $p < 0.01$ vs. DMSO-treated cells; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ vs. endosulfan-treated cells.



Supplementary Fig.2. Time-dependence of dieldrin-induced ERK1/2 (left) and Akt (right) phosphorylation. Cerebellar granule cells were exposed to DMSO for 24 h (C) or to 10 μM dieldrin for different times and then the protein levels of GAPDH, Akt, phospho-Akt, ERK1/2 and phospho-ERK1/2 were determined by Western Blot. Data are mean ± SE of 2-3 independent experiments. Statistical comparisons were made by one-way ANOVA: * $p < 0.05$, ** $p < 0.01$ vs. DMSO-treated cells.



Supplementary Fig.3. E_2 and insulin prevented endosulfan-induced reduction of Akt (left) and ERK1/2 (right) phosphorylation. Cerebellar granule cells were exposed to DMSO (C) or 10 μM endosulfán (E) for 24 h both in the absence and presence of E_2 (1nM) and insulin (500nM) and then the protein levels of Akt, phospho-Akt, ERK1/2 and phospho-ERK1/2 were determined by Western Blot. Densitometric quantification of the immunoblots is shown on the bottom and a representative immunoblot for the indicated proteins is shown on the top of the panel. Data are mean ± SE of 2 independent experiments. Statistical comparisons were made by one-way ANOVA: * $p < 0.05$, ** $p < 0.01$, vs. DMSO-treated cells; $^{\#}$ $p < 0.05$ vs. endosulfan-treated cells.

Supplementary methods

Determination of radical oxygen species

The fluorescent probe 2',7'-dichlorofluorescein diacetate (DCF-DA) (Sigma) was used quantify the generation of ROS. Cultured cortical neurons grown in 96-well plates were rinsed with pre-warmed Hanks solution and incubated with DCF-DA (10μM) prepared in Hanks solution for 30 min at 37ºC protected from the light. After measuring basal fluorescence in DMEM solution, cells were then treated with the chemicals (prepared in DMEM) and maintained at 37ºC in the darkness. Fluorescence was monitored at different times in a fluorimetric plate reader (SpectraMax GeminisXS; Molecular Devices, Sunnyvale, CA, USA) at an Ex/Em of 530/590 nm.

TRABAJO ANEXO

1. Deteminación de los pesticidas organoclorados en el medio extracelular tras su exposición prolongada en cultivos primarios de neuronas corticales.

En el trabajo nº 2 determinamos la cantidad de dieldrín, endosulfán y lindano que se había incorporado a las neuronas corticales tras 6 DIV de exposición, obteniendo, en todos los casos, una concentración intracelular que se correspondía, aproximadamente, al 10% de la concentración inicial. Paralelamente, determinamos la concentración de estos compuestos que quedaba en el medio extracelular tras los 6 DIV de exposición. Para ello, igualmente, hicimos uso de la cromatografía de gases acoplada a un detector de captura electrónica, descrito detalladamente en el trabajo Nº2. En la tabla 1 se muestran los resultados obtenidos.

Tabla 1

Pesticida	Concentración inicial (nM)	Concentración extracelular final (nM)	Porcentaje presente tras 6 DIV de exposición
Dieldrín	100	16 ± 11.2	16 ± 11.2
Endosulfán	1000	9.46	0.95
Lindano	10,000	590.41	5.90

Nota: Tras los tratamientos, los compuestos fueron extraídos en hexano y separados mediante cromatografía de gases. La concentración de cada uno de ellos fue determinada a través de un detector de captura electrónica (para una descripción detallada del procedimiento mirar la sección de métodos del trabajo nº 2). Los valores representan una sola medida en el caso de endosulfán y lindano, y tres medidas independientes (media±SD) en el caso de dieldrín.

Estos datos indican que la proporción de pesticida presente en el medio extracelular (respecto a la concentración inicial) tras 6 DIV de exposición es mayor para el dieldrín que para el lindano y mayor todavía respecto a la del endosulfán. Estos resultados se corresponden con el grado de degradación de cada pesticida en aguas y suelos (HSDB) así como en el medio de cultivo (sin células) tras 8 días a 37ºC observado en un trabajo previo (Babot, 2006), en donde la proporción presente era: 95% para dieldrín, 1,7% para endosulfán y 3,3% para lindano.

2. Afinidad del dieldrín por el receptor $GABA_A$ en neuronas corticales tras su exposición aguda y prolongada

En el trabajo nº 1 determinamos la potencia inhibitoria del dieldrín sobre el receptor $GABA_A$ en neuronas corticales tras 2 y 6 DIV de exposición mediante el ensayo de unión a [^{35}S]-TBPS. Al mismo tiempo, evaluamos la afinidad del dieldrín al receptor tras su exposición aguda. En la tabla 2 se muestra una comparativa de los valores de IC50 obtenidos en cada caso.

Table 2

Tiempo de exposición	Agudo	2 DIV	6 DIV
IC50 en el ensayo de unión a [^{35}S]-TBPS	26.3 ± 1.2	140.5 ± 9.2*	145.5 ± 46.3*

Nota: Cultivos primarios de neuronas corticales de 8 DIV fueron incubados con 1,5-3 nM de [^{35}S]-TBPS durante 30 minutos en presencia de diferentes concentraciones (10-200 nM) de dieldrín (agudo), o bien tras 2 o 6 DIV de exposición a 10-200 nM de dieldrín (para una descripción detallada del procedimiento mirar la sección de métodos del trabajo nº 1). Los valores de IC50 obtenidos de cada curva se determinaron mediante un ajuste por regresión no lineal y representan tres medidas independientes (media±SD). El análisis estadístico muestra diferencias significativas: * $p < 0.05$ vs. tratamiento agudo.

Los resultados indican que el bloqueo del receptor $GABA_A$ por parte del dieldrín es más efectivo tras su exposición aguda que prolongada. Sin embargo, no existe ninguna diferencia entre las exposiciones durante 2 y 6 DIV. Estos datos se correlacionan con la estabilidad del compuesto en el medio extracelular mencionada anteriormente; de manera que su concentración extracelular tras 6 DIV de exposición es unas 6 veces menor que la inicial y, de acuerdo con esto, su potencia de inhibición sobre el receptor $GABA_A$ tras 6 DIV es entre 5 y 6 veces inferior a observada en la exposición aguda.

3. Efectos de la exposición prolongada a los pesticidas organoclorados sobre los niveles de expresión de los receptores metabotrópicos de glutamato de tipo I en cultivos primarios neuronales

En el primer trabajo estudiamos, entre otras cosas, la expresión de los receptores metabotrópicos de glutamato de tipo I en neuronas corticales tras la exposición prolongada a dieldrín. Hemos querido ampliar el estudio a los otros dos pesticidas organoclorados, el endosulfán y el lindano, tanto en estos cultivos como en los de células granulares de cerebelo.

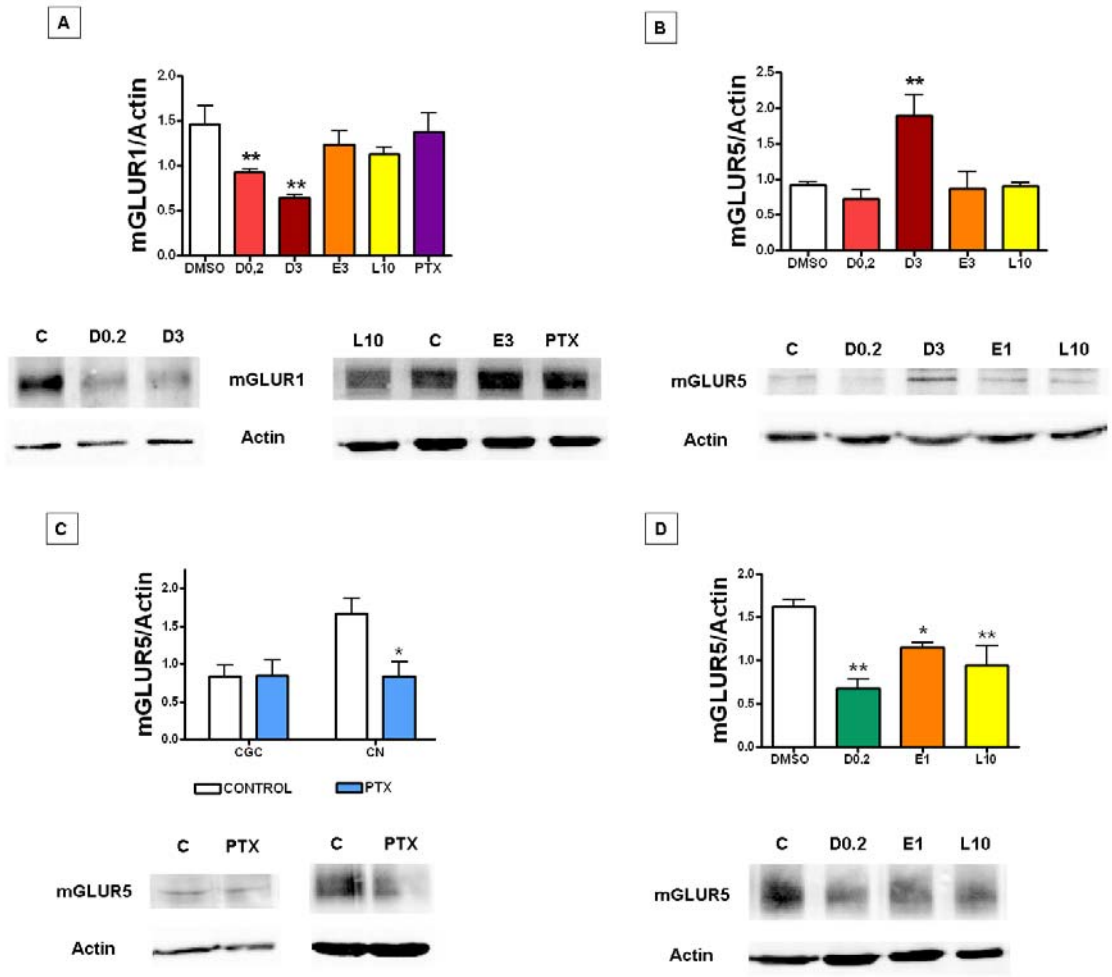


Fig. 1. **Efectos de los pesticidas organoclorados sobre los receptores metabotrópicos de glutamato de tipo I.** Cultivos primarios de células granulares de cerebelo (paneles A-C) y de neuronas corticales (paneles C-D) fueron tratados durante 6 DIV con DMSO (Control o C), 0,2 µM y/o 3µM de dieldrín (D0,2 o D3), 1 µM o 3µM de endosulfán (E1 o E3), 10µM de lindano y 100µM de picrotoxinina (PTX). Tras los tratamientos, los niveles de actina, mGLUR1 y mGLUR5 fueron determinados mediante *Western*

blot (para más detales sobre el procedimiento mirar la sección de métodos del trabajo nº 1). En la parte inferior de cada panel se muestra un ejemplo representativo de cada *inmunoblot* y en la parte superior su cuantificación densitométrica. Los resultados hacen referencia a la media ± SE de 2-5 experimentos independientes. La comparaciones estadísticas se realizaron mediante one-way ANOVA: * $p < 0.05$, ** $p < 0.01$, respecto al control.

A diferencia de lo observado en neuronas corticales, el tratamiento durante 6 DIV con dieldrín redujo los niveles de mGLUR1 en células granulares de cerebelo. En cambio, la exposición prolongada a endosulfán o lindano no produjo el mismo efecto. Con el fin de comprobar si los efectos del dieldrín estaban mediados por su acción sobre el receptor $GABA_A$, como sucedía en las neuronas corticales, utilizamos la picrotoxinina. Sin embargo, el tratamiento con ésta durante 6 DIV tampoco modificó la expresión de mGLUR1 (Fig. 1A). Llevamos a cabo el mismo procedimiento con mGLUR5, y observamos que, nuevamente, el dieldrín era el único de los pesticidas utilizados capaz de modificar la expresión de este receptor. No obstante, al contrario de lo sucedido con mGLUR1, la exposición prolongada a dieldrín incrementó los niveles de mGLUR5 (Fig. 1B). En este caso, el tratamiento con picrotoxinina tampoco alteró la expresión de mGLUR5 en células granulares de cerebelo. Por el contrario, la picrotoxinina sí que redujo la expresión de este receptor en neuronas corticales (Fig.1C). De manera similar, los niveles de mGLUR5 de vieron disminuidos tras la exposición durante 6 DIV a los tres pesticidas organoclorados, como ya habíamos observado en el trabajo nº 1 a una concentración inferior de dieldrín (Fig.1D).

Estos resultados indican, por un lado, que los pesticidas organoclorados tienen efectos similares sobre mGLUR5 en neuronas corticales, aunque el dieldrín a una concentración menor que el endosulfán y el lindano. Estos efectos están mediados, además, por su acción inhibitoria sobre el receptor $GABA_A$ puesto que son mimetizados por la picrotoxinina, algo que ya se intuía con el dieldrín en el primer trabajo, lo que sugiere que endosulfán y lindano podrían reproducir los efectos del dieldrín sobre el receptor NMDA descritos en el presente estudio. Por el contrario, los efectos del dieldrín sobre mGLUR1 y mGLUR5 en células granulares de cerebelo, no parecen mediados por el receptor $GABA_A$ ya que no se observaron ni con picrotoxinina ni con los otros pesticidas.

4. La alopregnanolona no previene la muerte neuronal inducida por dieldrín en células granulares de cerebelo.

Puesto que la alopregnanolona demostró tener propiedades neuroprotectoras frente a la toxicidad del dieldrín en neuronas corticales, comprobamos si esto también sucedía en células granulares de cerebelo. Sin embargo, la exposición el tratamiento con este neuroesteroide no impidió la muerte neuronal causada por la exposición prolongada a dieldrín, ya fuera en cultivos maduros o inmaduros (Fig. 2). Esto sugiere que el mecanismo de muerte inducida por el dieldrín es diferente entre los cultivos y por tanto la participación del receptor $GABA_A$ es menor al respecto en las células granulares de cerebelo, o bien que los receptores de estas neuronas tienen una sensibilidad menor hacia los neuroesteroides.

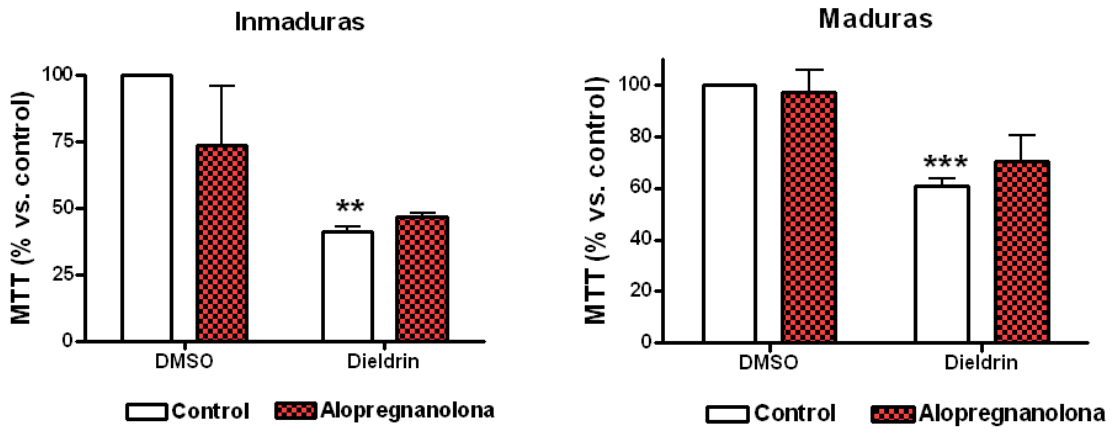


Fig. 2. La alopregnanolona no protégé frente al dieldrín en células granulares de cerebelo. Células granulares de cerebelo inmaduras (panel izquierdo) y maduras (panel derecho) fueron tratadas con DMSO 10μM de dieldrín de DIV 2 a DIV 4 y de DIV 6 a DIV 8, respectivamente, en presencia de 10μM de alopregnanolona. Los valores de MTT se expresan en % respecto a células incubadas con medio de cultivo DMEM (mean ± SE, N =2-5). El análisis estadístico fue realizado mediante two-way ANOVA: ** $p < 0.01$, *** $p < 0.001$ respecto al control.

IV. DISCUSIÓN

En este trabajo hemos estudiado los efectos de la exposición prolongada a bajas concentraciones de dieldrín, con el fin de reproducir *in vitro* unas condiciones experimentales que se aproximen, en la mayor medida posible, a la exposición ambiental a este contaminante que afecta a la población general. Teniendo en cuenta los niveles de este pesticida detectados en el cerebro humano (Fleming *et al.*, 1994; Corrigan *et al.*, 2000) y la proporción de lípidos en el tejido cerebral (6% en tejido húmedo, (Chavko *et al.*, 1993) puede hacerse una estimación de su concentración en el cerebro. De esta manera, 50-1000 ng/g de lípido para dieldrín corresponderían aproximadamente a 10-200 nM, que es el rango de concentraciones normalmente usado en los experimentos a largo plazo. Si bien son pocos los estudios que han determinado los niveles de pesticidas organoclorados en el cerebro y el número de sujetos analizados en estos es bajo, más numerosos y con muestras más amplias son los realizados en otros tejidos; habiéndose encontrado dieldrín, endosulfán y lindano en un alto porcentaje de individuos a niveles en torno a 20 ng/g de lípido en tejido adiposo, 1-50 nM en sangre y 30 nM de media en leche materna (Botella *et al.*, 2004; Cerrillo *et al.*, 2005; Mariscal-Arcas *et al.*, 2010). Esto indica que el cerebro acumula grandes cantidades de estos compuestos, de manera comparable al tejido adiposo, y por tanto se encuentra expuesto a niveles de pesticidas capaces de interferir con la neurotransmisión GABAérgica (Pomes *et al.*, 1993; Pomes *et al.*, 1994b; Vale *et al.*, 2003).

Estudios previos realizados en nuestro laboratorio mostraron que, a diferencia de endosulfán y lindano, la concentración de dieldrín se mantiene estable en el medio de cultivo a 37ºC durante al menos 8 DIV (Babot, 2006). La determinación de los pesticidas mediante GC-ECD (Gas Cromatography-Electron Capture Detector) tras 6 DIV de exposición en neuronas corticales mostró que la cantidad incorporada por éstas es la misma para todos ellos (en torno al 10%), probablemente porque en la célula se encuentran unidos de manera irreversible a sus dianas farmacológicas. Sin embargo, el dieldrín fue el menos degradado de los tres en el medio extracelular, lo que se corresponde con su mayor estabilidad en el medio ambiente y su mayor capacidad de bioacumulación (HSDB, 2011). La expresión constitutiva de algunas isoformas de las enzimas detoxificantes citocromo P450 en el cerebro (Warner & Gustafsson, 1999), unido al hecho de que los pesticidas organoclorados son inductores de éstas (Coumoul *et al.*, 2002; Johri *et al.*, 2008), puede explicar la degradación de la mayor parte de la cantidad inicial de pesticidas tras su exposición prolongada sobre los cultivos neuronales. El análisis de sus metabolitos podría confirmar esta idea.

