CLEYTON ROBERTO SOBRINHO

MECANISMOS PURINÉRGICOS NO BULBO VENTROLATERAL ROSTRAL MODULAM RESPOSTAS CARDIOVASCULARES E RESPIRATÓRIAS PROMOVIDAS PELA ATIVAÇÃO DOS QUIMIORRECEPTORES CENTRAIS E PERIFÉRICOS

Tese apresentada ao Programa de Pós-Graduação em Fisiologia Humana do Instituto de Ciências Biomédicas da Universidade de São Paulo, para obtenção do Título de Doutor em Ciências.

São Paulo
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Área de Concentração: Fisiologia Humana

Orientador: Prof. Dr. Thiago dos Santos Moreira

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CERTIFICADO

Certificamos que o protocolo registrado sob nº **118** nas fls. **109** do livro **02** para uso de animais em experimentação, sob a responsabilidade do Prof(a) Dr(a)) **Thiago dos Santos Moreira**, Coordenador (a) da Linha de pesquisa *"Interação entre astrócitos e neurônios do núcleo retotrapezóide nas respostas respiratórias promovidas pela ativação do quimiorreflexo central"* do qual participam o(s) aluno(s) **Cleyton Roberto Sobrinho e os pesquisadores Ana Carolina Thomaz Takakura, Vagner Roberto Antunes,** está de acordo com os Princípios Éticos de Experimentação Animal adotado pela Sociedade Brasileira de Ciência de Animais de Laboratório (SBCAL) e foi aprovado pela *COMISSÃO DE ÉTICA NO USO DE ANIMAIS* (CEUA) em **20.09.2011, com validade de 3 anos**.

São Paulo, 22 de setembro de 2011.

Prof.Dr.WOTHAN TAVARES DE LIMA
Coordenador
CEUA - ICB/USP

Prof. Dr. ARIEL MARIANO SILBER
Secretário
CEUA – ICB/USP

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Ostra feliz não faz pérola.
Rubem Alves

RESUMO

SOBRINHO, C. R. **Mecanismos purinérgicos no bulbo ventrolateral rostral modulam respostas cardiovasculares e respiratórias promovidas pela ativação dos quimiorreceptores centrais e periféricos.** 2015. 168 f. Tese (Doutorado em Fisiologia Humana) - Instituto de Ciências Biomédicas, Universidade de São Paulo, São Paulo, 2015.

Quimiorrecepção é o mecanismo pelo qual células especializadas detectam variações nos níveis de CO_2, O_2 e H^+ presentes no sangue, liquor e parênquima, onde dois sistemas principais são reconhecidos: quimiorreceptores periféricos, localizados na bifurcação da aorta e no corpúsculo carotídeo e quimiorreceptores centrais, representados por grupamentos de neurônios especializados em detectar apenas as variações de CO_2/H^+. Em comum, estas estruturas informam centros respiratórios e simpáticos no sistema nervoso central (SNC) gerando ajustes da atividade cardiorrespiratória. A região ventrolateral do bulbo contém neurônios bulbo-espinais que modulam atividade simpática (grupamento C1) e neurônios quimiossensíveis localizados no núcleo retrotrapezóide (RTN). Adicionalmente a quimiorrecepção intrínseca dos neurônios do RTN, tem sido demonstrado que outras células nesta região (possivelmente astrócitos) atuam como sensores de CO_2/H^+, liberando ATP para promover a ativação de neurônios via receptores purinérgicos P2, na tentativa de promover ajustes cardiorrespiratórios. No presente trabalho, ultilizamos ratos Wistar adultos, anestesiados e ventilados artificialmente para avaliar a ação da sinalização purinérgica em regiões encefálicas com características quimiossensíveis bem como o envolvimento dos receptores P2 durante as respostas cardiorrespiratórias promovidas pela ativação dos quimiorreceptores centrais e periféricos. Avaliamos também a participação de astrócitos neste processo. Os receptores P2, expressos em neurônios do RTN, modulam parcialmente a resposta quimiorreceptora central, enquanto que receptores P2Y1, expressos em neurônios C1, modulam parcialmente a resposta quimiorreceptora periférica, juntamente com receptores glutamatérgicos ionotrópicos. Adicionalmente, encontramos evidências convincentes de que a sinalização purinérgica na região do núcleo do trato solitário comissural (NTScom) ou na região da rafe pálido (RPa) não contribui para resposta quimiorreceptora central. Além disso, encontramos que varicosidades na região ventrolateral do bulbo rostral provenientes no NTScom expressam VNUT e VGLUT2, indicando a presença de vesículas de núcleotideos e glutamatérgicas, respectivamente. Finalmente, nossos experimentos revelaram que a manipulação farmacológica de astrócitos no RTN com a injeção da gliotoxina fluorocitrato, mas não da região do NTScom e RPa, produz alterações respiratórias mediadas por receptores P2. Nossos achados evidenciam a importância e contribuem para descriminação dos mecanismos de ação da sinalização purinérgica na região bulbo ventrolateral rostral durante a ativação de quimiorreceptores centrais e periféricos.