En el primer trabajo, hemos observado que el dieldrín mantiene parcialmente bloqueado el receptor $GABA_A$ de manera permanente, al menos durante 6 DIV, en neuronas corticales, siendo la IC50 en el ensayo de unión a [^{35}S]-TBPS (~150 nM) inversamente proporcional a su concentración en el medio extracelular. En cambio, el receptor $GABA_A$ no se encuentra inhibido tras la exposición prolongada a 300 nM de dieldrín en células granulares de cerebelo y solo a 3 μM se obtiene un bloqueo parcial (Babot *et al.*, 2007), lo que puede explicar su menor toxicidad sobre estas células a 6 DIV. A nivel molecular, la diferente composición de los receptores $GABA_A$ en unas y otras neuronas, dada la presencia de la subunidad *α*6 en células granulares de cerebelo, podría ser la causa de estas diferencias. Sin embargo, se ha descrito que el efecto inhibitorio del dieldrín sobre el receptor $GABA_A$ no parece depender de la composición de éste (Nagata *et al.*, 1994). No obstante, en este último estudio se han probado solo 3 combinaciones de receptores $GABA_A$ expresados de manera heteróloga (*α*1-*α*6/β/γ2) entre los múltiples existentes, a pesar de que las subunidades *α*6 se asocian exclusivamente a las δ *in vivo* (Barnard, 2001). Además, estos mismos autores han detectado receptores $GABA_A$ con afinidades al dieldrín menores a las anteriores (5 y 90 nM) en neuronas del ganglio dorsal (Nagata & Narahashi, 1994), y similar a la obtenida en este trabajo en neuronas corticales (~25 nM). Por otro lado, la regulación de las subunidades del receptor $GABA_A$ es diferente entre ambos cultivos. Así, el tratamiento con TBPS induce la expresión de las subunidades *α*3 y α5, entre otras, en neuronas corticales (Poulter *et al.*, 1997). En cambio, el crecimiento de las células granulares de cerebelo en presencia de 25 mM de K^+ favorece la expresión de *α*4 y δ (Payne *et al.*, 2008). Aunque se desconoce el efecto del dieldrín sobre las subunidades del receptor $GABA_A$ en estas neuronas, se han visto alteraciones en algunas de ellas en otros sistemas neuronales (Liu *et al.*, 1997b; Liu *et al.*, 1998). Así, su exposición a largo plazo podría modificar la composición de los receptores $GABA_A$ y, con ello, la sensibilidad de sus ligandos endógenos por éste.

La exposición prolongada a dieldrín redujo la funcionalidad y expresión de los receptores NMDA en la membrana de las neuronas corticales, de acuerdo con lo observado previamente en células granulares de cerebelo (Babot *et al.*, 2007). La inicial hiperexcitabilidad causada por el bloqueo de los receptores $GABA_A$, deviene al cabo de 2 DIV en una reducción de la respuesta postsináptica a glutamato (Turrigiano *et al.*, 1998). A pesar de que, en nuestro caso, la funcionalidad y expresión de los receptores no estaba alterada, sí que observamos una menor respuesta a glutamato (en relación a la

$[Ca^{2+}]_i$ y a la excitotoxicidad) en las células tratadas con dieldrín durante 2 DIV. Los experimentos de inmunocitoquímica sugieren la existencia de un acoplamiento funcional entre los receptores NMDA y mGLUR5 en neuronas corticales, como ha sido demostrado en otras regiones (Stoop *et al.*, 2003; Yang *et al.*, 2004; Guo *et al.*, 2004). Concretamente, son los receptores NR1/NR2B quienes interaccionan con mGLUR5 a través del complejo PSD95-*Shank-Homer1b/c* (Yang *et al.*, 2004; Guo *et al.*, 2004), lo que coincide con que sean estos mismos receptores los primeros afectados (junto a mGLUR5) por la exposición prolongada a dieldrín. En este sentido, se ha observado que la expresión de *Homer1a*, variante de *splicing* sin capacidad de formar complejos multiproteicos ni de transmitir la señalización intracelular, se incrementa en respuesta a la actividad sináptica y está implicada, además, en la plasticidad homeostática (Hu *et al.*, 2010), con lo que podría estar involucrada en los efectos del dieldrín sobre los receptores de glutamato. De esta manera, la asociación de la subunidad NR2B con PSD95 y nNOS mediaría los efectos neurotóxicos derivados de la activación del receptor NMDA (Cui *et al.*, 2007), y la supuesta perturbación de este complejo por *Homer1a* en las células expuestas a dieldrín podría prevenir la muerte neuronal causada por glutamato. Sin embargo, los experimentos de inmunoprecipitación muestran que NR2B no se encuentra asociada a PSD95 sino a SAP102 en neuronas corticales, como ha sido descrito en el cortex *in vivo* (van Zundert *et al.*, 2004). Además, el análisis inmunocitoquímico de mGLUR5 y NR1 indica que estas proteínas co-localizan principalmente en el soma de las células control, por lo que parece que se trata de receptores extrasinápticos, los cuales han sido relacionados a menudo con la excitotoxicidad (Hardingham, 2009). Por tanto, es más probable que la menor susceptibilidad a la muerte excitotóxica en las neuronas tratadas con dieldrín se deba a una disminución de los receptores NR1/NR2B y mGLUR5 extrasinápticos, no significativa a nivel individual pero sí conjuntamente, y, con ello, de su capacidad para incrementar la $[Ca^{2+}]_i$ y de activar las rutas de muerte celular.

El balance sináptico implica generalmente el tráfico de los receptores NMDA y/o AMPA/Kainato, entre otras proteínas, (Perez-Otano & Ehlers, 2005; Turrigiano, 2008) y, al menos en el caso de los antagonistas del receptor $GABA_A$, es dependiente de la concentración (Swann *et al.*, 2007). En los trabajos 1 y 3, hemos encontrado que la regulación de los receptores NMDA y mGLUR5 por dieldrín es también dependiente del tiempo y de la concentración. Sin embargo, ni los receptores AMPA/Kainato y mGLUR1 ni las proteínas *scaffold* PSD95 y SAP102 se vieron afectados por la

exposición prolongada a dieldrín, por lo que se trata de un efecto específico sobre los receptores NMDA y mGLUR5 y previo a la aparición de cualquier signo de neurodegeneración. No obstante, no pueden descartarse alteraciones en estas otras proteínas a concentraciones mayores de dieldrín como se ha descrito con el tratamiento crónico con bicuculina (Swann *et al.*, 2007).

Diversos factores participan en este tipo de plasticidad homeostática, aunque la mayoría de ellos han sido involucrados exclusivamente en el incremento de los receptores AMPA (BDNF, TNFα y CaMKIV) y NMDA (PKA y PKC) en situaciones de inhibición crónica de la actividad sináptica (Perez-Otano & Ehlers, 2005; Turrigiano, 2008). En cambio, un estudio reciente ha demostrado que la reducción de la respuesta postsináptica excitatoria tras 2 DIV de tratamiento con picrotoxinina está mediada por la fosforilación y posterior degradación de SPAR (Seeburg *et al.*, 2008), proteína *scaffold* asociada a PSD95 e implicada en la sinaptogénesis (Pak *et al.*, 2001). La disminución de esta proteína se ha relacionado también con la neurodegeneración causada por el glutamato (Wu *et al.*, 2007) y, tal vez, puede ser un mecanismo que enlace los efectos del dieldrín sobre los receptores de glutamato con sus efectos neurotóxicos. Perez-Otano & Ehlers (2005) han propuesto un modelo en el que la ubiquitinación y posterior degradación de componentes de la densidad postsináptica, incluidos los receptores NR1/NR2B, en respuesta a un estado mantenido de hiperexcitabilidad conduce a una mayor proporción de receptores NR1/NR2A y, consecuentemente, a una mayor activación de CREB pero menor de ERK1/2. Conviene tener en cuenta que la expresión de NR2A en neuronas corticales está regulada por la actividad sináptica a través de los receptores NR1/NR2B y los canales de Ca^{2+} de tipo L (Hoffmann *et al.*, 2000) y puede, además, ser reducida mediante la inhibición de la ruta MAPK (Numakawa *et al.*, 2007). Por tanto, la secuencia de eventos en nuestro modelo de exposición prolongada a dieldrín en neuronas corticales podría ser la siguiente: la hiperactivación de los receptores NR1/NR2B, predominantes en las sinapsis inmaduras, causa su internalización, lo que interfiere con el aumento de la expresión de NR2A e impide la maduración de las sinapsis (van Zundert *et al.*, 2004). En cambio, cuando el dieldrín es expuesto a neuronas corticales ya maduras, con receptores NR1/NR2A y/o triheteroméricos, la sobre-estimulación de los receptores de glutamato induce la activación de las calpaínas que degradan la subunidad NR2B, entre otras proteínas, impidiendo su interacción con SAP102 y la transducción de la señalización intracelular (Doshi & Lynch, 2009). El resultado será una disminución de la actividad nuclear de

ERK1/2 y, con ello, una disminución de la expresión de NR2A. No obstante, se ha propuesto que la fosforilación de ERK1/2 y CREB está regulada positiva y negativamente por los receptores NMDA sinápticos y extrasinápticos, respectivamente (Hardingham, 2009). Por tanto es posible que la exposición a 10μM concentración de dieldrín sea capaz de activar los receptores NR1/NR2B extrasinápticos e inhibir directamente la vía MAPK, como se ha descrito a altas concentraciones de NMDA (Chandler *et al.*, 2001).

Sin embargo, resulta contradictorio que el tratamiento crónico con otro antagonista del receptor $GABA_A$, bicuculina, no tenga un efecto tóxico sobre las neuronas, a pesar de afectar también a la neurotransmisión glutamatérgica (Swann *et al.*, 2007; Zhou *et al.*, 2007). Esto es probablemente debido a que el bloqueo del receptor $GABA_A$, aún siendo completo, causa la activación preferente de los receptores NMDA sinápticos (generalmente asociados a señales de supervivencia; (Hardingham, 2009) y, por lo tanto, no se desencadena una acción excitotóxica, a no ser que se inhiban los receptores NR1/NR2A (Liu *et al.*, 2007). Aunque otra posibilidad, es que la bicuculina, al ser un antagonista competitivo y reversible, no sea capaz de mantener bloqueado el receptor $GABA_A$ de manera permanente (como lo hace el dieldrín) y por ello no llegue a producir daño neuronal. Así, por ejemplo, el antagonista no competitivo picrotoxinina sí que causa neurodegeneración a altas concentraciones y, además, la allopregnanolona previene este efecto (Brinton, 1994). En este sentido, es también posible que la mayor toxicidad del dieldrín con respecto a endosulfán y lindano tras 6 DIV de exposición en neuronas corticales se deba a su mayor persistencia.

Por tanto, la toxicidad del dieldrín en neuronas corticales está probablemente mediada por su acción inhibitoria sobre el receptor $GABA_A$ y su posterior disminución de la neurotransmisión glutamatérgica, en la que la subunidad NR2A parece tener un papel clave. El neuroesteroide alopregnanolona, gracias a su modulación alostérica sobre los sitios de unión de la picrotoxinina y de las benzodiacepinas (Hawkinson *et al.*, 1994; Suñol *et al.*, 2006), impide el bloqueo del receptor $GABA_A$ por dieldrín cuando se añaden a la vez y, además, es capaz de revertir los efectos del pesticida sobre los receptores de glutamato incluso cuando es añadida *a posteriori*. La necesidad de una concentración alta de alopregnanolona (10μM) para que su acción neuroprotectora sea efectiva, sugiere que sus efectos activadores directos (GABA miméticos) sobre el receptor $GABA_A$ pueden ser requeridos al respecto, puesto que su modulación alostérica se produce a concentraciones menores (Hawkinson *et al.*, 1994; Lambert *et al.*, 2001;

Mellon, 2007). No obstante, esta es la concentración necesaria para obtener una potenciación total de la respuesta a GABA en neuronas corticales (Suñol *et al.*, 2006) y para prevenir la muerte causada por NMDA (Xilouri & Papazafiri, 2006). Además, la picrotoxinina inhibe la neuroprotección causada por el tratamiento con 1μM de alopregnanolona (Ardeshiri *et al.*, 2006), lo que refuerza lo observado con el dieldrín. En cambio, la alopregnanolona no tuvo efecto protector en células granulares de cerebelo, posiblemente porque ésta tiene una menor afinidad por los receptores $GABA_A$ que contienen las subunidades α6, α4 y δ allí expresados con respecto a (Zhu *et al.*, 1996; Hawkinson *et al.*, 1998). Este neuroesteroide comparte además otra diana molecular con el dieldrín, el receptor PXR (Coumoul *et al.*, 2002; Mellon, 2007), el cual requiere altas concentraciones de alopregnanolona para ser activado y, dada su baja expresión en el cerebro, es poco probable que medie sus efectos neuroprotectores. No obstante no se puede descartar dado que algunos neuroesteroides (alopregnanolona incluida) y otros derivados de pregnanos son capaces de regular la expresión de las proteínas de la familia Bcl-2, lo que contribuye a sus efectos antiapoptóticas en células PC12 (Charalampopoulos *et al.*, 2004). Por último, sus efectos neuroprotectores frente a la isquemia *in vivo* se han relacionado con su acción inhibitoria sobre el poro de transición de la permeabilidad mitocondrial (Sayeed *et al.*, 2009), aunque también con la estabilización del receptor $GABA_A$ en la membrana plasmática (Kelley *et al.*, 2008).

Los otros pesticidas organoclorados estudiados, endosulfán y lindano, parecen compartir con el dieldrín el mismo mecanismo de neurotoxicidad en neuronas corticales, puesto que ambos causaron un efecto similar al dieldrín sobre la expresión de mGLUR5, y a concentraciones proporcionales (10 veces menor para dieldrín y endosulfán) a sus LC50 tras 6 DIV de exposición. De acuerdo con esto, el daño celular provocado por el lindano en neuronas corticales ha sido atenuado con agonistas del receptor $GABA_A$ y con antagonistas de los de glutamato (Vale *et al.*, 1998b), aunque parece ser independiente del Ca^{2+} (Pomes *et al.*, 1994a), lo que excluye el componente excitotóxico pero es compatible con la hipótesis "hipoglutamatérgica" planteada anteriormente para el dieldrín. Por su parte, el endosulfán reduce la formación de neuritas en neuronas corticales inmaduras y su efecto citotóxico se ha asociado con la reducción de ERK1/2 (Kang *et al.*, 2001). Finalmente, el daño neuronal inducido por la epilepsia en el córtex y en el hipocampo inicialmente cursa con necrosis y apoptosis mediada por AIF (ambas causadas por la activación de las calpaínas), mientras que la apoptosis inducida por las caspasas prevalece a tiempos más largos (Kondratyev &

Gale, 2004; Fujikawa, 2005; Wang *et al.*, 2008). Siguiendo con la hipótesis planteada anteriormente, la activación de las calpaínas por la exposición a dieldrín en neuronas maduras degradaría algunos componentes de la membrana, como la subunidad NR2B y quizás mGLUR1, lo que supondría una menor activación de las rutas MAPK y PI3K/Akt (Thandi *et al.*, 2002; Yang *et al.*, 2004; Xu *et al.*, 2007b; Doshi & Lynch, 2009), y desencadenaría la activación de las caspasas en la fase final de la muerte.

En cambio, la toxicidad del lindano en células granulares de cerebelo se previene con agonistas del receptor $GABA_B$ y también con antagonistas de los de glutamato, aunque solo cuando las neuronas son inducidas a expresar receptores $GABA_B$ y $GABA_A$ insensibles a las benzodiazepinas (Vale *et al.*, 1998a), mientras que, en condiciones normales, está mediada por la movilización de los depósitos intracelulares de Ca^{2+} (Rosa *et al.*, 1996). Además, tanto lindano como endosulfan alteran el potencial de membrana mitocondrial pero esto no viene acompañado de estrés oxidativo (Rosa *et al.*, 1996). Los resultados derivados de este trabajo indican que la toxicidad del dieldrín y el endosulfán en estas neuronas tampoco es de tipo excitotóxica ni debida a la producción de radicales libres, sino que implica la inhibición de las rutas de supervivencia neuronal PI3K/Akt y MAPK y la consecuente apoptosis mediada por la caspasa-3. Este tipo de muerte apoptótica causada por la inactivación de dichas rutas ha sido también descrita en células granulares de cerebelo privadas de suero o expuestas a un medio con baja $[K^+]$ (Linseman *et al.*, 2002; Barneda-Zahonero *et al.*, 2009), aunque la estimulación sostenida de ERK1/2 también se ha observado bajo estas mismas condiciones (Subramaniam *et al.*, 2005). Aunque *a priori* contradictorios, estos efectos pueden explicarse en base a la localización y duración de la actividad de ERK1/2. Por un lado, se ha demostrado que la activación transitoria de la ruta MAPK tiene efectos neuroprotectores e implica la regulación génica de proteínas pro- y antiapoptóticas de la familia Bcl-2 (Hardingham, 2009), mientras que la activación crónica se ha relacionado con diversas patologías (Colucci-D'Amato *et al.*, 2003). En el último caso, la retención de la forma activa de ERK1/2 en el núcleo se produce en situaciones de estrés oxidativo, como en la isquemia, mientras que su confinamiento en mitocondrias o autofagosomas está asociado a enfermedades neurodegenerativas, como el Alzheimer o el Parkinson (Chu *et al.*, 2004). En los experimentos de inmunocitoquímica realizados en el presente estudio se puede observar que, si bien la activación de Akt se ve reducida de manera general tras la exposición a dieldrín y endosulfán, el efecto sobre ERK1/2 no está tan claro. Tras la exposición a ambos pesticidas parece haber menos células positivas para

fosfo-ERK1/2; sin embargo, el marcaje en los axones y dendritas es más intenso en las neuronas tratadas con dieldrín, incluso con respecto al control. La activación excesiva de ERK1/2 en zonas sinápticas se ha implicado en la muerte necrótica bajo un modelo de epilepsia (Murray *et al.*, 1998) y también produce daño en la membrana plasmática en las células granulares de cerebelo(Subramaniam *et al.*, 2005), por lo que puede ser otro mecanismo implicado en la toxicidad del dieldrín en estas neuronas. Asimismo, la activación transitoria de ERK1/2 por estradiol e ICI182780 causa muerte necrótica en células granulares de cerebelo inmaduras (Wong *et al.*, 2003), efecto aparentemente mediado a través de ERβ (Le & Belcher, 2010). Esto podría explicar la mayor susceptibilidad de estas neuronas al dieldrín durante los primeros DIV. En cambio, el aumento de viabilidad observado tras la exposición a bajas concentraciones de dieldrín (200-600 nM) durante 6DIV podría ser debido a su antagonismo sobre el receptor $GABA_A$, al promover la supervivencia de estas neuronas, que depende de la actividad sináptica (Gault & Siegel, 1998). Sin embargo, este efecto solo se observó en el caso del dieldrín, por lo que podría tratarse de un efecto estrogénico sobre la mitosis, como ha sido descrito para el estradiol en estas misma células (Wong *et al.*, 2003).