Palavras-Chave: Núcleo retrotrapezóide. Grupamento C1. Quimiorrecepção central. Quimiorrecepção periférica. Sinalização purinérgica. Receptores purinérgicos P2.

ABSTRACT

SOBRINHO, C. R. **Purinergic mechanism in rostroventrolateral medulla modulate cardiovascular and respiratory responses promoted by central and peripheral chemoreceptors activation.** 2015. 168 p. Ph. D. Thesis (Human Phisiology) - Instituto de Ciências Biomédicas, Universidade de São Paulo, São Paulo, 2015.

Chemoreception is the mechanism by which specialized cells detect changes in the levels of CO_2, O_2 and H^+ in the blood, cerebrospinal fluid and brain, where two main systems are recognized: peripheral chemoreceptors are located at the the aorta and carotid body, and the central chemoreceptors groups represented by specialized neurons detect only changes in CO_2/H^+. In common, these structures inform respiratory and sympathetic centers in the central nervous system (CNS), generating adjustments of cardiorrespiratory activity. The rostral ventrolateral medulla contains neurons that project to spinal cord to modulate sympathetic activity (C1 group) and intrinsic chemoreceptors neurons in the RTN neurons. In addition, it has been shown that other cells in the RTN region (possibly astrocytes) act as CO_2 sensors by releasing ATP to produce neuronal activation via P2 purinergic receptors. In this study, we used male adult Wistar rats, anesthetized and artificially ventilated to evaluate the effect of purinergic signaling in regions with chemosensitive characteristics and the involvement of P2 receptors in cardiorespiratory responses elicited by activation of central and peripheral chemoreceptors. We also evaluated the role of astrocytes in this process. The P2 receptors are expressed in RTN neurons and partially modulate the central chemoreceptors cardiorespiratory responses, whereas P2Y1 receptors are expressed in C1 neurons, and partially modulate peripheral chemoreceptor cardiorespiratory responses, together with ionotropic glutamate receptors. Additionally, we found convinced evidences that the purinergic signaling in the commissural aspect of the nucleus of the solitary tract (NTScom) or in the raphe pallidus (RPA/PPy) region did not contribute to central chemoreceptor response. Furthermore, we fond that varicosidades in the rostral ventrolateral medulla from the commNTS express VNUT and VGLUT2, indicating the presence of nucleotides and glutamate vesicles, respectively. Finally, our experiments have shown that pharmacological manipulation of astrocytes within the RTN with the gliotoxin fluorocitrate injection produces respiratory changes mediated by P2 receptors. Our findings show the importance and contribute to discrimination of the mechanisms of purinergic signaling in the rostral ventrolateral medulla during activation of central and peripheral chemoreceptors.

Key words: Retrotrapezoid nucleus. C1 group. Central chemoreception. Peripheral chemoreception. Purinergic signaling. P2 Purinergic receptors.

LISTA DE FIGURAS

LISTA DE ABREVIATURAS E SIGLAS

7 ou VII - núcleo motor do nervo facial

7n - nervo facial

XII - núcleo hipoglosso

A5 - grupamento noradrenérgico A5

A6 - grupamento noradrenérgico A6 ou locus coeruleus

ADP - difosfato de adenosina

ADO - adenosina

AMP - monofosfato de adenosina

anti-DβH-SAP - Neurotoxina Saporina conjulgada com dopamina β-hidroxilase

AP - Anteroposterior

ATP - Trifosfato de Adenosina

BDA - Biotina Dextrana Amina

BötC - Complexo de Bötzinger

C1 - Grupamento adrenérgico C1

cc - canal central;

CO_2 - Dióxido de Carbono

DV - Dorsoventral

E-NTPDases - ectonucleotidases trifosfato de difosfohidrolase

FCt - Fluorocitrato

GPR4 - receptores ativados por prótons ligados a proteína G

Gr - Núcleo Grácil

GRD - Grupamento Respiratório Dorsal

GRV - Grupamento Respiratório Ventral

GRVc - Grupamento Respiratório Ventrolateral caudal

GRVr- Grupamento Respiratório Ventrolateral rostral

H^+ - Íons de Hidrogênio

IO, núcleo olivar inferior

ISN, núcleo salivatório inferior

KCN - Cianeto de potássio

KF - Kolliker-Fuse

Kyn – ácido quinurênico

ML – Médio lateral

mmHg - milímetros de mercúrio

nA, núcleo ambíguo;

NSO, núcleo olivar superior;

NTS - Núcleo do trato solitário

NTScom - porção comissural do núcleo do trato solitário

O_2 - Oxigênio

PAM - pressão arterial média

P_{CO2} – Pressão Parcial de Oxigênio

P_{O2} - Pressão Parcial de Dióxido de Carbono

PNA - Atividade do nervo frênico

pré-BötC - complexo de pré-Bötzinger

py - trato piramidal

RPa- núcleo pálido da rafe

RPa/PPy - núcleo pálido da rafe/ região parapiramidal

RTN - núcleo retrotrapezóide

RVL – Bulbo ventrolateral rostral

SNC - Sistema Nervoso Central

sSNA - Atividade do nervo simpático esplâncnico

SO, oliva superior

Sp5, trato espinal do trigêmeo

SSN, núcleo salivatório superior

tz - corpo trapezoide

TASK – canais de potássio sensíveis a pH

TH – Tirosina Hidroxilase

VNUT - transportador vesicular de nucleotídeos

VGLUT2 - transportador vesicular de glutamato

SUMÁRIO

1 INTRODUÇÃO

1.1 Função e controle neural da respiração

O sistema respiratório possui diversas funções, dentre as quais podemos destacar sua participação em processos mastigatórios e de deglutição, na fonação e principalmente no processo de trocas gasosas pulmonares, de forma a garantir o fornecimento adequado de oxigênio (O_2) para os tecidos assim como a remoção de dióxido de carbono (CO_2). De particular interesse, esta última função promove ainda a regulação do pH plasmático, mantendo-o dentro dos limites de normalidade fisiológica.

A musculatura respiratória trabalha de forma coordenada para assegurar o fluxo de ar adequado para os pulmões. Para tanto, o sistema nervoso central (SNC) participa ativamente no controle da musculatura respiratória, podendo exercer sua atividade de forma voluntária por comandos gerados em áreas corticais superiores. Em geral esses comandos podem ser recrutados de forma consciente durante manobras específicas como, por exemplo, durante uma apnéia voluntária, porém, o controle motor é essencialmente gerado de forma involuntária e, assim como a maioria dos comandos fundamentais para assegurar a sobrevivência ou integridade do individuo, são gerados ou modulados por estruturas subcorticais, localizadas no diencéfalo e/ou tronco encefálico (BARNA; TAKAKURA; MOREIRA, 2012; CEZARIO et al., 2008; GUYENET; BAYLISS, 2015; GUYENET; STORNETTA; BAYLISS, 2010; MOTTA et al., 2009; SCHREIHOFER; GUYENET, 1997).

Dentre os grupamentos neurais que controlam a atividade respiratória podemos destacar regiões dorsais e ventrais distribuídas ao longo do eixo rostrocaudal do tronco encefálico.