La neuroprotección inducida por la activación del receptor de IGF-I frente a varios modelos de apoptosis se ha asociado a la estabilización de los niveles basales de fosforilación de estas kinasas (Linseman *et al.*, 2002; Subramaniam *et al.*, 2005; Willaime-Morawek *et al.*, 2005). Por el contrario, la incapacidad del estradiol de activar Akt en células granulares de cerebelo se ha visto responsable de su falta de efectos neuroprotectores ante la disminución de la $[K^+]$ y frente a la excitotoxicidad (Miñano *et al.*, 2007). En cualquier caso, el tratamiento con estradiol e insulina por separado no recuperó los niveles basales de Akt o ERK1/2 ni la muerte neuronal causada por dieldrin, y tan solo el tratamiento combinado con éstas hormonas tuvo efectos significativos sobre dichos parámetros celulares, lo que está de acuerdo con los efectos sinérgicos de estradiol e IGF-I sobre la neuroprotección mediada por estas rutas de señalización, previamente reportados bajo otros modelos de muerte celular (Mendez *et al.*, 2005). En cambio, el tratamiento con insulina sí revirtió parcialmente la toxicidad del endosulfán, tal vez debido a que esta hormona incrementa por sí sola la fosforilación de Akt, aunque no de manera significativa, en presencia de dieldrín y por el contrario, no tiene ningún efecto sobre fosfo-ERK1/2; lo que sugiere que la toxicidad del endosulfán depende más de su efecto sobre Akt. De acuerdo con esto, el dieldrín pero no el endosulfán estimula la fosforilación prolongada de ERK1/2 en neuritas y esto

puede contribuir a su muerte, como hemos comentado antes. Además, la activación de Akt en células granulares de cerebelo puede tener efectos tóxicos bajo determinadas circunstancias, como la inducida por NMDA, a través de la estimulación de nNOS (Llansola & Felipo, 2010). Estas últimas observaciones nos llevan a la hipótesis de que la activación sostenida (durante al menos 5 horas) de las rutas PI3K/Akt y MAPK por el dieldrín y mediada a través de su interacción con los receptores de estrógenos, puede ser responsables de su propia inhibición observada tras 24 horas de tratamiento. A este respecto, se sabe que la activación mantenida de ERK1/2 estimula fuertemente la expresión de las fosfatasas MKP encargadas de inhibir a la propia ruta MAPK, en un mecanismo de autorregulación o retroalimentación negativa (Colucci-D'Amato *et al.*, 2003), razón por la cual estas proteínas pueden explicar los efectos del dieldrín sobre ERK1/2. Otro candidato es la fosfatasa PP2A, que media la desactivación de Akt frente a estímulos apoptóticos (Mora *et al.*, 2002) pero también la de ERK1/2 y cuya actividad y expresión están reguladas por el estradiol (Belcher *et al.*, 2005; Yi & Simpkins, 2008). Así, esta acción genómica sobre PP2A podría ser mimetizada por los pesticidas organoclorados a través de los receptores de estrógenos. Además, la actividad de PP2A ha sido implicada en la muerte de las células granulares de cerebelo inducida por xenoestrógenos (Le & Belcher, 2010), aunque también en la neuroprotección inducida por estradiol (Yi & Simpkins, 2008). La activación rápida de PP2A por estradiol puede ser responsable también de su inhabilidad de estimular la fosforilación de Akt en células granulares de cerebelo. Por el contrario, este efecto no es reproducido por ICI182780 (Belcher *et al.*, 2005), lo que podría explicar, a su vez, que el endosulfán y el dieldrín sí sean capaces de activar Akt.

De manera similar, la fosforilación de ERK1/2 inducida por los pesticidas organoclorados en neuronas corticales maduras, mediada por la inhibición del receptor $GABA_A$ y posterior activación de los receptores de glutamato, puede conducir a la sobreexpresión de las MKPs y a la desativación de la ruta MAPK, regulando de esa manera la expresión de NR2A, como se ha postulado anteriormente. Puesto que la exposición prolongada a dieldrín también causa la internalización de los receptores NMDA en células granulares de cerebelo (Babot *et al.*, 2007), los receptores $GABA_A$ también podrían estar implicados en la desactivación de estas vías de señalización y, por ende, en la toxicidad de los pesticidas en estas células. En este sentido, la neuroprotección por parte de estradiol e insulina en modelos de epilepsia e isquemia *in vitro* se ha asociado a su potenciación de la neurotransmisión GABAérgica, al

incrementar la síntesis de GABA y la inserción de receptores $GABA_A$ en la membrana, respectivamente (Mielke & Wang, 2005; Zhou *et al.*, 2007), siendo este último efecto mediado por Akt (Fujii *et al.*, 2010). Por tanto, la acción combinada de estradiol e insulina podría contrarrestar la inhibición del receptor $GABA_A$ causada por los pesticidas organoclorados. Comparando los valores de LC50 de estos contaminantes en células granulares de cerebelo con las IC50 sobre los receptores $GABA_A$ y de estrógenos, observamos que la toxicidad de dieldrín y endosulfán es similar en los dos tiempos de exposición estudiados, y superior a la de lindano, lo que se corresponde con su potencia en la inhibición del receptor $GABA_A$ (Vale *et al.*, 2003) y su letalidad aguda en animales (Lawrence & Casida, 1984; Bloomquist, 2002).

En cambio, el dieldrín es más afín que el endosulfán y el lindano por los receptores de estrógenos, lo que sugiere una implicación menor de estos últimos en la toxicidad de los pesticidas organoclorados en las células granulares de cerebelo. Otros estudios *in vitro* han observado también una mayor potencia del dieldrín con respecto a los otros dos xenoestrógenos sobre ERα recombinante (Scippo *et al.*, 2004; Sumbayev *et al.*, 2005). Esto, sumado a la mayor expresión de ERα en las células granulares de cerebelo podría contribuir al efecto observado. En concordancia con esto, también se ha descrito una afinidad superior para el dieldrín sobre ERα respecto a ERβ (Gale *et al.*, 2004). No obstante, el hecho de que el dieldrín tenga más sitios de unión (al actuar tanto sobre ERα y ERβ como sobre GPR30) que el endosulfan y el lindano, puede ser la razón de estas diferencias. A pesar de que la expresión de GPR30 no se ha demostrado en células granulares de cerebelo, sí que se ha detectado en la capa de Purkinje y en el cerebelo de ratones adultos y de crías de 7 días (Canonaco *et al.*, 2008; Hazell *et al.*, 2009). Además, la activación de ERK1/2 por estradiol e ICI182780 en las células granulares del cerebelo es dependiente de un GPR de membrana sin identificar (Wong *et al.*, 2003; Belcher *et al.*, 2005), aunque los mismos autores han observado que puede ser mediada a través de ERβ (Le & Belcher, 2010). Los datos obtenidos con el uso del antagonista específico de GPR30, G-15, indican no sólo que este receptor debe estar presente en las células granulares del cerebelo, sino que además media, al menos en parte, las acciones no genómicas del dieldrín estudiadas en estas neuronas.

Los efectos de dieldrín y endosulfán sobre Akt en neuronas corticales fueron mediados por su acción sobre ERβ, lo que podría deberse a la mayor expresión de esta isoforma en dichos cultivos. Más difícil de explicar es el efecto de endosulfan sobre Akt en células granulares de cerebelo, mediado también por ERβ. No obstante tanto dieldrín

como endosulfan son capaces de producir cambios conformacionales ya sea sobre ERα como en ERβ a concentraciones bajas (Sumbayev *et al.*, 2005), con lo cual incluso una moderada expresión de ERβ podría ser suficiente para activar Akt. Por otro lado, el endosulfán y, en mayor medida, el lindano bloquean totalmente los efectos del estradiol sobre estas vías de señalización en ambos cultivos, lo que sugiere que ambos compuestos son también capaces de actuar sobre GPR30, si asumimos que este receptor es responsable la activación de ERK1/2 por estradiol en el cerebelo. En cualquier caso, las interacciones moleculares de los pesticidas organoclorados con los receptores de estrógenos son muy diferente a las observadas con estradiol o con los SERMs tamoxifen e ICI182780 (Sumbayev *et al.*, 2005). Aparentemente, esto es debido a la estructura química no planar de los pesticidas organoclorados, que les permite activar sólo la región AF1, a diferencia de los compuestos planos, como los estrógenos naturales, que pueden activar AF1 y AF2 (Bentrem *et al.*, 2003). Aunque se desconocen las implicaciones que esto pueda tener en lo referente a los efectos no genómicos, es de suponer que modificará la señalización mediada por el receptor de estrógenos y tendrá consecuencias fisiológicas indeseadas. Así por ejemplo, se han observado alteraciones en la plasticidad sináptica y en la sinaptogénesis en presencia de ciertos disruptores endocrinos que pueden contribuir a sus efectos cognitivos (Schantz & Widholm, 2001; Ogiue-Ikeda *et al.*, 2008).

La exposición prolongada a los pesticidas organoclorados redujo los niveles de ERα pero no de ERβ en ambos cultivos neuronales, de acuerdo con lo observado en células MCF-7 (Grunfeld & Bonefeld-Jorgensen, 2004). La autorregulación de ERα parece responsable de los efectos de los pesticidas organoclorados en células granulares de cerebelo. De la misma manera, otros efectos genómicos descritos para estos contaminantes se atribuido a la *trans*-activación de ERα, con la excepción del lindano, a quien se ha asignado actividad sobre ERβ o ninguna actividad dependiendo del ensayo y del tipo celular (Soto *et al.*, 1995; Lemaire *et al.*, 2006; Maranghi *et al.*, 2007; Li *et al.*, 2008). A este respecto, podría argumentarse que los efectos genómicos del lindano tal vez se deban a su acción estimuladora sobre la aromatasa (Nativelle-Serpentini *et al.*, 2003) o incluso mediante un mecanismo no convencional, como ha sido demostrado para su isómero β-HCH (Silva *et al.*, 2010). Si bien los efectos sobre ERα en células granulares de cerebelo parecen deberse a este mecanismo de regulación autóloga, en neuronas corticales es el bloqueo del receptor $GABA_A$ el mecanismo más probablemente implicado, puesto que el tratamiento crónico con picrotoxinina redujo

los niveles de ERα, a diferencia de lo ocurrido en células granulares de cerebelo. En concordancia con esto, recientemente se ha detectado una menor expresión de ERα en pacientes epilépticos y una recuperación de estos con benzodiacepinas (Killer *et al.*, 2009), lo que refuerza la implicación del receptor $GABA_A$. No obstante, otros mecanismos no excluyentes pueden estar involucrados al respecto, tales como: la activación de la ruta MAPK, que estimula la actividad transcripcional del receptor de estrógenos a través de AF1 (Kato *et al.*, 1995); la inhibición de Akt, que disminuiría la estabilidad de ERα (Mendez & Garcia-Segura, 2006); e incluso podrían intervenir mecanismos de regulación epigenéticos, como se ha observado a lo largo del desarrollo y tras el daño neuronal (Wilson & Westberry, 2009).

La disminución de los niveles de ERα en las neuronas corticales producida por la exposición prolongada a los pesticidas organoclorados podría interferir con los efectos del estradiol regulados a través de este receptor, tales como la modulación de los receptores AMPA y NMDA (Brann *et al.*, 1993; Numakawa *et al.*, 2007; Morissette *et al.*, 2008b) y posiblemente de los mGLUR de tipo I (Mermelstein, 2009). Si este es un efecto general de los pesticidas organoclorados en todo el cerebro, la reducción de ERα afectaría a procesos tan importantes como la sináptogénesis y la supervivencia neuronal (Jelks *et al.*, 2007; Zhao & Brinton, 2007; Ogiue-Ikeda *et al.*, 2008; Morissette *et al.*, 2008a). Además, tal vez pueda explicar la ausencia de neuroprotección frente al dieldrín por parte del estradiol en neuronas corticales, aunque el efecto negativo de esta hormona sobre los niveles corticales de GABA, mediados a través de ERβ, también puede contribuir (Blurton-Jones & Tuszynski, 2006). Por el contrario, el incremento de las subunidades α1, α2 y γ2 inducido por el estradiol no parece ser suficientemente efectivo, quizás porque al mismo tiempo disminuye los niveles corticales de allopregnanolona y, con ello, la estimulación endógena del receptor $GABA_A$ (Calza *et al.*, 2010). Debe hacerse constancia de que el estradiol fue capaz de incrementar la expresión de los receptores NR1/NR2B en neuronas corticales expuestas a 60 nM de dieldrín. Esto es posible dado a esta concentración el pesticida no tiene efectos significativos sobre ERα. Sería interesante comprobar si el estradiol conserva su capacidad de incrementar estos receptores NMDA en células expuestas a 200 nM de dieldrín.

En el trabajo anexo a este estudio hemos comprobado que el dieldrín regula de manera opuesta la expresión de los receptores metabotrópicos de glutamato de tipo I en células granulares de cerebelo. Sin embargo, ningún otro de los antagonistas del receptor

$GABA_A$ probados mostró efectos significativos sobre estos receptores. Asimismo, se ha comprobado que el dieldrín regula la expresión de los receptores de glutamato en células carentes del receptor $GABA_A$ (Slotkin & Seidler, 2009). Aunque se requerirían más experimentos para confirmarlo, es posible que los receptores de estrógenos sean responsables de esta regulación, dado que, como hemos observado en el segundo trabajo, el dieldrín tiene una mayor afinidad por ellos que el endosulfán y el lindano en estas neuronas. Sin embargo, a pesar de que las concentraciones de estos últimos usadas en estos experimentos pueden no ser suficientes para activar el receptor de estrógenos, lo cierto es que sí lo fueron para disminuir los niveles de ERα en estas mismas neuronas, efecto que hemos atribuido a su regulación autóloga. Por tanto, es posible que otros mecanismos estén implicados, como la inhibición o estimulación de la actividad transcripcional de ERβ, aunque estos efectos también han sido descritos para endosulfán y lindano, respectivamente (Lemaire *et al.*, 2006; Maranghi *et al.*, 2007). Una opción exclusiva para dieldrín sería, por lo observado en este trabajo, la activación de GPR30 y, por ende, de la ruta MAPK, la cual se ha visto implicada en la regulación de numerosos genes a través de CREB o de los receptores de estrógenos (Kato *et al.*, 1995; Hardingham, 2009). Para añadir mayor complejidad al asunto, entre los efectos no genómicos atribuidos a los receptores de estrógenos está, precisamente, la activación de los receptores metabotrópicos de glutamato de tipos I y II. Así, la activación de ERα puede estimular la ruta MAPK mediante mGLUR1/5, o inhibirla a través de mGLUR2/3 (Mermelstein, 2009). Dado que dieldrín fue el único de los pesticidas que fue capaz de activar ERK1/2 en células granulares de cerebelo, esta vía de señalización es una candidata probable a mediar sus efectos a largo plazo sobre los receptores metabotrópicos de glutamato de tipo I. Se puede incluso postular que la fosforilación de ERK1/2 inducida por dieldrín en estas células y, en parte, mediada por ERα podría también involucrar la activación de mGLUR1 y/o mGLUR5. En cambio, la falta de efecto de endosulfán y lindano sobre la ruta MAPK podría deberse a la activación por éstos de ERβ y, en consecuencia de los receptores metabotrópicos de glutamato de tipo II. En cualquier caso, la exposición prolongada a dieldrín parece interferir con el cambio de predominancia en la expresión entre mGLUR5 y mGLUR1 que se da a lo largo del desarrollo (Catania *et al.*, 1994), y probablemente causará un desajuste en sus vías de señalización, implicadas en la supervivencia y en la excitotoxicidad (Xu *et al.*, 2007b; Pshenichkin *et al.*, 2008; Llansola & Felipo, 2010). Además, la pérdida de mGLUR1

puede conducir a una disfunción crónica del cerebelo, como puede ser la ataxia cerebelar (Nakanishi, 2005).

En este trabajo también mostramos que las neuronas glutamatérgicas (en especial las corticales inmaduras) son particularmente sensibles a la exposición prolongada a los pesticidas organoclorados, al menos tanto como lo son las monoaminérgicas (Liu *et al.*, 1997a; Sanchez-Ramos *et al.*, 1998; Chan *et al.*, 2006). En cambio, las neuronas corticales GABAérgicas son más resistentes frente a la excitotoxicidad (Tecoma & Choi, 1989) y, por el contrario, más susceptibles a los antagonistas del receptor NMDA. Esto es, al parecer, debido a que expresan una mayor proporción de receptores NMDA que contienen la subunidad NR2A con respecto a las neuronas piramidales (Kinney *et al.*, 2006). Esta podría ser la razón por la cual las neuronas GABAérgicas también se ven menos afectadas por los pesticidas que las glutamatérgicas (y posiblemente también que las colinérgicas). Es interesante resaltar que todos los receptores afectados en primer lugar por la exposición prolongada a dieldrín, NR1/NR2B, mGLUR5 (puesto que co-localiza con NR1/NR2B) y ERα, se expresan preferentemente en las neuronas piramidales (Monyer *et al.*, 1994; Kinney *et al.*, 2006; Blurton-Jones & Tuszynski, 2006; Ogiue-Ikeda *et al.*, 2008).

Numerosos estudios con animales han demostrado que la exposición crónica a los pesticidas organoclorados tiene efectos perjudiciales sobre las funciones cognitivas y sobre el comportamiento (Smith *et al.*, 1976; Topinka *et al.*, 1984; Tilson *et al.*, 1987; Paul *et al.*, 1994; Schantz & Widholm, 2001; Mariussen & Fonnum, 2006). Sin embargo, muy pocos trabajos se han centrado en los posibles efectos neurológicos de estos contaminantes durante el desarrollo (para revisión consultar (Evangelista de Duffard & Duffard, 1996). Los resultados derivados de este trabajo pueden contribuir al entendimiento de los mecanismos moleculares implicados en los efectos crónicos de los pesticidas organoclorados sobre la memoria y el aprendizaje así como sus alteraciones locomotoras, citados anteriormente, y en los que el córtex y el cerebelo, respectivamente, tienen un papel clave. Finalmente, este estudio pone de manifiesto la vulnerabilidad del cerebro en desarrollo frente a la exposición a estos productos sintéticos.

V. CONCLUSIONES

1. La exposición prolongada a dieldrín causa la internalización de los receptores NMDA que contienen la subunidad NR2B y reduce la expresión de mGLUR5 y de la subunidad NR2A del receptor NMDA en neuronas corticales.

2. La exposición prolongada a dieldrín reduce los niveles de mGLUR1 y aumenta los de mGLUR5 en células granulares de cerebelo.

3. El tratamiento con alopregnanolona restablece la funcionalidad de los receptores NMDA y $GABA_A$ tras la exposición a dieldrín en neuronas corticales y previene la muerte neuronal inducida por el pesticida.

4. Los pesticidas organoclorados dieldrín, endosulfán y lindano interaccionan de manera diferente con los receptores de estrógenos en neuronas corticales y en células granulares de cerebelo y son capaces de activar las vías de señalización MAPK y PI3K/Akt en ambos tipos neuronales, aunque por distintos mecanismos.

5. La exposición prolongada a estos pesticidas reduce los niveles de ERα pero no de ERβ en células granulares de cerebelo y en neuronas corticales gracias a su interacción con los receptores de estrógenos y con el receptor $GABA_A$, respectivamente.

6. Las neuronas corticales y granulares de cerebelo inmaduras son más vulnerables a la toxicidad de los pesticidas organoclorados que las maduras. Además, las neuronas corticales glutamatérgicas son más sensibles a la toxicidad del dieldrín que las neuronas corticales GABAérgicas.

7. El tratamiento con estradiol e insulina previene la apoptosis mediada por caspasas inducida por dieldrín y endosulfán en células granulares de cerebelo a través de la activación de las rutas PI3K/Akt y MAPK.

VI. BIBLIOGRAFÍA

Aamodt, S. M., Shi, J., Colonnese, M. T., Veras, W., and Constantine-Paton, M. (2000). Chronic NMDA exposure accelerates development of GABAergic inhibition in the superior colliculus. *J.Neurophysiol.* 83, 1580-1591.