O sub-núcleo ventrolateral dos núcleos do trato solitário (NTS) contém neurônios predominantemente inspiratórios e corresponde à subdivisão chamada de Grupamento Respiratório Dorsal (GRD) (CASTRO; LIPSKI; KANJHAN, 1994). O Grupamento Respiratório Ventral (GRV) contém importantes grupamentos neurais envolvidos no controle respiratório. A região caudal à área postrema contém neurônios pré-motores expiratórios e recebe o nome de Grupamento Respiratório Ventrolateral caudal (GRVc) (FORTUNA et al., 2008). O Grupamento Respiratório Ventrolateral rostral (GRVr), localizado ao nível da área postrema, apresenta neurônios pré-motores inspiratórios que projetam para neurônios motores que dão origem ao nervo frênico e inervam o principal músculo respiratório, o diafragma (STORNETTA; SEVIGNY; GUYENET, 2003). Imediatamente adjacente ao GRVr está localizado o grupamento pré-Bötzinger (pré-BötC), onde evidências experimentais apontam para atividade marcapasso destes neurônios e, desta forma, seriam responsáveis por gerar o ritmo inspiratório (FELDMAN; DEL NEGRO; GRAY, 2012; FELDMAN; DEL NEGRO,

2006; SMITH et al., 1991). O grupamento denominado de complexo Bötzinger (BötC), localizado imediatamente rostral ao pré-BötC, contém interneurônios inibitórios, envolvidos no processo expiratório (SCHREIHOFER; GUYENET, 1997). Ainda na região ventrolateral do bulbo, existe um conjunto de neurônios localizados ventralmente ao núcleo motor do nervo facial chamado de núcleo retrotrapezóide (RTN) (CONNELLY; ELLENBERGER; FELDMAN, 1990). A particularidade mais relevante apresentada pelos neurônios do RTN é a capacidade de detectar o aumento da pressão parcial de CO_2 (P_{CO2}) plasmática e do parênquima encefálico assim como a consequente redução do pH, gerando rapidamente o aumento da atividade respiratória (GUYENET; BAYLISS, 2015; KUMAR et al., 2015; MOREIRA et al., 2006; MULKEY et al., 2004; TAKAKURA et al., 2006). Mais recentemente também tem sido proposto que este núcleo poderia modular a expiração ativa auxiliando na eliminação do CO_2 (ABDALA et al., 2009; HUCKSTEPP et al., 2015; JANCZEWSKI; FELDMAN, 2006b).

Além desses grupamentos, existem evidências bastante convincentes na literatura sobre a participação de outros núcleos na modulação da atividade respiratória como, por exemplo, os núcleos serotonérgicos da rafe, localizados na linha mediana do bulbo (DEPUY et al., 2011; MESSIER; LI; NATTIE, 2004; RICHERSON, 2004), os grupamentos catecolaminérgicos pontinos A5 e A6 (BIANCARDI et al., 2008; TAXINI et al., 2011), o núcleo fastigial do cerebelo (MARTINO et al., 2006a, 2006b) e os neurônios orexinérgicos do hipotálamo (DENG et al., 2007; WILLIAMS et al., 2007).

1.2 Bulbo ventrolateral rostral (RVL)

1.2.1 Caracterização do RTN e do grupamento catecolaminérgico C1

Como mencionado anteriormente, o RTN está localizado muito próximo da superfície ventrolateral do bulbo (CONNELLY; ELLENBERGER; FELDMAN, 1990; MULKEY et al., 2004). Este núcleo é constituído por aproximadamente 2100 neurônios no rato, os quais se estendem ventralmente ao núcleo motor do facial desde sua porção mais caudal até a porção caudal do corpo trapezóide, englobando uma distância de aproximadamente 2,0 mm no sentido rostro-caudal (Figura 1) (TAKAKURA et al., 2008, 2014).

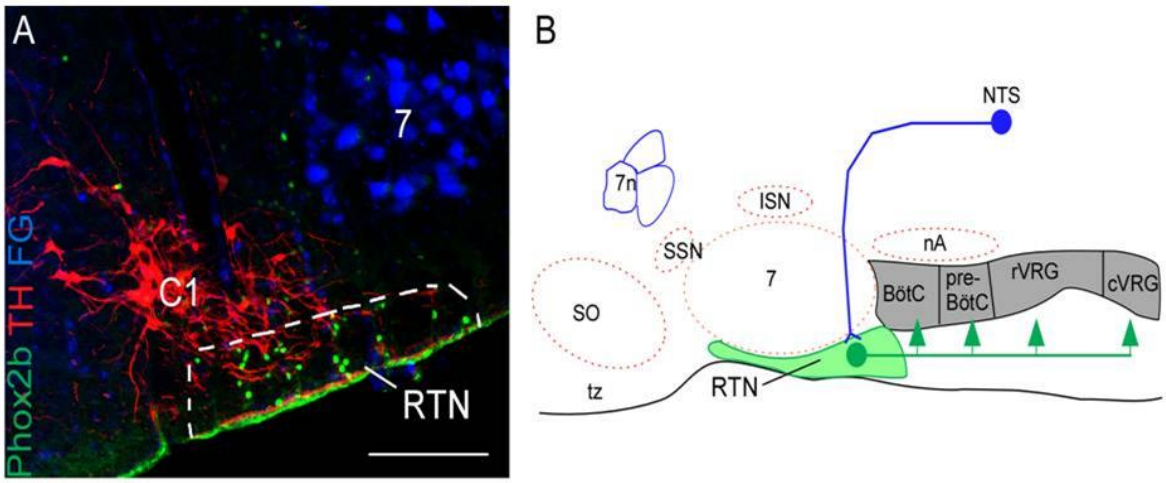

Figura 1: Bulbo ventrolateral rostral – RTN, C1 e a organização dos grupamentos respiratórios

A: Fotomicrografia ilustrando a imununorreatividade nuclear para o gene PHOX2B (Alexa 488, verde) no núcleo retrotrapezóide (RTN) em uma secção coronal do bulbo de um rato. A localização do RTN está no mesmo nível do neurônio representado em verde na figura B. Imunorreatividade para Tirosina Hidroxilase (Cy3, vermelho), indicando a localização do grupamento C1. Imunorreatividade dos neurônios motores donúcleo facial (7) após a injeção do traçador retrógrado Fluoro-Gold intraperitonialmente (Stornetta et al.; 2006) Escala: 100 µm. **B:** Figura esquemática ilustrando uma secção parassagital de um encéfalo de rato desde a ponte até o bulbo (modificado de Feldman e Del Negro, 2006; Guyenet e Bayliss, 2015). O RTN está marcado em verde e as outras regiões da coluna respiratória estão em cinza. Abreviaturas: 7, núcleo facial; 7n nervo facial; nA, núcleo ambíguo; BötC, complexo Bötzinger; pre- BötC complexo pré-Bötzinger, rVRG, coluna respiratória ventral rostral; cVRG, coluna respiratória ventral caudal; NTS, núcleo do trato solitário, ISN, núcleo salivatório inferior; SSN, núcleo salivatório superior; SO, oliva superior; tz, corpo trapezóide.

Até o presente momento, sabe-se que os neurônios do RTN são neurônios não colinérgicos e não aminérgicos, altamente responsivos à substância P e predominantemente glutamatérgicos (GUYENET; BAYLISS, 2015; GUYENET; STORNETTA; BAYLISS, 2010; GUYENET et al., 2008; LAZARENKO et al., 2011; MULKEY et al., 2004; ROSIN; CHANG; GUYENET, 2006). Essas assinaturas bioquímicas do RTN podem ser identificadas por imunoistoquímica e utilizadas como referência para identificação e delimitação do núcleo. O fator de transcrição Phox2b constitui outra importante característica dos neurônios do RTN, e também pode ser identificado por imunoistoquímica para identificação dos neurônios quimiossensíveis (ABBOTT et al., 2009a; AMIEL et al., 2003; BURKE et al., 2015; MULKEY et al., 2004; TAKAKURA et al., 2008). Esse fator de transcrição é responsável por modular a diferenciação celular e a sobrevida de restritos grupamentos neuronais localizados na ponte e no bulbo, incluindo o RTN (AMIEL et al., 2003). Parece estar bem estabelecido na literatura de que os neurônios do RTN estão envolvidos em basicamente três processos neurais relacionados ao controle respiratório, como: a) controle do movimento inspiratório, já que eles se projetam para regiões mais caudais da coluna respiratória ventral e para os neurônios motores que controlam a inspiração (DOBBINS; FELDMAN, 1994); b) estão envolvidos na quimiorrecepção central, pois respondem frente uma situação de hipercapnia (aumento da concentração de CO_2) (FELDMAN; MITCHELL; NATTIE, 2003; KUMAR et al., 2015; NATTIE; LI; ST JOHN, 1991; NATTIE; LI, 1994, 2002a; OKADA et al., 2002; PUTNAM; FILOSA; RITUCCI, 2004; SATO; SEVERINGHAUS; BASBAUM, 1992) e c) por fim, estudos mais recentes têm procurado relacionar o RTN também à geração da expiração ativa (ABDALA et al., 2009; JANCZEWSKI; FELDMAN, 2006a; PAGLIARDINI et al., 2011).