Aguirre, C., Jayaraman, A., Pike, C., and Baudry, M. (2010). Progesterone inhibits estrogen-mediated neuroprotection against excitotoxicity by down-regulating estrogen receptor-beta. *J.Neurochem.* 115, 1277-1287.

Alagarsamy, S., Saugstad, J., Warren, L., Mansuy, I. M., Gereau, R. W., and Conn, P. J. (2005). NMDA-induced potentiation of mGluR5 is mediated by activation of protein phosphatase 2B/calcineurin. *Neuropharmacology* 49 Suppl 1, 135-145.

Aldea, M., Sobrado, M., Fuentealba, J., Arroyo, G., and García, A. G. (2002). Neurotransmisión, señales de calcio y enfermedad de Alzheimer. In Fronteras en la enfermedad de Alzheimer (A. G. García, and L. Gandía, Eds.), pp. 11-32. Farmaindustria.

Alger, B. E., and Le Beau, F. E. N. (2001). Physiology of the GABA and Glycine Systems. In Pharmacology of GABA and Glycine Neurotransmission (H. Möhler, Ed.), pp. 3-76. Springer-Verlag.

Amateau, S. K., Alt, J. J., Stamps, C. L., and McCarthy, M. M. (2004). Brain estradiol content in newborn rats: sex differences, regional heterogeneity, and possible de novo synthesis by the female telencephalon. *Endocrinology* 145, 2906-2917.

Andersen, H. R., Andersson, A. M., Arnold, S. F., Autrup, H., Barfoed, M., Beresford, N. A., Bjerregaard, P., Christiansen, L. B., Gissel, B., Hummel, R., Jorgensen, E. B., Korsgaard, B., Le Guevel, R., Leffers, H., McLachlan, J., Moller, A., Nielsen, J. B., Olea, N., Oles-Karasko, A., Pakdel, F., Pedersen, K. L., Perez, P., Skakkeboek, N. E., Sonnenschein, C., Soto, A. M., and . (1999). Comparison of short-term estrogenicity tests for identification of hormone-disrupting chemicals. *Environ.Health Perspect.* 107 Suppl 1, 89-108.

Andersen, H. R., Vinggaard, A. M., Rasmussen, T. H., Gjermandsen, I. M., and Bonefeld-Jorgensen, E. C. (2002). Effects of currently used pesticides in assays for estrogenicity, androgenicity, and aromatase activity in vitro. *Toxicol.Appl.Pharmacol.* 179, 1-12.

Araque, A., Sanzgiri, R. P., Parpura, V., and Haydon, P. G. (1999). Astrocyte-induced modulation of synaptic transmission. *Can.J.Physiol Pharmacol.* 77, 699-706.

Ardeshiri, A., Kelley, M. H., Korner, I. P., Hurn, P. D., and Herson, P. S. (2006). Mechanism of progesterone neuroprotection of rat cerebellar Purkinje cells following oxygen-glucose deprivation. *Eur.J.Neurosci.* 24, 2567-2574.

ATSDR. Agency for Toxic Substances and Disease Registry . 2002.

Babot, Z. Implicació dels sistemes glutamatèrgic i GABAèrgic en la mort per excitotoxicitat en cultius de cèl·lules granulars de cerebel. Alteracions d'aquests sistemes pel contaminant orgànic persistent dieldrín. 74-75. 2006.
Ref Type: Thesis/Dissertation

Babot, Z., Cristofol, R., and Sunol, C. (2005). Excitotoxic death induced by released glutamate in depolarized primary cultures of mouse cerebellar granule cells is dependent on GABAA receptors and niflumic acid-sensitive chloride channels. *Eur.J.Neurosci.* 21, 103-112.

Babot, Z., Vilaro, M. T., and Sunol, C. (2007). Long-term exposure to dieldrin reduces gamma-aminobutyric acid type A and N-methyl-D-aspartate receptor function in primary cultures of mouse cerebellar granule cells. *J.Neurosci.Res.* 85, 3687-3695.

Barnard, E. A. (2001). The Molecualr Architecture of GABAA Receptors. In Pharmacology of GABA and Glycine Neurotransmission (H. Möhler, Ed.), pp. 79-99. Springer-Verlag.

Barneda-Zahonero, B., Minano-Molina, A., Badiola, N., Fado, R., Xifro, X., Saura, C. A., and Rodriguez-Alvarez, J. (2009). Bone morphogenetic protein-6 promotes cerebellar granule neurons survival by activation of the MEK/ERK/CREB pathway. *Mol.Biol.Cell* 20, 5051-5063.

Battaglia, G., Bruno, V., Pisani, A., Centonze, D., Catania, M. V., Calabresi, P., and Nicoletti, F. (2001). Selective blockade of type-1 metabotropic glutamate receptors induces neuroprotection by enhancing gabaergic transmission. *Mol.Cell Neurosci.* 17, 1071-1083.

Behringer, K. A., Gault, L. M., and Siegel, R. E. (1996). Differential regulation of GABA A receptor subunit mRNAs in rat cerebellar granule neurons: importance of environmental cues. *J.Neurochem.* 66, 1347-1353.

Belcher, S. M. (1999). Regulated expression of estrogen receptor alpha and beta mRNA in granule cells during development of the rat cerebellum. *Brain Res.Dev.Brain Res.* 115, 57-69.

Belcher, S. M., Le, H. H., Spurling, L., and Wong, J. K. (2005). Rapid estrogenic regulation of extracellular signal- regulated kinase 1/2 signaling in cerebellar granule cells involves a G protein- and protein kinase A-dependent mechanism and intracellular activation of protein phosphatase 2A. *Endocrinology* 146, 5397-5406.

Bentrem, D., Fox, J. E., Pearce, S. T., Liu, H., Pappas, S., Kupfer, D., Zapf, J. W., and Jordan, V. C. (2003). Distinct molecular conformations of the estrogen receptor alpha complex exploited by environmental estrogens. *Cancer Res.* 63, 7490-7496.

Berthele, A., Platzer, S., Laurie, D. J., Weis, S., Sommer, B., Zieglgansberger, W., Conrad, B., and Tolle, T. R. (1999). Expression of metabotropic glutamate receptor subtype mRNA (mGluR1-8) in human cerebellum. *Neuroreport* 10, 3861-3867.

Bi, R., Broutman, G., Foy, M. R., Thompson, R. F., and Baudry, M. (2000). The tyrosine kinase and mitogen-activated protein kinase pathways mediate multiple effects of estrogen in hippocampus. *Proc.Natl.Acad.Sci.U.S.A* 97, 3602-3607.

Bloomquist, J. R. (2002). Agents affecting chloride channels. In Handbook of Neurotoxicology (E. Massaro, Ed.), pp. 65-77. Humana Press.

Blurton-Jones, M., and Tuszynski, M. H. (2006). Estradiol-induced modulation of estrogen receptor-beta and GABA within the adult neocortex: a potential transsynaptic mechanism for estrogen modulation of BDNF. *J.Comp Neurol.* 499, 603-612.

Bonci, A., Grillner, P., Siniscalchi, A., Mercuri, N. B., and Bernardi, G. (1997). Glutamate metabotropic receptor agonists depress excitatory and inhibitory transmission on rat mesencephalic principal neurons. *Eur.J.Neurosci.* 9, 2359-2369.

Bortolotto, Z. A., Clarke, V. R., Delany, C. M., Parry, M. C., Smolders, I., Vignes, M., Ho, K. H., Miu, P., Brinton, B. T., Fantaske, R., Ogden, A., Gates, M., Ornstein, P. L., Lodge, D., Bleakman, D., and Collingridge, G. L. (1999). Kainate receptors are involved in synaptic plasticity. *Nature* 402, 297-301.

Botella, B., Crespo, J., Rivas, A., Cerrillo, I., Olea-Serrano, M. F., and Olea, N. (2004). Exposure of women to organochlorine pesticides in Southern Spain. *Environ.Res.* 96, 34-40.

Bourque, M., Dluzen, D. E., and Di Paolo, T. (2009). Neuroprotective actions of sex steroids in Parkinson's disease. *Front Neuroendocrinol.* 30, 142-157.

Brandoli, C., Sanna, A., De Bernardi, M. A., Follesa, P., Brooker, G., and Mocchetti, I. (1998). Brain-derived neurotrophic factor and basic fibroblast growth factor downregulate NMDA receptor function in cerebellar granule cells. *J.Neurosci.* 18, 7953-7961.

Brann, D. W., Zamorano, P. L., Chorich, L. P., and Mahesh, V. B. (1993). Steroid hormone effects on NMDA receptor binding and NMDA receptor mRNA levels in the hypothalamus and cerebral cortex of the adult rat. *Neuroendocrinology* 58, 666-672.

Brinton, R. D. (1994). The neurosteroid 3 alpha-hydroxy-5 alpha-pregnan-20-one induces cytoarchitectural regression in cultured fetal hippocampal neurons. *J.Neurosci.* 14, 2763-2774.

Brock, C., Oueslati, N., Soler, S., Boudier, L., Rondard, P., and Pin, J. P. (2007). Activation of a dimeric metabotropic glutamate receptor by intersubunit rearrangement. *J.Biol.Chem.* 282, 33000-33008.

Bruno, V., Battaglia, G., Copani, A., Cespedes, V. M., Galindo, M. F., Cena, V., Sanchez-Prieto, J., Gasparini, F., Kuhn, R., Flor, P. J., and Nicoletti, F. (2001). An activity-dependent switch from facilitation to inhibition in the control of excitotoxicity by group I metabotropic glutamate receptors. *Eur.J.Neurosci.* 13, 1469-1478.

Bruno, V., Copani, A., Knopfel, T., Kuhn, R., Casabona, G., Dell'Albani, P., Condorelli, D. F., and Nicoletti, F. (1995). Activation of metabotropic glutamate receptors coupled to inositol phospholipid hydrolysis amplifies NMDA-induced neuronal degeneration in cultured cortical cells. *Neuropharmacology* 34, 1089-1098.

Bulleit, R. F., and Hsieh, T. (2000). MEK inhibitors block BDNF-dependent and -independent expression of GABA(A) receptor subunit mRNAs in cultured mouse cerebellar granule neurons. *Brain Res.Dev.Brain Res.* 119, 1-10.

Calza, A., Sogliano, C., Santoru, F., Marra, C., Angioni, M. M., Mostallino, M. C., Biggio, G., and Concas, A. (2010). Neonatal exposure to estradiol in rats influences neuroactive steroid concentrations, GABAA receptor expression, and behavioral sensitivity to anxiolytic drugs. *J.Neurochem.* 113, 1285-1295.

Canonaco, M., Giusi, G., Madeo, A., Facciolo, R. M., Lappano, R., Canonaco, A., and Maggiolini, M. (2008). A sexually dimorphic distribution pattern of the novel estrogen receptor G-protein-coupled receptor 30 in some brain areas of the hamster. *J.Endocrinol.* 196, 131-138.

Carlson, B. X., Belhage, B., Hansen, G. H., Elster, L., Olsen, R. W., and Schousboe, A. (1997). Expression of the GABA(A) receptor alpha6 subunit in cultured cerebellar granule cells is developmentally regulated by activation of GABA(A) receptors. *J.Neurosci.Res.* 50, 1053-1062.

Carriedo, S. G., Yin, H. Z., Sensi, S. L., and Weiss, J. H. (1998). Rapid Ca2+ entry through Ca2+-permeable AMPA/Kainate channels triggers marked intracellular Ca2+ rises and consequent oxygen radical production. *J.Neurosci.* 18, 7727-7738.

Catania, M. V., Landwehrmeyer, G. B., Testa, C. M., Standaert, D. G., Penney, J. B., Jr., and Young, A. B. (1994). Metabotropic glutamate receptors are differentially regulated during development. *Neuroscience* 61, 481-495.

Cebers, G., Cebere, A., Wagner, A., and Liljequist, S. (1999). Prolonged inhibition of glutamate reuptake down-regulates NMDA receptor functions in cultured cerebellar granule cells. *J.Neurochem.* 72, 2181-2190.

Cerrillo, I., Granada, A., Lopez-Espinosa, M. J., Olmos, B., Jimenez, M., Cano, A., Olea, N., and Fatima Olea-Serrano, M. (2005). Endosulfan and its metabolites in fertile women, placenta, cord blood, and human milk. *Environ.Res.* 98, 233-239.

Chan, M. P., Morisawa, S., Nakayama, A., Kawamoto, Y., and Yoneda, M. (2006). Development of an in vitro blood-brain barrier model to study the effects of endosulfan on the permeability of tight junctions and a comparative study of the cytotoxic effects of endosulfan on rat and human glial and neuronal cell cultures. *Environ.Toxicol.* 21, 223-235.

Chandler, L. J., Sutton, G., Dorairaj, N. R., and Norwood, D. (2001). N-methyl D-aspartate receptor-mediated bidirectional control of extracellular signal-regulated kinase activity in cortical neuronal cultures. *J.Biol.Chem.* 276, 2627-2636.

Charalampopoulos, I., Tsatsanis, C., Dermitzaki, E., Alexaki, V. I., Castanas, E., Margioris, A. N., and Gravanis, A. (2004). Dehydroepiandrosterone and allopregnanolone protect sympathoadrenal medulla cells against apoptosis via antiapoptotic Bcl-2 proteins. *Proc.Natl.Acad.Sci.U.S.A* 101, 8209-8214.

Chavko, M., Nemoto, E. M., and Melick, J. A. (1993). Regional lipid composition in the rat brain. *Mol.Chem.Neuropathol.* 18, 123-131.

Chen, P., Gu, Z., Liu, W., and Yan, Z. (2007). Glycogen synthase kinase 3 regulates N-methyl-D-aspartate receptor channel trafficking and function in cortical neurons. *Mol.Pharmacol.* 72, 40-51.

Cheyne, J. E., and Montgomery, J. M. (2008). Plasticity-dependent changes in metabotropic glutamate receptor expression at excitatory hippocampal synapses. *Mol.Cell Neurosci.* 37, 432-439.

Choi, D. W. (1987). Ionic dependence of glutamate neurotoxicity. *J.Neurosci.* 7, 369-379.

Chopra, A. K., Sharma, M. K., and Chamoli, S. (2011). Bioaccumulation of organochlorine pesticides in aquatic system--an overview. *Environ.Monit.Assess.* 173, 905-916.

Chu, C. T., Levinthal, D. J., Kulich, S. M., Chalovich, E. M., and DeFranco, D. B. (2004). Oxidative neuronal injury. The dark side of ERK1/2. *Eur.J.Biochem.* 271, 2060-2066.

Chun, H. S., Gibson, G. E., DeGiorgio, L. A., Zhang, H., Kidd, V. J., and Son, J. H. (2001). Dopaminergic cell death induced by MPP(+), oxidant and specific neurotoxicants shares the common molecular mechanism. *J.Neurochem.* 76, 1010-1021.

Chung, H. J., Huang, Y. H., Lau, L. F., and Huganir, R. L. (2004). Regulation of the NMDA receptor complex and trafficking by activity-dependent phosphorylation of the NR2B subunit PDZ ligand. *J.Neurosci.* 24, 10248-10259.

Colucci-D'Amato, L., Perrone-Capano, C., and di Porzio, U. (2003). Chronic activation of ERK and neurodegenerative diseases. *Bioessays* 25, 1085-1095.

Coosen, R., and van Velsen, F. L. (1989). Effects of the beta-isomer of hexachlorocyclohexane on estrogen-sensitive human mammary tumor cells. *Toxicol.Appl.Pharmacol.* 101, 310-318.

Cordey, M., Gundimeda, U., Gopalakrishna, R., and Pike, C. J. (2003). Estrogen activates protein kinase C in neurons: role in neuroprotection. *J.Neurochem.* 84, 1340-1348.

Corrigan, F. M., Wienburg, C. L., Shore, R. F., Daniel, S. E., and Mann, D. (2000). Organochlorine insecticides in substantia nigra in Parkinson's disease. *J.Toxicol.Environ.Health A* 59, 229-234.

Coumoul, X., Diry, M., and Barouki, R. (2002). PXR-dependent induction of human CYP3A4 gene expression by organochlorine pesticides. *Biochem.Pharmacol.* 64, 1513-1519.

Crump, F. T., Dillman, K. S., and Craig, A. M. (2001). cAMP-dependent protein kinase mediates activity-regulated synaptic targeting of NMDA receptors. *J.Neurosci.* 21, 5079-5088.

Cui, H., Hayashi, A., Sun, H. S., Belmares, M. P., Cobey, C., Phan, T., Schweizer, J., Salter, M. W., Wang, Y. T., Tasker, R. A., Garman, D., Rabinowitz, J., Lu, P. S., and Tymianski, M. (2007). PDZ protein interactions underlying NMDA receptor-mediated excitotoxicity and neuroprotection by PSD-95 inhibitors. *J.Neurosci.* 27, 9901-9915.

Cull-Candy, S., Brickley, S., and Farrant, M. (2001). NMDA receptor subunits: diversity, development and disease. *Curr.Opin.Neurobiol.* 11, 327-335.

d'Anglemont, d. T., X, Campagne, C., Dehouck, B., Leroy, D., Holstein, G. R., Beauvillain, J. C., Buee-Scherrer, V., and Prevot, V. (2007). Coupling of neuronal nitric oxide synthase to NMDA receptors via postsynaptic density-95 depends on estrogen and contributes to the central control of adult female reproduction. *J.Neurosci.* 27, 6103-6114.

d'Anglemont, d. T., X, Campagne, C., Steculorum, S., and Prevot, V. (2009). Estradiol induces physical association of neuronal nitric oxide synthase with NMDA receptor and promotes nitric oxide formation via estrogen receptor activation in primary neuronal cultures. *J.Neurochem.* 109, 214-224.

D'Onofrio, M., Cuomo, L., Battaglia, G., Ngomba, R. T., Storto, M., Kingston, A. E., Orzi, F., De Blasi, A., Di Iorio, P., Nicoletti, F., and Bruno, V. (2001). Neuroprotection mediated by glial group-II metabotropic glutamate receptors requires the activation of the MAP kinase and the phosphatidylinositol-3-kinase pathways. *J.Neurochem.* 78, 435-445.

Daniel, J. M., and Dohanich, G. P. (2001). Acetylcholine mediates the estrogen-induced increase in NMDA receptor binding in CA1 of the hippocampus and the associated improvement in working memory. *J.Neurosci.* 21, 6949-6956.

Das, S., Sasaki, Y. F., Rothe, T., Premkumar, L. S., Takasu, M., Crandall, J. E., Dikkes, P., Conner, D. A., Rayudu, P. V., Cheung, W., Chen, H. S., Lipton, S. A., and Nakanishi, N. (1998). Increased NMDA current and spine density in mice lacking the NMDA receptor subunit NR3A. *Nature* 393, 377-381.

Ding, S., Fellin, T., Zhu, Y., Lee, S. Y., Auberson, Y. P., Meaney, D. F., Coulter, D. A., Carmignoto, G., and Haydon, P. G. (2007). Enhanced astrocytic Ca2+ signals contribute to neuronal excitotoxicity after status epilepticus. *J.Neurosci.* 27, 10674-10684.

Dong, Y. N., Waxman, E. A., and Lynch, D. R. (2004). Interactions of postsynaptic density-95 and the NMDA receptor 2 subunit control calpain-mediated cleavage of the NMDA receptor. *J.Neurosci.* 24, 11035-11045.

Doshi, S., and Lynch, D. R. (2009). Calpain and the glutamatergic synapse. *Front Biosci.(Schol.Ed)* 1, 466-476.

Duggan, R. E., Corneliussen, P. E., Duggan, M. B., McMahon, B. M., and Martin, R. J. (1983). Pesticide residue levels in foods in the United States from July 1, 1969, to June 30, 1976: Summary. *J.Assoc.Off Anal.Chem.* 66, 1534-1535.

Duguid, I., and Sjostrom, P. J. (2006). Novel presynaptic mechanisms for coincidence detection in synaptic plasticity. *Curr.Opin.Neurobiol.* 16, 312-322.

Durand, D., Pampillo, M., Caruso, C., and Lasaga, M. (2008). Role of metabotropic glutamate receptors in the control of neuroendocrine function. *Neuropharmacology* 55, 577-583.

Ehlers, M. D. (2000). Reinsertion or degradation of AMPA receptors determined by activity-dependent endocytic sorting. *Neuron* 28, 511-525.

Elias, G. M., Elias, L. A., Apostolides, P. F., Kriegstein, A. R., and Nicoll, R. A. (2008). Differential trafficking of AMPA and NMDA receptors by SAP102 and PSD-95 underlies synapse development. *Proc.Natl.Acad.Sci.U.S.A* 105, 20953-20958.

Elster, L., Hansen, G. H., Belhage, B., Fritschy, J. M., Mohler, H., and Schousboe, A. (1995). Differential distribution of GABAA receptor subunits in soma and processes of cerebellar granule cells: effects of maturation and a GABA agonist. *Int.J.Dev.Neurosci.* 13, 417-428.

Evangelista de Duffard, A. M., and Duffard, R. (1996). Behavioral toxicology, risk assessment, and chlorinated hydrocarbons. *Environ.Health Perspect.* 104 Suppl 2, 353-360.

Evinger, A. J., III, and Levin, E. R. (2005). Requirements for estrogen receptor alpha membrane localization and function. *Steroids* 70, 361-363.

FAO/UNEP. Decision Guidance Documents: Dieldrin, Endosulfan, Lindane. Joint Food and Agriculture Organization/United Nations Environment Programme. 1991.

Ferraguti, F., and Shigemoto, R. (2006). Metabotropic glutamate receptors. *Cell Tissue Res.* 326, 483-504.

ffrench-Constant, R. H., Rocheleau, T. A., Steichen, J. C., and Chalmers, A. E. (1993). A point mutation in a Drosophila GABA receptor confers insecticide resistance. *Nature* 363, 449-451.

Filardo, E. J., Quinn, J. A., Bland, K. I., and Frackelton, A. R., Jr. (2000). Estrogen-induced activation of Erk-1 and Erk-2 requires the G protein-coupled receptor homolog, GPR30, and occurs via trans-activation of the epidermal growth factor receptor through release of HB-EGF. *Mol.Endocrinol.* 14, 1649-1660.

Fleming, L., Mann, J. B., Bean, J., Briggle, T., and Sanchez-Ramos, J. R. (1994). Parkinson's disease and brain levels of organochlorine pesticides. *Ann.Neurol.* 36, 100-103.

Fogal, B., Trettel, J., Uliasz, T. F., Levine, E. S., and Hewett, S. J. (2005). Changes in secondary glutamate release underlie the developmental regulation of excitotoxic neuronal cell death. *Neuroscience* 132, 929-942.

Francesconi, A., Kumari, R., and Zukin, R. S. (2009). Regulation of group I metabotropic glutamate receptor trafficking and signaling by the caveolar/lipid raft pathway. *J.Neurosci.* 29, 3590-3602.

Frandsen, A., and Schousboe, A. (1990). Development of excitatory amino acid induced cytotoxicity in cultured neurons. *Int.J.Dev.Neurosci.* 8, 209-216.

Fujii, M., Kanematsu, T., Ishibashi, H., Fukami, K., Takenawa, T., Nakayama, K. I., Moss, S. J., Nabekura, J., and Hirata, M. (2010). Phospholipase C-related but catalytically inactive protein is required for insulin-induced cell surface expression of gamma-aminobutyric acid type A receptors. *J.Biol.Chem.* 285, 4837-4846.

Fujikawa, D. G. (2005). Prolonged seizures and cellular injury: understanding the connection. *Epilepsy Behav.* 7 Suppl 3, S3-11.

Fujita, K., Nakanishi, K., Sobue, K., Ueki, T., Asai, K., and Kato, T. (1998). Astrocytic gap junction blockage and neuronal Ca2+ oscillation in neuron-astrocyte cocultures in vitro. *Neurochem.Int.* 33, 41-49.

Gale, W. L., Patino, R., and Maule, A. G. (2004). Interaction of xenobiotics with estrogen receptors alpha and beta and a putative plasma sex hormone-binding globulin from channel catfish (Ictalurus punctatus). *Gen.Comp Endocrinol.* 136, 338-345.

Gallo, V., and Russell, J. T. (1995). Excitatory amino acid receptors in glia: different subtypes for distinct functions? *J.Neurosci.Res.* 42, 1-8.

Gant, D. B., Eldefrawi, M. E., and Eldefrawi, A. T. (1987). Cyclodiene insecticides inhibit GABAA receptor-regulated chloride transport. *Toxicol.Appl.Pharmacol.* 88, 313-321.

Garcia-Segura, L. M., Chowen, J. A., Naftolin, F., and Torres-Aleman, I. (1999). Steroid Effects on Brain Plasticity: Role of Glial Cells and Trophic Factors. In Neurosteroids: A New Regulatory Function in the Nervous System (E. Baulieu, P. Robel, and M. Schumacher, Eds.), pp. 255-268. Humana Press.

Garcia-Segura, L. M., Veiga, S., Sierra, A., Melcangi, R. C., and Azcoitia, I. (2003). Aromatase: a neuroprotective enzyme. *Prog.Neurobiol.* 71, 31-41.

Gardoni, F., Mauceri, D., Malinverno, M., Polli, F., Costa, C., Tozzi, A., Siliquini, S., Picconi, B., Cattabeni, F., Calabresi, P., and Di Luca, M. (2009). Decreased NR2B subunit synaptic levels cause impaired long-term potentiation but not long-term depression. *J.Neurosci.* 29, 669-677.

Gardoni, F., Polli, F., Cattabeni, F., and Di Luca, M. (2006). Calcium-calmodulin-dependent protein kinase II phosphorylation modulates PSD-95 binding to NMDA receptors. *Eur.J.Neurosci.* 24, 2694-2704.

Gault, L. M., and Siegel, R. E. (1998). NMDA receptor stimulation selectively initiates GABA(A) receptor delta subunit mRNA expression in cultured rat cerebellar granule neurons. *J.Neurochem.* 70, 1907-1915.

Gazzaley, A. H., Weiland, N. G., McEwen, B. S., and Morrison, J. H. (1996). Differential regulation of NMDAR1 mRNA and protein by estradiol in the rat hippocampus. *J.Neurosci.* 16, 6830-6838.

Gillespie, D. C., Kim, G., and Kandler, K. (2005). Inhibitory synapses in the developing auditory system are glutamatergic. *Nat.Neurosci.* 8, 332-338.

Gonzalez, M., Miglioranza, K. S., Aizpun De Moreno, J. E., and Moreno, V. J. (2003). Occurrence and distribution of organochlorine pesticides (OCPs) in tomato (Lycopersicon esculentum) crops from organic production. *J.Agric.Food Chem.* 51, 1353-1359.

Grunfeld, H. T., and Bonefeld-Jorgensen, E. C. (2004). Effect of in vitro estrogenic pesticides on human oestrogen receptor alpha and beta mRNA levels. *Toxicol.Lett.* 151, 467-480.

Guo, W., Wei, F., Zou, S., Robbins, M. T., Sugiyo, S., Ikeda, T., Tu, J. C., Worley, P. F., Dubner, R., and Ren, K. (2004). Group I metabotropic glutamate receptor NMDA receptor coupling and signaling cascade mediate spinal dorsal horn NMDA receptor 2B tyrosine phosphorylation associated with inflammatory hyperalgesia. *J.Neurosci.* 24, 9161-9173.

Habas, A., Kharebava, G., Szatmari, E., and Hetman, M. (2006). NMDA neuroprotection against a phosphatidylinositol-3 kinase inhibitor, LY294002 by NR2B-mediated suppression of glycogen synthase kinase-3beta-induced apoptosis. *J.Neurochem.* 96, 335-348.

Hall, J. M., and McDonnell, D. P. (1999). The estrogen receptor beta-isoform (ERbeta) of the human estrogen receptor modulates ERalpha transcriptional activity and is a key regulator of the cellular response to estrogens and antiestrogens. *Endocrinology* 140, 5566-5578.

Hardingham, G. E. (2009). Coupling of the NMDA receptor to neuroprotective and neurodestructive events. *Biochem.Soc.Trans.* 37, 1147-1160.

Hatcher, J. M., Delea, K. C., Richardson, J. R., Pennell, K. D., and Miller, G. W. (2008). Disruption of dopamine transport by DDT and its metabolites. *Neurotoxicology* 29, 682-690.

Hawkinson, J. E., Acosta-Burruel, M., Yang, K. C., Hogenkamp, D. J., Chen, J. S., Lan, N. C., Drewe, J. A., Whittemore, E. R., Woodward, R. M., Carter, R. B., and Upasani, R. B. (1998). Substituted 3beta-phenylethynyl derivatives of 3alpha-hydroxy-5alpha-pregnan-20-one: remarkably potent neuroactive steroid modulators of gamma-aminobutyric acidA receptors. *J.Pharmacol.Exp.Ther.* 287, 198-207.

Hawkinson, J. E., Kimbrough, C. L., Belelli, D., Lambert, J. J., Purdy, R. H., and Lan, N. C. (1994). Correlation of neuroactive steroid modulation of [35S]t-butylbicyclophosphorothionate and [3H]flunitrazepam binding and gamma-aminobutyric acidA receptor function. *Mol.Pharmacol.* 46, 977-985.

Hayashi, T., Thomas, G. M., and Huganir, R. L. (2009). Dual palmitoylation of NR2 subunits regulates NMDA receptor trafficking. *Neuron* 64, 213-226.

Hazell, G. G., Yao, S. T., Roper, J. A., Prossnitz, E. R., O'Carroll, A. M., and Lolait, S. J. (2009). Localisation of GPR30, a novel G protein-coupled oestrogen receptor, suggests multiple functions in rodent brain and peripheral tissues. *J.Endocrinol.* 202, 223-236.

Heinz, G. H., Hill, E. F., and Contrera, J. F. (1980). Dopamine and norepinephrine depletion in ring doves fed DDE, dieldrin, and Aroclor 1254. *Toxicol.Appl.Pharmacol.* 53, 75-82.

Herrero, I., Miras-Portugal, M. T., and Sanchez-Prieto, J. (1998). Functional switch from facilitation to inhibition in the control of glutamate release by metabotropic glutamate receptors. *J.Biol.Chem.* 273, 1951-1958.

Hilber, I., Mader, P., Schulin, R., and Wyss, G. S. (2008). Survey of organochlorine pesticides in horticultural soils and there grown Cucurbitaceae. *Chemosphere* 73, 954-961.

Hille, B. (1968). Pharmacological modifications of the sodium channels of frog nerve. *J.Gen.Physiol* 51, 199-219.

Hilton, G. D., Nunez, J. L., Bambrick, L., Thompson, S. M., and McCarthy, M. M. (2006). Glutamate-mediated excitotoxicity in neonatal hippocampal neurons is mediated by mGluR-induced release of Ca++ from intracellular stores and is prevented by estradiol. *Eur.J.Neurosci.* 24, 3008-3016.

Hites, R. A., Foran, J. A., Carpenter, D. O., Hamilton, M. C., Knuth, B. A., and Schwager, S. J. (2004). Global assessment of organic contaminants in farmed salmon. *Science* 303, 226-229.

Hoffmann, H., Gremme, T., Hatt, H., and Gottmann, K. (2000). Synaptic activity-dependent developmental regulation of NMDA receptor subunit expression in cultured neocortical neurons. *J.Neurochem.* 75, 1590-1599.

Hojo, Y., Hattori, T. A., Enami, T., Furukawa, A., Suzuki, K., Ishii, H. T., Mukai, H., Morrison, J. H., Janssen, W. G., Kominami, S., Harada, N., Kimoto, T., and Kawato, S. (2004). Adult male rat hippocampus synthesizes estradiol from pregnenolone by cytochromes P45017alpha and P450 aromatase localized in neurons. *Proc.Natl.Acad.Sci.U.S.A* 101, 865-870.

Hollmann, M., Hartley, M., and Heinemann, S. (1991). Ca2+ permeability of KA-AMPA--gated glutamate receptor channels depends on subunit composition. *Science* 252, 851-853.

Hosie, A. M., Wilkins, M. E., da Silva, H. M., and Smart, T. G. (2006). Endogenous neurosteroids regulate GABAA receptors through two discrete transmembrane sites. *Nature* 444, 486-489.

HSDB. Hazardous Substances Data Bank. http://toxnet.nlm.nih.gov.Last update: 24-5-2011.

Hu, J. H., Park, J. M., Park, S., Xiao, B., Dehoff, M. H., Kim, S., Hayashi, T., Schwarz, M. K., Huganir, R. L., Seeburg, P. H., Linden, D. J., and Worley, P. F. (2010). Homeostatic scaling requires group I mGluR activation mediated by Homer1a. *Neuron* 68, 1128-1142.

IARC. Evaluation of the carcinogenic risk of chemicals to humans. Summaries & Evaluations: Dieldrin. International Agency for Research on Cancer . 1987.

Ikeda, T., Matsuki, N., and Yamada, M. K. (2006). Estrogen produced in cultured hippocampal neurons is a functional regulator of a GABAergic machinery. *J.Neurosci.Res.* 84, 1771-1777.

Inoue, H., and Okada, Y. (2007). Roles of volume-sensitive chloride channel in excitotoxic neuronal injury. *J.Neurosci.* 27, 1445-1455.

Jelks, K. B., Wylie, R., Floyd, C. L., McAllister, A. K., and Wise, P. (2007). Estradiol targets synaptic proteins to induce glutamatergic synapse formation in cultured hippocampal neurons: critical role of estrogen receptor-alpha. *J.Neurosci.* 27, 6903-6913.

Jia, Z., and Misra, H. P. (2007a). Developmental exposure to pesticides zineb and/or endosulfan renders the nigrostriatal dopamine system more susceptible to these environmental chemicals later in life. *Neurotoxicology* 28, 727-735.

Jia, Z., and Misra, H. P. (2007b). Reactive oxygen species in in vitro pesticide-induced neuronal cell (SH-SY5Y) cytotoxicity: role of NFkappaB and caspase-3. *Free Radic.Biol.Med.* 42, 288-298.

Jin, L., Tran, D. Q., Ide, C. F., McLachlan, J. A., and Arnold, S. F. (1997). Several synthetic chemicals inhibit progesterone receptor-mediated transactivation in yeast. *Biochem.Biophys.Res.Commun.* 233, 139-146.

Johnson, D. C., Sen, M., and Dey, S. K. (1992). Differential effects of dichlorodiphenyltrichloroethane analogs, chlordecone, and 2,3,7,8-tetrachlorodibenzo-p-dioxin on establishment of pregnancy in the hypophysectomized rat. *Proc.Soc.Exp.Biol.Med.* 199, 42-48.

Johnston, G. A. (2005). GABA(A) receptor channel pharmacology. *Curr.Pharm.Des* 11, 1867-1885.

Johri, A., Dhawan, A., Singh, R. L., and Parmar, D. (2008). Persistence in alterations in the ontogeny of cerebral and hepatic cytochrome P450s following prenatal exposure to low doses of lindane. *Toxicol.Sci.* 101, 331-340.

Jong, Y. J., Schwetye, K. E., and O'Malley, K. L. (2007). Nuclear localization of functional metabotropic glutamate receptor mGlu1 in HEK293 cells and cortical neurons: role in nuclear calcium mobilization and development. *J.Neurochem.* 101, 458-469.

Kalantzi, O. I., Alcock, R. E., Johnston, P. A., Santillo, D., Stringer, R. L., Thomas, G. O., and Jones, K. C. (2001). The global distribution of PCBs and organochlorine pesticides in butter. *Environ.Sci.Technol.* 35, 1013-1018.

Kang, K. S., Park, J. E., Ryu, D. Y., and Lee, Y. S. (2001). Effects and neuro-toxic mechanisms of 2, 2', 4, 4', 5, 5'-hexachlorobiphenyl and endosulfan in neuronal stem cells. *J.Vet.Med.Sci.* 63, 1183-1190.

Kanthasamy, A. G., Kitazawa, M., Kaul, S., Yang, Y., Lahiri, D. K., Anantharam, V., and Kanthasamy, A. (2003). Proteolytic activation of proapoptotic kinase PKCdelta is regulated by overexpression of Bcl-2: implications for oxidative stress and environmental factors in Parkinson's disease. *Ann.N.Y.Acad.Sci.* 1010, 683-686.

Kato, A., Rouach, N., Nicoll, R. A., and Bredt, D. S. (2005). Activity-dependent NMDA receptor degradation mediated by retrotranslocation and ubiquitination. *Proc.Natl.Acad.Sci.U.S.A* 102, 5600-5605.

Kato, S., Endoh, H., Masuhiro, Y., Kitamoto, T., Uchiyama, S., Sasaki, H., Masushige, S., Gotoh, Y., Nishida, E., Kawashima, H., Metzger, D., and Chambon, P. (1995). Activation of the estrogen receptor through phosphorylation by mitogen-activated protein kinase. *Science* 270, 1491-1494.

Kelley, M. H., Taguchi, N., Ardeshiri, A., Kuroiwa, M., Hurn, P. D., Traystman, R. J., and Herson, P. S. (2008). Ischemic insult to cerebellar Purkinje cells causes diminished GABAA receptor function and allopregnanolone neuroprotection is associated with GABAA receptor stabilization. *J.Neurochem.* 107, 668-678.

Kelly, M. J., and Ronnekleiv, O. K. (2009). Control of CNS neuronal excitability by estrogens via membrane-initiated signaling. *Mol.Cell Endocrinol.* 308, 17-25.

Killer, N., Hock, M., Gehlhaus, M., Capetian, P., Knoth, R., Pantazis, G., Volk, B., and Meyer, R. P. (2009). Modulation of androgen and estrogen receptor expression by antiepileptic drugs and steroids in hippocampus of patients with temporal lobe epilepsy. *Epilepsia* 50, 1875-1890.

Kim, E. K., and Choi, E. J. (2010). Pathological roles of MAPK signaling pathways in human diseases. *Biochim.Biophys.Acta* 1802, 396-405.

King, A. E., Chung, R. S., Vickers, J. C., and Dickson, T. C. (2006). Localization of glutamate receptors in developing cortical neurons in culture and relationship to susceptibility to excitotoxicity. *J.Comp Neurol.* 498, 277-294.

Kinney, J. W., Davis, C. N., Tabarean, I., Conti, B., Bartfai, T., and Behrens, M. M. (2006). A specific role for NR2A-containing NMDA receptors in the maintenance of parvalbumin and GAD67 immunoreactivity in cultured interneurons. *J.Neurosci.* 26, 1604-1615.

Kitazawa, M., Anantharam, V., and Kanthasamy, A. G. (2001). Dieldrin-induced oxidative stress and neurochemical changes contribute to apoptopic cell death in dopaminergic cells. *Free Radic.Biol.Med.* 31, 1473-1485.

Kleckner, N. W., and Dingledine, R. (1988). Requirement for glycine in activation of NMDA-receptors expressed in Xenopus oocytes. *Science* 241, 835-837.

Koh, J. Y., and Choi, D. W. (1991). Selective blockade of non-NMDA receptors does not block rapidly triggered glutamate-induced neuronal death. *Brain Res.* 548, 318-321.

Kondo, M., Sumino, R., and Okado, H. (1997). Combinations of AMPA receptor subunit expression in individual cortical neurons correlate with expression of specific calcium-binding proteins. *J.Neurosci.* 17, 1570-1581.

Kondratyev, A., and Gale, K. (2004). Latency to onset of status epilepticus determines molecular mechanisms of seizure-induced cell death. *Brain Res.Mol.Brain Res.* 121, 86-94.

Kornau, H. C., Schenker, L. T., Kennedy, M. B., and Seeburg, P. H. (1995). Domain interaction between NMDA receptor subunits and the postsynaptic density protein PSD-95. *Science* 269, 1737-1740.

Koroshetz, W. J., Freese, A., and DiFiglia, M. (1990). The correlation between excitatory amino acid-induced current responses and excitotoxicity in striatal cultures. *Brain Res.* 521, 265-272.

Korpi, E. R., and Luddens, H. (1993). Regional gamma-aminobutyric acid sensitivity of t-butylbicyclophosphoro[35S]thionate binding depends on gamma-aminobutyric acidA receptor alpha subunit. *Mol.Pharmacol.* 44, 87-92.

Kuiper, G. G., Enmark, E., Pelto-Huikko, M., Nilsson, S., and Gustafsson, J. A. (1996). Cloning of a novel receptor expressed in rat prostate and ovary. *Proc.Natl.Acad.Sci.U.S.A* 93, 5925-5930.

Lambert, J. J., Peters, J. A., Harney, S. C., and Belelli, D. (2001). Steroid Modulation of GABAA Receptors. In Pharmacology of GABA and Glycine Neurotransmission (H. Möhler, Ed.), pp. 117-140. Springer-Verlag.

Lan, J. Y., Skeberdis, V. A., Jover, T., Zheng, X., Bennett, M. V., and Zukin, R. S. (2001). Activation of metabotropic glutamate receptor 1 accelerates NMDA receptor trafficking. *J.Neurosci.* 21, 6058-6068.

Lavezzari, G., McCallum, J., Dewey, C. M., and Roche, K. W. (2004). Subunit-specific regulation of NMDA receptor endocytosis. *J.Neurosci.* 24, 6383-6391.

Law, R. J., and Lightstone, F. C. (2008). Gaba receptor insecticide non-competitive antagonists may bind at allosteric modulator sites. *Int.J.Neurosci.* 118, 705-734.

Lawrence, L. J., and Casida, J. E. (1984). Interactions of lindane, toxaphene and cyclodienes with brain-specific t-butylbicyclophosphorothionate receptor. *Life Sci.* 35, 171-178.

Le Bras, M., Rouy, I., and Brenner, C. (2006). The modulation of inter-organelle cross-talk to control apoptosis. *Med.Chem.* 2, 1-12.

Le, H. H., and Belcher, S. M. (2010). Rapid signaling actions of environmental estrogens in developing granule cell neurons are mediated by estrogen receptor ss. *Endocrinology* 151, 5689-5699.

Lee, S. H., Simonetta, A., and Sheng, M. (2004). Subunit rules governing the sorting of internalized AMPA receptors in hippocampal neurons. *Neuron* 43, 221-236.

Lemaire, G., Balaguer, P., Michel, S., and Rahmani, R. (2005). Activation of retinoic acid receptor-dependent transcription by organochlorine pesticides. *Toxicol.Appl.Pharmacol.* 202, 38-49.

Lemaire, G., Mnif, W., Mauvais, P., Balaguer, P., and Rahmani, R. (2006). Activation of alpha- and beta-estrogen receptors by persistent pesticides in reporter cell lines. *Life Sci.* 79, 1160-1169.

Leveille, F., Papadia, S., Fricker, M., Bell, K. F., Soriano, F. X., Martel, M. A., Puddifoot, C., Habel, M., Wyllie, D. J., Ikonomidou, C., Tolkovsky, A. M., and Hardingham, G. E. (2010). Suppression of the intrinsic apoptosis pathway by synaptic activity. *J.Neurosci.* 30, 2623-2635.

Li, J., Li, N., Ma, M., Giesy, J. P., and Wang, Z. (2008). In vitro profiling of the endocrine disrupting potency of organochlorine pesticides. *Toxicol.Lett.* 183, 65-71.

Li, J. H., Wang, Y. H., Wolfe, B. B., Krueger, K. E., Corsi, L., Stocca, G., and Vicini, S. (1998). Developmental changes in localization of NMDA receptor subunits in primary cultures of cortical neurons. *Eur.J.Neurosci.* 10, 1704-1715.

Li, X., Zhang, S., and Safe, S. (2006). Activation of kinase pathways in MCF-7 cells by 17beta-estradiol and structurally diverse estrogenic compounds. *J.Steroid Biochem.Mol.Biol.* 98, 122-132.

Linseman, D. A., Phelps, R. A., Bouchard, R. J., Le, S. S., Laessig, T. A., McClure, M. L., and Heidenreich, K. A. (2002). Insulin-like growth factor-I blocks Bcl-2 interacting mediator of cell death (Bim) induction and intrinsic death signaling in cerebellar granule neurons. *J.Neurosci.* 22, 9287-9297.

Liu, F., Day, M., Muniz, L. C., Bitran, D., Arias, R., Revilla-Sanchez, R., Grauer, S., Zhang, G., Kelley, C., Pulito, V., Sung, A., Mervis, R. F., Navarra, R., Hirst, W. D., Reinhart, P. H., Marquis, K. L., Moss, S. J., Pangalos, M. N., and Brandon, N. J. (2008). Activation of estrogen receptor-beta regulates hippocampal synaptic plasticity and improves memory. *Nat.Neurosci.* 11, 334-343.

Liu, J., Brannen, K. C., Grayson, D. R., Morrow, A. L., Devaud, L. L., and Lauder, J. M. (1998). Prenatal exposure to the pesticide dieldrin or the GABA(A) receptor antagonist bicuculline differentially alters expression of GABA(A) receptor subunit mRNAs in fetal rat brainstem. *Dev.Neurosci.* 20, 83-92.

Liu, J., Morrow, A. L., Devaud, L., Grayson, D. R., and Lauder, J. M. (1997a). GABAA receptors mediate trophic effects of GABA on embryonic brainstem monoamine neurons in vitro. *J.Neurosci.* 17, 2420-2428.

Liu, J., Morrow, A. L., Devaud, L. L., Grayson, D. R., and Lauder, J. M. (1997b). Regulation of GABA(A) receptor subunit mRNA expression by the pesticide dieldrin in embryonic brainstem cultures: a quantitative, competitive reverse transcription-polymerase chain reaction study. *J.Neurosci.Res.* 49, 645-653.

Liu, X. B., Murray, K. D., and Jones, E. G. (2004). Switching of NMDA receptor 2A and 2B subunits at thalamic and cortical synapses during early postnatal development. *J.Neurosci.* 24, 8885-8895.

Liu, Y., Wong, T. P., Aarts, M., Rooyakkers, A., Liu, L., Lai, T. W., Wu, D. C., Lu, J., Tymianski, M., Craig, A. M., and Wang, Y. T. (2007). NMDA receptor subunits have differential roles in mediating excitotoxic neuronal death both in vitro and in vivo. *J.Neurosci.* 27, 2846-2857.

Llansola, M., and Felipo, V. (2010). Metabotropic glutamate receptor 5, but not 1, modulates NMDA receptor-mediated activation of neuronal nitric oxide synthase. *Neurochem.Int.* 56, 535-545.

Lu, Y. M., Jia, Z., Janus, C., Henderson, J. T., Gerlai, R., Wojtowicz, J. M., and Roder, J. C. (1997). Mice lacking metabotropic glutamate receptor 5 show impaired learning and reduced CA1 long-term potentiation (LTP) but normal CA3 LTP. *J.Neurosci.* 17, 5196-5205.

Lujan, R., Roberts, J. D., Shigemoto, R., Ohishi, H., and Somogyi, P. (1997). Differential plasma membrane distribution of metabotropic glutamate receptors mGluR1 alpha, mGluR2 and mGluR5, relative to neurotransmitter release sites. *J.Chem.Neuroanat.* 13, 219-241.

Luo, Y., and DeFranco, D. B. (2006). Opposing roles for ERK1/2 in neuronal oxidative toxicity: distinct mechanisms of ERK1/2 action at early versus late phases of oxidative stress. *J.Biol.Chem.* 281, 16436-16442.

Madsen, K. K., Larsson, O. M., and Schousboe, A. (2008). Regulation of excitation by GABA neurotransmission: focus on metabolism and transport. *Results Probl.Cell Differ.* 44, 201-221.

Maher, P., and Davis, J. B. (1996). The role of monoamine metabolism in oxidative glutamate toxicity. *J.Neurosci.* 16, 6394-6401.

Mannella, P., and Brinton, R. D. (2006). Estrogen receptor protein interaction with phosphatidylinositol 3-kinase leads to activation of phosphorylated Akt and extracellular signal-regulated kinase 1/2 in the same population of cortical neurons: a unified mechanism of estrogen action. *J.Neurosci.* 26, 9439-9447.

Maranghi, F., Rescia, M., Macri, C., Di Consiglio, E., De Angelis, G., Testai, E., Farini, D., De Felici, M., Lorenzetti, S., and Mantovani, A. (2007). Lindane may modulate the female reproductive development through the interaction with ER-beta: an in vivo-in vitro approach. *Chem.Biol.Interact.* 169, 1-14.

Mariscal-Arcas, M., Lopez-Martinez, C., Granada, A., Olea, N., Lorenzo-Tovar, M. L., and Olea-Serrano, F. (2010). Organochlorine pesticides in umbilical cord blood serum of women from Southern Spain and adherence to the Mediterranean diet. *Food Chem.Toxicol.* 48, 1311-1315.

Mariussen, E., and Fonnum, F. (2006). Neurochemical targets and behavioral effects of organohalogen compounds: an update. *Crit Rev.Toxicol.* 36, 253-289.

Martin, D. L., and Rimvall, K. (1993). Regulation of gamma-aminobutyric acid synthesis in the brain. *J.Neurochem.* 60, 395-407.

Massey, P. V., Johnson, B. E., Moult, P. R., Auberson, Y. P., Brown, M. W., Molnar, E., Collingridge, G. L., and Bashir, Z. I. (2004). Differential roles of NR2A and NR2B-containing NMDA receptors in cortical long-term potentiation and long-term depression. *J.Neurosci.* 24, 7821-7828.

Masuko, N., Makino, K., Kuwahara, H., Fukunaga, K., Sudo, T., Araki, N., Yamamoto, H., Yamada, Y., Miyamoto, E., and Saya, H. (1999). Interaction of NE-dlg/SAP102, a neuronal and endocrine tissue-specific membrane-associated guanylate kinase protein, with calmodulin and PSD-95/SAP90. A possible regulatory role in molecular clustering at synaptic sites. *J.Biol.Chem.* 274, 5782-5790.

Matsuda, K., Sakamoto, H., Mori, H., Hosokawa, K., Kawamura, A., Itose, M., Nishi, M., Prossnitz, E. R., and Kawata, M. (2008). Expression and intracellular distribution of the G protein-coupled receptor 30 in rat hippocampal formation. *Neurosci.Lett.* 441, 94-99.

Mattson, M. P. (2008). Glutamate and neurotrophic factors in neuronal plasticity and disease. *Ann.N.Y.Acad.Sci.* 1144, 97-112.

McDonald, J. W., and Schoepp, D. D. (1992). The metabotropic excitatory amino acid receptor agonist 1S,3R-ACPD selectively potentiates N-methyl-D-aspartate-induced brain injury. *Eur.J.Pharmacol.* 215, 353-354.

McEwen, B. S. (1999). Gonadal Hormone Regulation of Synaptic Plasticity in the Brain: What is the mechanism? In Neurosteroids: A New Regulatory Function in the Nervous System (E. Baulieu, P. Robel, and M. Schumacher, Eds.), pp. 233-253. Humana Press.

McEwen, B. S., Gould, E., Orchinik, M., Weiland, N. G., and Woolley, C. S. (1995). Oestrogens and the structural and functional plasticity of neurons: implications for memory, ageing and neurodegenerative processes. *Ciba Found.Symp.* 191, 52-66.

Mehrotra, B. D., Bansal, S. K., and Desaiah, D. (1982). Comparative effects of structurally related cyclodiene pesticides on ATPases. *J.Appl.Toxicol.* 2, 278-283.

Mehrotra, B. D., Moorthy, K. S., Reddy, S. R., and Desaiah, D. (1989). Effects of cyclodiene compounds on calcium pump activity in rat brain and heart. *Toxicology* 54, 17-29.

Meli, E., Pangallo, M., Picca, R., Baronti, R., Moroni, F., and Pellegrini-Giampietro, D. E. (2004). Differential role of poly(ADP-ribose) polymerase-1in apoptotic and necrotic neuronal death induced by mild or intense NMDA exposure in vitro. *Mol.Cell Neurosci.* 25, 172-180.

Mellon, S. H. (2007). Neurosteroid regulation of central nervous system development. *Pharmacol.Ther.* 116, 107-124.

Mellon, S. H., and Compagnone, N. A. (1999). Molecular Biology and Developmental Regulation of the Enzymes Involved in the Biosynthesis and Metabolism of Neurosteroids. In Neurosteroids: A New Regulatory Function in the Nervous System (E. Baulieu, P. Robel, and M. Schumacher, Eds.), pp. 27-49. Humana Press.

Mendez, P., Azcoitia, I., and Garcia-Segura, L. M. (2005). Interdependence of oestrogen and insulin-like growth factor-I in the brain: potential for analysing neuroprotective mechanisms. *J.Endocrinol.* 185, 11-17.

Mendez, P., and Garcia-Segura, L. M. (2006). Phosphatidylinositol 3-kinase and glycogen synthase kinase 3 regulate estrogen receptor-mediated transcription in neuronal cells. *Endocrinology* 147, 3027-3039.

Mendez, P., Wandosell, F., and Garcia-Segura, L. M. (2006). Cross-talk between estrogen receptors and insulin-like growth factor-I receptor in the brain: cellular and molecular mechanisms. *Front Neuroendocrinol.* 27, 391-403.

Mermelstein, P. G. (2009). Membrane-localised oestrogen receptor alpha and beta influence neuronal activity through activation of metabotropic glutamate receptors. *J.Neuroendocrinol.* 21, 257-262.

Michaels, R. L., and Rothman, S. M. (1990). Glutamate neurotoxicity in vitro: antagonist pharmacology and intracellular calcium concentrations. *J.Neurosci.* 10, 283-292.

Mielke, J. G., and Wang, Y. T. (2005). Insulin exerts neuroprotection by counteracting the decrease in cell-surface GABA receptors following oxygen-glucose deprivation in cultured cortical neurons. *J.Neurochem.* 92, 103-113.

Migaud, M., Charlesworth, P., Dempster, M., Webster, L. C., Watabe, A. M., Makhinson, M., He, Y., Ramsay, M. F., Morris, R. G., Morrison, J. H., O'Dell, T. J., and Grant, S. G. (1998). Enhanced long-term potentiation and impaired learning in mice with mutant postsynaptic density-95 protein. *Nature* 396, 433-439.

Mihalek, R. M., Banerjee, P. K., Korpi, E. R., Quinlan, J. J., Firestone, L. L., Mi, Z. P., Lagenaur, C., Tretter, V., Sieghart, W., Anagnostaras, S. G., Sage, J. R., Fanselow, M. S., Guidotti, A., Spigelman, I., Li, Z., DeLorey, T. M., Olsen, R. W., and Homanics, G. E. (1999). Attenuated sensitivity to neuroactive steroids in gamma-aminobutyrate type A receptor delta subunit knockout mice. *Proc.Natl.Acad.Sci.U.S.A* 96, 12905-12910.

Miller, S., Kesslak, J. P., Romano, C., and Cotman, C. W. (1995). Roles of metabotropic glutamate receptors in brain plasticity and pathology. *Ann.N.Y.Acad.Sci.* 757, 460-474.

Milner, T. A., Lubbers, L. S., Alves, S. E., and McEwen, B. S. (2008). Nuclear and extranuclear estrogen binding sites in the rat forebrain and autonomic medullary areas. *Endocrinology* 149, 3306-3312.

Miñano, A., Cerbon, M. A., Xifro, X., Malagelada, C., Aguilera, J., and Rodriguez-Alvarez, J. (2007). 17beta-estradiol does not protect cerebellar granule cells from excitotoxicity or apoptosis. *J.Neurochem.* 102, 354-364.

Mishina, M., Sakimura, K., Mori, H., Kushiya, E., Harabayashi, M., Uchino, S., and Nagahari, K. (1991). A single amino acid residue determines the Ca2+ permeability of AMPA-selective glutamate receptor channels. *Biochem.Biophys.Res.Commun.* 180, 813-821.

Mitra, S. W., Hoskin, E., Yudkovitz, J., Pear, L., Wilkinson, H. A., Hayashi, S., Pfaff, D. W., Ogawa, S., Rohrer, S. P., Schaeffer, J. M., McEwen, B. S., and Alves, S. E. (2003). Immunolocalization of estrogen receptor beta in the mouse brain: comparison with estrogen receptor alpha. *Endocrinology* 144, 2055-2067.

Mizuta, I., Katayama, M., Watanabe, M., Mishina, M., and Ishii, K. (1998). Developmental expression of NMDA receptor subunits and the emergence of glutamate neurotoxicity in primary cultures of murine cerebral cortical neurons. *Cell Mol.Life Sci.* 54, 721-725.

Möhler, H., Alger, B. E., Le Beau, F. E. N., Barnard, E. A., and Lambert, J. J. (2001). *Pharmacology of GABA and Glycine Neurotransmission*. Springer-Verlag.

Monyer, H., Burnashev, N., Laurie, D. J., Sakmann, B., and Seeburg, P. H. (1994). Developmental and regional expression in the rat brain and functional properties of four NMDA receptors. *Neuron* 12, 529-540.

Mora, A., Sabio, G., Risco, A. M., Cuenda, A., Alonso, J. C., Soler, G., and Centeno, F. (2002). Lithium blocks the PKB and GSK3 dephosphorylation induced by ceramide through protein phosphatase-2A. *Cell Signal.* 14, 557-562.

Morissette, M., Le Saux, M., D'Astous, M., Jourdain, S., Al Sweidi, S., Morin, N., Estrada-Camarena, E., Mendez, P., Garcia-Segura, L. M., and Di Paolo, T. (2008a). Contribution of estrogen receptors alpha and beta to the effects of estradiol in the brain. *J.Steroid Biochem.Mol.Biol.* 108, 327-338.

Morissette, M., Le Saux, M., and Di Paolo, T. (2008b). Effect of oestrogen receptor alpha and beta agonists on brain N-methyl-D-aspartate receptors. *J.Neuroendocrinol.* 20, 1006-1014.

Mu, Y., Otsuka, T., Horton, A. C., Scott, D. B., and Ehlers, M. D. (2003). Activity-dependent mRNA splicing controls ER export and synaptic delivery of NMDA receptors. *Neuron* 40, 581-594.

Muller, B. M., Kistner, U., Kindler, S., Chung, W. J., Kuhlendahl, S., Fenster, S. D., Lau, L. F., Veh, R. W., Huganir, R. L., Gundelfinger, E. D., and Garner, C. C. (1996). SAP102, a novel postsynaptic protein that interacts with NMDA receptor complexes in vivo. *Neuron* 17, 255-265.

Mundell, S. J., Matharu, A. L., Pula, G., Roberts, P. J., and Kelly, E. (2001). Agonist-induced internalization of the metabotropic glutamate receptor 1a is arrestin- and dynamin-dependent. *J.Neurochem.* 78, 546-551.

Murphy, D. D., Cole, N. B., Greenberger, V., and Segal, M. (1998). Estradiol increases dendritic spine density by reducing GABA neurotransmission in hippocampal neurons. *J.Neurosci.* 18, 2550-2559.

Murphy, T. H., Miyamoto, M., Sastre, A., Schnaar, R. L., and Coyle, J. T. (1989). Glutamate toxicity in a neuronal cell line involves inhibition of cystine transport leading to oxidative stress. *Neuron* 2, 1547-1558.

Murphy, T. H., Schnaar, R. L., and Coyle, J. T. (1990). Immature cortical neurons are uniquely sensitive to glutamate toxicity by inhibition of cystine uptake. *FASEB J.* 4, 1624-1633.

Murray, B., Alessandrini, A., Cole, A. J., Yee, A. G., and Furshpan, E. J. (1998). Inhibition of the p44/42 MAP kinase pathway protects hippocampal neurons in a cell-culture model of seizure activity. *Proc.Natl.Acad.Sci.U.S.A* 95, 11975-11980.

Musante, V., Neri, E., Feligioni, M., Puliti, A., Pedrazzi, M., Conti, V., Usai, C., Diaspro, A., Ravazzolo, R., Henley, J. M., Battaglia, G., and Pittaluga, A. (2008). Presynaptic mGlu1 and mGlu5 autoreceptors facilitate glutamate exocytosis from mouse cortical nerve endings. *Neuropharmacology* 55, 474-482.

Nagata, K., Hamilton, B. J., Carter, D. B., and Narahashi, T. (1994). Selective effects of dieldrin on the GABAA receptor-channel subunits expressed in human embryonic kidney cells. *Brain Res.* 645, 19-26.

Nagata, K., and Narahashi, T. (1994). Dual action of the cyclodiene insecticide dieldrin on the gamma-aminobutyric acid receptor-chloride channel complex of rat dorsal root ganglion neurons. *J.Pharmacol.Exp.Ther.* 269, 164-171.

Nakanishi, N., Tu, S., Shin, Y., Cui, J., Kurokawa, T., Zhang, D., Chen, H. S., Tong, G., and Lipton, S. A. (2009). Neuroprotection by the NR3A subunit of the NMDA receptor. *J.Neurosci.* 29, 5260-5265.

Nakanishi, S. (2005). Synaptic mechanisms of the cerebellar cortical network. *Trends Neurosci.* 28, 93-100.

Naqvi, S. M., and Vaishnavi, C. (1993). Bioaccumulative potential and toxicity of endosulfan insecticide to non-target animals. *Comp Biochem.Physiol C.* 105, 347-361.

Nativelle-Serpentini, C., Richard, S., Seralini, G. E., and Sourdaine, P. (2003). Aromatase activity modulation by lindane and bisphenol-A in human placental JEG-3 and transfected kidney E293 cells. *Toxicol.In Vitro* 17, 413-422.

Nicoletti, F., Bruno, V., Catania, M. V., Battaglia, G., Copani, A., Barbagallo, G., Cena, V., Sanchez-Prieto, J., Spano, P. F., and Pizzi, M. (1999). Group-I metabotropic glutamate receptors: hypotheses to explain their dual role in neurotoxicity and neuroprotection. *Neuropharmacology* 38, 1477-1484.

Nishi, M., Hinds, H., Lu, H. P., Kawata, M., and Hayashi, Y. (2001). Motoneuron-specific expression of NR3B, a novel NMDA-type glutamate receptor subunit that works in a dominant-negative manner. *J.Neurosci.* 21, RC185.

Nishimaru, H., Restrepo, C. E., Ryge, J., Yanagawa, Y., and Kiehn, O. (2005). Mammalian motor neurons corelease glutamate and acetylcholine at central synapses. *Proc.Natl.Acad.Sci.U.S.A* 102, 5245-5249.

Numakawa, Y., Matsumoto, T., Yokomaku, D., Taguchi, T., Niki, E., Hatanaka, H., Kunugi, H., and Numakawa, T. (2007). 17beta-estradiol protects cortical neurons against oxidative stress-induced cell death through reduction in the activity of mitogen-activated protein kinase and in the accumulation of intracellular calcium. *Endocrinology* 148, 627-637.

Nuñez, J. L., Bambrick, L. L., Krueger, B. K., and McCarthy, M. M. (2005). Prolongation and enhancement of gamma-aminobutyric acid receptor mediated excitation by chronic treatment with estradiol in developing rat hippocampal neurons. *Eur.J.Neurosci.* 21, 3251-3261.

O'Malley, K. L., Jong, Y. J., Gonchar, Y., Burkhalter, A., and Romano, C. (2003). Activation of metabotropic glutamate receptor mGlu5 on nuclear membranes mediates intranuclear Ca2+ changes in heterologous cell types and neurons. *J.Biol.Chem.* 278, 28210-28219.

Ogiue-Ikeda, M., Tanabe, N., Mukai, H., Hojo, Y., Murakami, G., Tsurugizawa, T., Takata, N., Kimoto, T., and Kawato, S. (2008). Rapid modulation of synaptic plasticity by estrogens as well as endocrine disrupters in hippocampal neurons. *Brain Res.Rev.* 57, 363-375.

Olney, J. W. (1969). Brain lesions, obesity, and other disturbances in mice treated with monosodium glutamate. *Science* 164, 719-721.

Ostlund, H., Keller, E., and Hurd, Y. L. (2003). Estrogen receptor gene expression in relation to neuropsychiatric disorders. *Ann.N.Y.Acad.Sci.* 1007, 54-63.

Ozawa, S., Kamiya, H., and Tsuzuki, K. (1998). Glutamate receptors in the mammalian central nervous system. *Prog.Neurobiol.* 54, 581-618.

Paech, K., Webb, P., Kuiper, G. G., Nilsson, S., Gustafsson, J., Kushner, P. J., and Scanlan, T. S. (1997). Differential ligand activation of estrogen receptors ERalpha and ERbeta at AP1 sites. *Science* 277, 1508-1510.

Pak, D. T., Yang, S., Rudolph-Correia, S., Kim, E., and Sheng, M. (2001). Regulation of dendritic spine morphology by SPAR, a PSD-95-associated RapGAP. *Neuron* 31, 289-303.

Palmada, M., and Centelles, J. J. (1998). Excitatory amino acid neurotransmission. Pathways for metabolism, storage and reuptake of glutamate in brain. *Front Biosci.* 3, d701-d718.

Parpura, V., Basarsky, T. A., Liu, F., Jeftinija, K., Jeftinija, S., and Haydon, P. G. (1994). Glutamate-mediated astrocyte-neuron signalling. *Nature* 369, 744-747.

Paul, V., Balasubramaniam, E., and Kazi, M. (1994). The neurobehavioural toxicity of endosulfan in rats: a serotonergic involvement in learning impairment. *Eur.J.Pharmacol.* 270, 1-7.

Payne, H. L., Ives, J. H., Sieghart, W., and Thompson, C. L. (2008). AMPA and kainate receptors mediate mutually exclusive effects on GABA(A) receptor expression in cultured mouse cerebellar granule neurones. *J.Neurochem.* 104, 173-186.

Peineau, S., Bradley, C., Taghibiglou, C., Doherty, A., Bortolotto, Z. A., Wang, Y. T., and Collingridge, G. L. (2008). The role of GSK-3 in synaptic plasticity. *Br.J.Pharmacol.* 153 Suppl 1, S428-S437.

Perez-Otano, I., and Ehlers, M. D. (2004). Learning from NMDA receptor trafficking: clues to the development and maturation of glutamatergic synapses. *Neurosignals.* 13, 175-189.

Perez-Otano, I., and Ehlers, M. D. (2005). Homeostatic plasticity and NMDA receptor trafficking. *Trends Neurosci.* 28, 229-238.

Perroy, J., Raynaud, F., Homburger, V., Rousset, M. C., Telley, L., Bockaert, J., and Fagni, L. (2008). Direct interaction enables cross-talk between ionotropic and group I metabotropic glutamate receptors. *J.Biol.Chem.* 283, 6799-6805.

Pizzi, M., Galli, P., Consolandi, O., Arrighi, V., Memo, M., and Spano, P. F. (1996). Metabotropic and ionotropic transducers of glutamate signal inversely control cytoplasmic Ca2+ concentration and excitotoxicity in cultured cerebellar granule cells: pivotal role of protein kinase C. *Mol.Pharmacol.* 49, 586-594.

Pomes, A., Frandsen, A., Sunol, C., Sanfeliu, C., Rodriguez-Farre, E., and Schousboe, A. (1994a). Lindane cytotoxicity in cultured neocortical neurons is ameliorated by GABA and flunitrazepam. *J.Neurosci.Res.* 39, 663-668.

Pomes, A., Rodriguez-Farre, E., and Sunol, C. (1993). Inhibition of t-[35S]butylbicyclophosphorothionate binding by convulsant agents in primary cultures of cerebellar neurons. *Brain Res.Dev.Brain Res.* 73, 85-90.

Pomes, A., Rodriguez-Farre, E., and Sunol, C. (1994b). Disruption of GABA-dependent chloride flux by cyclodienes and hexachlorocyclohexanes in primary cultures of cortical neurons. *J.Pharmacol.Exp.Ther.* 271, 1616-1623.

Popoli, P., Pintor, A., Tebano, M. T., Frank, C., Pepponi, R., Nazzicone, V., Grieco, R., Pezzola, A., Reggio, R., Minghetti, L., De Berardinis, M. A., Martire, A., Potenza, R. L., Domenici, M. R., and Massotti, M. (2004). Neuroprotective effects of the mGlu5R antagonist MPEP towards quinolinic acid-induced striatal toxicity: involvement of pre- and post-synaptic mechanisms and lack of direct NMDA blocking activity. *J.Neurochem.* 89, 1479-1489.

POPs. Stockholm Convention on Persistent Organic Pollutants. Last Update: 24-5-2011.

Poulter, M. O., and Brown, L. A. (1999). Transient expression of GABAA receptor subunit mRNAs in the cellular processes of cultured cortical neurons and glia. *Brain Res.Mol.Brain Res.* 69, 44-52.

Poulter, M. O., Ohannesian, L., Larmet, Y., and Feltz, P. (1997). Evidence that GABAA receptor subunit mRNA expression during development is regulated by GABAA receptor stimulation. *J.Neurochem.* 68, 631-639.

Prange-Kiel, J., Wehrenberg, U., Jarry, H., and Rune, G. M. (2003). Para/autocrine regulation of estrogen receptors in hippocampal neurons. *Hippocampus* 13, 226-234.

Prewitt, A. K., and Wilson, M. E. (2007). Changes in estrogen receptor-alpha mRNA in the mouse cortex during development. *Brain Res.* 1134, 62-69.

Prybylowski, K., Chang, K., Sans, N., Kan, L., Vicini, S., and Wenthold, R. J. (2005). The synaptic localization of NR2B-containing NMDA receptors is controlled by interactions with PDZ proteins and AP-2. *Neuron* 47, 845-857.

Pshenichkin, S., Dolinska, M., Klauzinska, M., Luchenko, V., Grajkowska, E., and Wroblewski, J. T. (2008). Dual neurotoxic and neuroprotective role of metabotropic glutamate receptor 1 in conditions of trophic deprivation - possible role as a dependence receptor. *Neuropharmacology* 55, 500-508.

Raiteri, L., Zappettini, S., Stigliani, S., Paluzzi, S., Raiteri, M., and Bonanno, G. (2005). Glutamate release induced by activation of glycine and GABA transporters in spinal cord is enhanced in a mouse model of amyotrophic lateral sclerosis. *Neurotoxicology* 26, 883-892.

Rao, A., and Craig, A. M. (1997). Activity regulates the synaptic localization of the NMDA receptor in hippocampal neurons. *Neuron* 19, 801-812.

Razandi, M., Pedram, A., and Levin, E. R. (2000a). Estrogen signals to the preservation of endothelial cell form and function. *J.Biol.Chem.* 275, 38540-38546.

Razandi, M., Pedram, A., and Levin, E. R. (2000b). Plasma membrane estrogen receptors signal to antiapoptosis in breast cancer. *Mol.Endocrinol.* 14, 1434-1447.

Richardson, J. R., Caudle, W. M., Wang, M., Dean, E. D., Pennell, K. D., and Miller, G. W. (2006). Developmental exposure to the pesticide dieldrin alters the dopamine system and increases neurotoxicity in an animal model of Parkinson's disease. *FASEB J.* 20, 1695-1697.

Rivera, C., Voipio, J., Payne, J. A., Ruusuvuori, E., Lahtinen, H., Lamsa, K., Pirvola, U., Saarma, M., and Kaila, K. (1999). The K+/Cl- co-transporter KCC2 renders GABA hyperpolarizing during neuronal maturation. *Nature* 397, 251-255.

Robel, P., Schumacher, M., and Baulieu, E. (1999). Neurosteroids: From definition and Biochemistry to Physiopathologic Function. In Neurosteroids: A New Regulatory Function in the Nervous System (E. Baulieu, P. Robel, and M. Schumacher, Eds.), pp. 1-25. Humana Press.

Romano, C., Yang, W. L., and O'Malley, K. L. (1996). Metabotropic glutamate receptor 5 is a disulfide-linked dimer. *J.Biol.Chem.* 271, 28612-28616.

Rong, Y., Lu, X., Bernard, A., Khrestchatisky, M., and Baudry, M. (2001). Tyrosine phosphorylation of ionotropic glutamate receptors by Fyn or Src differentially modulates their susceptibility to calpain and enhances their binding to spectrin and PSD-95. *J.Neurochem.* 79, 382-390.

Rosa, R., Rodriguez-Farre, E., and Sanfeliu, C. (1996). Cytotoxicity of hexachlorocyclohexane isomers and cyclodienes in primary cultures of cerebellar granule cells. *J.Pharmacol.Exp.Ther.* 278, 163-169.

Rosa, R., Sanfeliu, C., Sunol, C., Pomes, A., Rodriguez-Farre, E., Schousboe, A., and Frandsen, A. (1997). The mechanism for hexachlorocyclohexane-induced cytotoxicity and changes in intracellular Ca2+ homeostasis in cultured cerebellar granule neurons is different for the gamma- and delta-isomers. *Toxicol.Appl.Pharmacol.* 142, 31-39.

Roselli, C. E., Ellinwood, W. E., and Resko, J. A. (1984). Regulation of brain aromatase activity in rats. *Endocrinology* 114, 192-200.

Rudick, C. N., and Woolley, C. S. (2001). Estrogen regulates functional inhibition of hippocampal CA1 pyramidal cells in the adult female rat. *J.Neurosci.* 21, 6532-6543.

Sabelhaus, C. F., Schroder, U. H., Breder, J., Henrich-Noack, P., and Reymann, K. G. (2000). Neuroprotection against hypoxic/hypoglycaemic injury after the insult by the group III metabotropic glutamate receptor agonist (R, S)-4-phosphonophenylglycine. *Br.J.Pharmacol.* 131, 655-658.

Sagara, Y., and Schubert, D. (1998). The activation of metabotropic glutamate receptors protects nerve cells from oxidative stress. *J.Neurosci.* 18, 6662-6671.

Sahoo, A., Samanta, L., Das, A., Patra, S. K., and Chainy, G. B. (1999). Hexachlorocyclohexane-induced behavioural and neurochemical changes in rat. *J.Appl.Toxicol.* 19, 13-18.

Sanchez-Ramos, J., Facca, A., Basit, A., and Song, S. (1998). Toxicity of dieldrin for dopaminergic neurons in mesencephalic cultures. *Exp.Neurol.* 150, 263-271.

Sans, N., Prybylowski, K., Petralia, R. S., Chang, K., Wang, Y. X., Racca, C., Vicini, S., and Wenthold, R. J. (2003). NMDA receptor trafficking through an interaction between PDZ proteins and the exocyst complex. *Nat.Cell Biol.* 5, 520-530.

Sayeed, I., Parvez, S., Wali, B., Siemen, D., and Stein, D. G. (2009). Direct inhibition of the mitochondrial permeability transition pore: a possible mechanism for better neuroprotective effects of allopregnanolone over progesterone. *Brain Res.* 1263, 165-173.

Schafer, K. S., and Kegley, S. E. (2002). Persistent toxic chemicals in the US food supply. *J.Epidemiol.Community Health* 56, 813-817.

Schantz, S. L., and Widholm, J. J. (2001). Cognitive effects of endocrine-disrupting chemicals in animals. *Environ.Health Perspect.* 109, 1197-1206.

Schubert, D., Kimura, H., and Maher, P. (1992). Growth factors and vitamin E modify neuronal glutamate toxicity. *Proc.Natl.Acad.Sci.U.S.A* 89, 8264-8267.

Schwarz, J. M., Liang, S. L., Thompson, S. M., and McCarthy, M. M. (2008). Estradiol induces hypothalamic dendritic spines by enhancing glutamate release: a mechanism for organizational sex differences. *Neuron* 58, 584-598.

Scippo, M. L., Argiris, C., Van De, W. C., Muller, M., Willemsen, P., Martial, J., and Maghuin-Rogister, G. (2004). Recombinant human estrogen, androgen and progesterone receptors for detection of potential endocrine disruptors. *Anal.Bioanal.Chem.* 378, 664-669.

Scott, D. B., Blanpied, T. A., and Ehlers, M. D. (2003). Coordinated PKA and PKC phosphorylation suppresses RXR-mediated ER retention and regulates the surface delivery of NMDA receptors. *Neuropharmacology* 45, 755-767.

Seeburg, D. P., Feliu-Mojer, M., Gaiottino, J., Pak, D. T., and Sheng, M. (2008). Critical role of CDK5 and Polo-like kinase 2 in homeostatic synaptic plasticity during elevated activity. *Neuron* 58, 571-583.

Sharma, H., Zhang, P., Barber, D. S., and Liu, B. (2010). Organochlorine pesticides dieldrin and lindane induce cooperative toxicity in dopaminergic neurons: role of oxidative stress. *Neurotoxicology* 31, 215-222.

Sheldahl, L. C., Shapiro, R. A., Bryant, D. N., Koerner, I. P., and Dorsa, D. M. (2008). Estrogen induces rapid translocation of estrogen receptor beta, but not estrogen receptor alpha, to the neuronal plasma membrane. *Neuroscience* 153, 751-761.

Shen, H., Gong, Q. H., Yuan, M., and Smith, S. S. (2005). Short-term steroid treatment increases delta GABAA receptor subunit expression in rat CA1 hippocampus: pharmacological and behavioral effects. *Neuropharmacology* 49, 573-586.

Sheng, M., and Kim, M. J. (2002). Postsynaptic signaling and plasticity mechanisms. *Science* 298, 776-780.

Shi, J., Aamodt, S. M., Townsend, M., and Constantine-Paton, M. (2001). Developmental depression of glutamate neurotransmission by chronic low-level activation of NMDA receptors. *J.Neurosci.* 21, 6233-6244.

Shibata, H., Spencer, T. E., Onate, S. A., Jenster, G., Tsai, S. Y., Tsai, M. J., and O'Malley, B. W. (1997). Role of co-activators and co-repressors in the mechanism of steroid/thyroid receptor action. *Recent Prog.Horm.Res.* 52, 141-164.

Silva, E., Kabil, A., and Kortenkamp, A. (2010). Cross-talk between non-genomic and genomic signalling pathways--distinct effect profiles of environmental estrogens. *Toxicol.Appl.Pharmacol.* 245, 160-170.

Sinor, J. D., Du, S., Venneti, S., Blitzblau, R. C., Leszkiewicz, D. N., Rosenberg, P. A., and Aizenman, E. (2000). NMDA and glutamate evoke excitotoxicity at distinct cellular locations in rat cortical neurons in vitro. *J.Neurosci.* 20, 8831-8837.

Skeberdis, V. A., Lan, J., Zheng, X., Zukin, R. S., and Bennett, M. V. (2001). Insulin promotes rapid delivery of N-methyl-D- aspartate receptors to the cell surface by exocytosis. *Proc.Natl.Acad.Sci.U.S.A* 98, 3561-3566.

Slotkin, T. A., and Seidler, F. J. (2008). Developmental neurotoxicants target neurodifferentiation into the serotonin phenotype: Chlorpyrifos, diazinon, dieldrin and divalent nickel. *Toxicol.Appl.Pharmacol.* 233, 211-219.

Slotkin, T. A., and Seidler, F. J. (2009). Oxidative and excitatory mechanisms of developmental neurotoxicity: transcriptional profiles for chlorpyrifos, diazinon, dieldrin, and divalent nickel in PC12 cells. *Environ.Health Perspect.* 117, 587-596.

Smith, C. C., Vedder, L. C., and McMahon, L. L. (2009). Estradiol and the relationship between dendritic spines, NR2B containing NMDA receptors, and the magnitude of long-term potentiation at hippocampal CA3-CA1 synapses. *Psychoneuroendocrinology* 34 Suppl 1, S130-S142.

Smith, R. M., Cunningham, W. L., Jr., van Gelder, G. A., and Karas, G. G. (1976). Dieldrin toxicity and successive discrimation reversal in squirrel monkeys (Saimiri sciureus). *J.Toxicol.Environ.Health* 1, 737-747.

Smith, S. B. (2002). Diabetic Retinopathy and the NMDA Receptor. *Drug News Perspect.* 15, 226-232.

Solà, C., Martinez, E., Camon, L., Pazos, A., and Rodriguez-Farre, E. (1993). Lindane administration to the rat induces modifications in the regional cerebral binding of [3H]Muscimol, [3H]-flunitrazepam, and t-[35S]butylbicyclophosphorothionate: an autoradiographic study. *J.Neurochem.* 60, 1821-1834.

Sonnewald, U., Olstad, E., Qu, H., Babot, Z., Cristofol, R., Sunol, C., Schousboe, A., and Waagepetersen, H. (2004). First direct demonstration of extensive GABA synthesis in mouse cerebellar neuronal cultures. *J.Neurochem.* 91, 796-803.

Sonntag, W. E. (2002). Neuroendocrine System. In Encyclopedia of Aging (D. J. Ekerdt, Ed.), Macmillan Reference USA.

Soriano, F. X., Papadia, S., Hofmann, F., Hardingham, N. R., Bading, H., and Hardingham, G. E. (2006). Preconditioning doses of NMDA promote neuroprotection by enhancing neuronal excitability. *J.Neurosci.* 26, 4509-4518.

Soto, A. M., Chung, K. L., and Sonnenschein, C. (1994). The pesticides endosulfan, toxaphene, and dieldrin have estrogenic effects on human estrogen-sensitive cells. *Environ.Health Perspect.* 102, 380-383.

Soto, A. M., Sonnenschein, C., Chung, K. L., Fernandez, M. F., Olea, N., and Serrano, F. O. (1995). The E-SCREEN assay as a tool to identify estrogens: an update on estrogenic environmental pollutants. *Environ.Health Perspect.* 103 Suppl 7, 113-122.

Steinmetz, R., Young, P. C., Caperell-Grant, A., Gize, E. A., Madhukar, B. V., Ben Jonathan, N., and Bigsby, R. M. (1996). Novel estrogenic action of the pesticide residue beta-hexachlorocyclohexane in human breast cancer cells. *Cancer Res.* 56, 5403-5409.

Stoop, R., Conquet, F., and Pralong, E. (2003). Determination of group I metabotropic glutamate receptor subtypes involved in the frequency of epileptiform activity in vitro using mGluR1 and mGluR5 mutant mice. *Neuropharmacology* 44, 157-162.

Subramaniam, S., Shahani, N., Strelau, J., Laliberte, C., Brandt, R., Kaplan, D., and Unsicker, K. (2005). Insulin-like growth factor 1 inhibits extracellular signal-regulated kinase to promote neuronal survival via the phosphatidylinositol 3-kinase/protein kinase A/c-Raf pathway. *J.Neurosci.* 25, 2838-2852.

Sumbayev, V. V., Bonefeld-Jorgensen, E. C., Wind, T., and Andreasen, P. A. (2005). A novel pesticide-induced conformational state of the oestrogen receptor ligand-binding domain, detected by conformation-specific peptide binding. *FEBS Lett.* 579, 541-548.

Suñol, C., Garcia, D. A., Bujons, J., Kristofikova, Z., Matyas, L., Babot, Z., and Kasal, A. (2006). Activity of B-nor analogues of neurosteroids on the GABA(A) receptor in primary neuronal cultures. *J.Med.Chem.* 49, 3225-3234.

Suñol, C., Tusell, J. M., Gelpi, E., and Rodriguez-Farre, E. (1988). Regional concentrations of GABA, serotonin and noradrenaline in brain at onset of seizures induced by lindane (gamma-hexachlorocyclohexane). *Neuropharmacology* 27, 677-681.

Swann, J. W., Le, J. T., Lam, T. T., Owens, J., and Mayer, A. T. (2007). The impact of chronic network hyperexcitability on developing glutamatergic synapses. *Eur.J.Neurosci.* 26, 975-991.

Tecoma, E. S., and Choi, D. W. (1989). GABAergic neocortical neurons are resistant to NMDA receptor-mediated injury. *Neurology* 39, 676-682.

Terasawa, E., Noel, S. D., and Keen, K. L. (2009). Rapid action of oestrogen in luteinising hormone-releasing hormone neurones: the role of GPR30. *J.Neuroendocrinol.* 21, 316-321.

Thandi, S., Blank, J. L., and Challiss, R. A. (2002). Group-I metabotropic glutamate receptors, mGlu1a and mGlu5a, couple to extracellular signal-regulated kinase (ERK) activation via distinct, but overlapping, signalling pathways. *J.Neurochem.* 83, 1139-1153.

Thomas, P., and Dong, J. (2006). Binding and activation of the seven-transmembrane estrogen receptor GPR30 by environmental estrogens: a potential novel mechanism of endocrine disruption. *J.Steroid Biochem.Mol.Biol.* 102, 175-179.

Tiemann, U. (2008). In vivo and in vitro effects of the organochlorine pesticides DDT, TCPM, methoxychlor, and lindane on the female reproductive tract of mammals: a review. *Reprod.Toxicol.* 25, 316-326.

Tilson, H. A., Shaw, S., and McLamb, R. L. (1987). The effects of lindane, DDT, and chlordecone on avoidance responding and seizure activity. *Toxicol.Appl.Pharmacol.* 88, 57-65.

Topinka, M. A., Eisenstein, E. M., Siegel, D., Trofeli, R., and Barraco, D. (1984). The effects of dieldrin and chlordimeform on learning and memory in the cockroach, Periplaneta americana: a study in behavioral toxicology. *J.Toxicol.Environ.Health* 13, 705-719.

Toran-Allerand, C. D. (1999). Novel Mechanisms of Estrogen Action in the Developing Brain: Role of Steroid/Neurotrophin Interactions. In Neurosteroids: A New Regulatory Function in the Nervous System (E. Baulieu, P. Robel, and M. Schumacher, Eds.), pp. 293-315. Humana Press.

Toran-Allerand, C. D. (2005). Estrogen and the brain: beyond ER-alpha, ER-beta, and 17beta-estradiol. *Ann.N.Y.Acad.Sci.* 1052, 136-144.

Tovar, K. R., and Westbrook, G. L. (1999). The incorporation of NMDA receptors with a distinct subunit composition at nascent hippocampal synapses in vitro. *J.Neurosci.* 19, 4180-4188.

Trudeau, L. E. (2004). Glutamate co-transmission as an emerging concept in monoamine neuron function. *J.Psychiatry Neurosci.* 29, 296-310.

Tu, J. C., Xiao, B., Naisbitt, S., Yuan, J. P., Petralia, R. S., Brakeman, P., Doan, A., Aakalu, V. K., Lanahan, A. A., Sheng, M., and Worley, P. F. (1999). Coupling of mGluR/Homer and PSD-95 complexes by the Shank family of postsynaptic density proteins. *Neuron* 23, 583-592.

Turrigiano, G. G. (2008). The self-tuning neuron: synaptic scaling of excitatory synapses. *Cell* 135, 422-435.

Turrigiano, G. G., Leslie, K. R., Desai, N. S., Rutherford, L. C., and Nelson, S. B. (1998). Activity-dependent scaling of quantal amplitude in neocortical neurons. *Nature* 391, 892-896.

Tusell, J. M., Vendrell, M., Serratosa, J., and Trullas, R. (1992). Lindane-induced convulsions in NMRI and OF1 mice: antagonism with (+)MK-801 and voltage-dependent calcium channel blockers. *Brain Res.* 593, 209-214.

Tymianski, M., Charlton, M. P., Carlen, P. L., and Tator, C. H. (1993). Source specificity of early calcium neurotoxicity in cultured embryonic spinal neurons. *J.Neurosci.* 13, 2085-2104.

US EPA. Carcinogenicity assessment of Dieldrin and Aldrin. Environmental Protection Agency . 1987.

Vale, C., Damgaard, I., Sunol, C., Rodriguez-Farre, E., and Schousboe, A. (1998a). Cytotoxic action of lindane in cerebellar granule neurons is mediated by interaction with inducible GABA(B) receptors. *J.Neurosci.Res.* 52, 286-294.

Vale, C., Damgaard, I., Sunol, C., Rodriguez-Farre, E., and Schousboe, A. (1998b). Cytotoxic action of lindane in neocortical GABAergic neurons is primarily mediated by interaction with flunitrazepam-sensitive GABA(A) receptors. *J.Neurosci.Res.* 52, 276-285.

Vale, C., Fonfria, E., Bujons, J., Messeguer, A., Rodriguez-Farre, E., and Sunol, C. (2003). The organochlorine pesticides gamma-hexachlorocyclohexane (lindane), alpha-endosulfan and dieldrin differentially interact with GABA(A) and glycine-gated chloride channels in primary cultures of cerebellar granule cells. *Neuroscience* 117, 397-403.

Vale, C., Pomes, A., Rodriguez-Farre, E., and Sunol, C. (1997). Allosteric interactions between gamma-aminobutyric acid, benzodiazepine and picrotoxinin binding sites in primary cultures of cerebellar granule cells. Differential effects induced by gamma- and delta-hexachlorocyclohexane. *Eur.J.Pharmacol.* 319, 343-353.

van den Pol, A. N., Gao, X. B., Patrylo, P. R., Ghosh, P. K., and Obrietan, K. (1998). Glutamate inhibits GABA excitatory activity in developing neurons. *J.Neurosci.* 18, 10749-10761.

van den, B. J., and Narahashi, T. (1974). Effects of aldrin-transdiol--a metabolite of the insecticide dieldrin--on nerve membrane. *Eur.J.Pharmacol.* 27, 255-258.

van Zundert, B., Yoshii, A., and Constantine-Paton, M. (2004). Receptor compartmentalization and trafficking at glutamate synapses: a developmental proposal. *Trends Neurosci.* 27, 428-437.

von Engelhardt, J., Coserea, I., Pawlak, V., Fuchs, E. C., Kohr, G., Seeburg, P. H., and Monyer, H. (2007). Excitotoxicity in vitro by NR2A- and NR2B-containing NMDA receptors. *Neuropharmacology* 53, 10-17.

Wang, M. M., Traystman, R. J., Hurn, P. D., and Liu, T. (2004). Non-classical regulation of estrogen receptor-alpha by ICI182,780. *J.Steroid Biochem.Mol.Biol.* 92, 51-62.

Wang, S., Wang, S., Shan, P., Song, Z., Dai, T., Wang, R., and Chi, Z. (2008). Mu-calpain mediates hippocampal neuron death in rats after lithium-pilocarpine-induced status epilepticus. *Brain Res.Bull.* 76, 90-96.

Warner, M., and Gustafsson, J. (1999). Cytochrome P450 in the Central Nervous System. In Neurosteroids: A New Regulatory Function in the Nervous System (E. Baulieu, P. Robel, and M. Schumacher, Eds.), pp. 51-65. Humana Press.

Waters, K. M., Safe, S., and Gaido, K. W. (2001). Differential gene expression in response to methoxychlor and estradiol through ERalpha, ERbeta, and AR in reproductive tissues of female mice. *Toxicol.Sci.* 63, 47-56.

Watson, C. S., Bulayeva, N. N., Wozniak, A. L., and Alyea, R. A. (2007). Xenoestrogens are potent activators of nongenomic estrogenic responses. *Steroids* 72, 124-134.

Weaver, C. E., Jr., Park-Chung, M., Gibbs, T. T., and Farb, D. H. (1997). 17beta-Estradiol protects against NMDA-induced excitotoxicity by direct inhibition of NMDA receptors. *Brain Res.* 761, 338-341.

WHO. Environmental Health Criteria: dieldrin, endosulfan, lindane. World Health Organization . 2004.

Wijetunge, L. S., Till, S. M., Gillingwater, T. H., Ingham, C. A., and Kind, P. C. (2008). mGluR5 regulates glutamate-dependent development of the mouse somatosensory cortex. *J.Neurosci.* 28, 13028-13037.

Willaime-Morawek, S., Arbez, N., Mariani, J., and Brugg, B. (2005). IGF-I protects cortical neurons against ceramide-induced apoptosis via activation of the PI-3K/Akt and ERK pathways; is this protection independent of CREB and Bcl-2? *Brain Res.Mol.Brain Res.* 142, 97-106.

Wilson, M. E., and Westberry, J. M. (2009). Regulation of oestrogen receptor gene expression: new insights and novel mechanisms. *J.Neuroendocrinol.* 21, 238-242.

Wohlfarth, K. M., Bianchi, M. T., and Macdonald, R. L. (2002). Enhanced neurosteroid potentiation of ternary GABA(A) receptors containing the delta subunit. *J.Neurosci.* 22, 1541-1549.

Wong, J. K., Le, H. H., Zsarnovszky, A., and Belcher, S. M. (2003). Estrogens and ICI182,780 (Faslodex) modulate mitosis and cell death in immature cerebellar neurons via rapid activation of p44/p42 mitogen-activated protein kinase. *J.Neurosci.* 23, 4984-4995.

Wu, H. Y., and Lynch, D. R. (2006). Calpain and synaptic function. *Mol.Neurobiol.* 33, 215-236.

Wu, L. X., Sun, C. K., Zhang, Y. M., Fan, M., Xu, J., Ma, H., and Zhang, J. (2007). Involvement of the Snk-SPAR pathway in glutamate-induced excitotoxicity in cultured hippocampal neurons. *Brain Res.* 1168, 38-45.

Xilouri, M., and Papazafiri, P. (2006). Anti-apoptotic effects of allopregnanolone on P19 neurons. *Eur.J.Neurosci.* 23, 43-54.

Xu, J., Zhang, Q. G., Li, C., and Zhang, G. Y. (2007a). Subtoxic N-methyl-D-aspartate delayed neuronal death in ischemic brain injury through TrkB receptor- and calmodulin-mediated PI-3K/Akt pathway activation. *Hippocampus* 17, 525-537.

Xu, W., Wong, T. P., Chery, N., Gaertner, T., Wang, Y. T., and Baudry, M. (2007b). Calpain-mediated mGluR1alpha truncation: a key step in excitotoxicity. *Neuron* 53, 399-412.

Xu, X., Ye, Y., Li, T., Chen, L., Tian, D., Luo, Q., and Lu, M. (2010). Bisphenol-A rapidly promotes dynamic changes in hippocampal dendritic morphology through estrogen receptor-mediated pathway by concomitant phosphorylation of NMDA receptor subunit NR2B. *Toxicol.Appl.Pharmacol.* 249, 188-196.

Yang, L., Mao, L., Tang, Q., Samdani, S., Liu, Z., and Wang, J. Q. (2004). A novel Ca2+-independent signaling pathway to extracellular signal-regulated protein kinase by coactivation of NMDA receptors and metabotropic glutamate receptor 5 in neurons. *J.Neurosci.* 24, 10846-10857.

Yao, H. H., Ding, J. H., Zhou, F., Wang, F., Hu, L. F., Sun, T., and Hu, G. (2005). Enhancement of glutamate uptake mediates the neuroprotection exerted by activating group II or III metabotropic glutamate receptors on astrocytes. *J.Neurochem.* 92, 948-961.

Yi, K. D., and Simpkins, J. W. (2008). Protein phosphatase 1, protein phosphatase 2A, and calcineurin play a role in estrogen-mediated neuroprotection. *Endocrinology* 149, 5235-5243.

Young, K. C., McGehee, D. S., and Brorson, J. R. (2007). Glutamate receptor expression and chronic glutamate toxicity in rat motor cortex. *Neurobiol.Dis.* 26, 78-85.

Zhao, L., and Brinton, R. D. (2007). Estrogen receptor alpha and beta differentially regulate intracellular Ca(2+) dynamics leading to ERK phosphorylation and estrogen neuroprotection in hippocampal neurons. *Brain Res.* 1172, 48-59.

Zhou, L., Lehan, N., Wehrenberg, U., Disteldorf, E., von Lossow, R., Mares, U., Jarry, H., and Rune, G. M. (2007). Neuroprotection by estradiol: a role of aromatase against spine synapse loss after blockade of GABA(A) receptors. *Exp.Neurol.* 203, 72-81.

Zhu, D., Wu, X., Strauss, K. I., Lipsky, R. H., Qureshi, Z., Terhakopian, A., Novelli, A., Banaudha, K., and Marini, A. M. (2005). N-methyl-D-aspartate and TrkB receptors protect neurons against glutamate excitotoxicity through an extracellular signal-regulated kinase pathway. *J.Neurosci.Res.* 80, 104-113.

Zhu, W. J., Wang, J. F., Krueger, K. E., and Vicini, S. (1996). Delta subunit inhibits neurosteroid modulation of GABAA receptors. *J.Neurosci.* 16, 6648-6656.

Zhuo, M., and Hawkins, R. D. (1995). Long-term depression: a learning-related type of synaptic plasticity in the mammalian central nervous system. *Rev.Neurosci.* 6, 259-277.