Dados da literatura demonstraram que uma subpopulação de células do RTN em ratos adultos estão inativas em situações basais e tornam-se ritmicamente ativas durante hipercapnia (MARINA et al., 2010) ou desinibições locais (MOLKOV et al., 2010; PAGLIARDINI et al., 2011). Experimentos realizados por Pagliardini e colaboradores (2011) sugerem que o RTN contém um oscilador condicional que gera uma expiração ativa; entretanto, existe uma inibição sináptica para o RTN que suprime a expiração ativa, e pode ser desinibida durante uma situação de hipercapnia, hipóxia ou qualquer situação em que existe a necessidade da expiração ativa. Entretanto, até o presente momento, não se sabe a procedência desta inibição para o RTN. A literatura sugere algumas possíveis regiões como o NTS e a região do Kolliker-Fuse (KF) (MOREIRA et al., 2007; ROSIN; CHANG;

GUYENET, 2006; TAKAKURA et al., 2007), e o complexo de Botzinger (CREAM; LI; NATTIE, 2002; ROSIN; CHANG; GUYENET, 2006).

Imediatamente adjacente ao RTN, encontra-se um grupamento de neurônios que atuam no controle do tônus simpático mediante sua influência sobre neurônios pré-ganglionares da coluna intermédio-lateral, localizados na medula espinal (DAMPNEY, 1994; GUYENET, 2006). Em condições de repouso, os neurônios pré-motores simpáticos do bulbo ventrolateral são a fonte primária do tônus simpático, sendo considerados os geradores da atividade simpática (DAMPNEY et al., 2003; FELDBERG; GUERTZENSTEIN, 1972). Acredita-se que todos os neurônios bulbo-espinhais pré-simpáticos do bulbo ventrolateral liberem glutamato na coluna intermédio-lateral da medula espinhal, mas sabe-se que eles também sintetizam outros neurotransmissores, incluindo adrenalina, encefalina, serotonina entre outros (SUN, 1996). Os neurônios que sintetizam adrenalina correspondem a mais de 70% dos neurônios pré-simpáticos do bulbo ventrolateral e pertencem, por definição, ao grupamento C1 (ROSS et al., 1984; SCHREIHOFER; GUYENET, 1997). Estudos neuroanatômicos e funcionais descreveram que esses neurônios do bulbo ventrolateral rostral estão localizados imediatamente caudais ao limite posterior do núcleo motor do nervo facial, ventralmente ao núcleo ambíguo e não somam mais do que algumas poucas centenas de corpos celulares (SUN, 1996).

Resumidamente, a área C1 constitui parte da região rostroventrolateral do bulbo onde estão localizados os neurônios vasomotores simpáticos e contém neurônios catecolaminérgicos, mas também é capaz de liberar o glutamato como neurotransmissor, além de outros neuropeptídeos (GUYENET et al., 2004; SCHREIHOFER; GUYENET, 1997; STORNETTA; MCQUISTON; GUYENET, 2004). Sabe-se que além da clássica contribuição dos neurônios C1 no controle da pressão arterial e atividade simpática, esse importante grupamento neuronal pode influenciar diversas funções fisiológicas, incluindo respostas neuroendócrinas devido um processo inflamatório, homeostase de glicose, processo reprodutivo, respiração, termorregulação, ativação do eixo hipotalâmico e ingestão de alimentos (GUYENET et al., 2013).

1.3 Quimiorrecepção central e periférica

Para que o ritmo e a amplitude respiratória sejam ajustados de forma a assegurar a homeostase gasométrica, é necessário que o SNC receba informações refinadas e precisas dos valores arteriais de O_2 e CO_2. Esse papel é atribuído às células conhecidas como

quimiorreceptores, que são estruturas especializadas, sensíveis as alterações químicas no sangue (FELDMAN et al., 2003; GUYENET et al., 2010). Em condições normais, essas células realizam o monitoramento contínuo, informando ao SNC sobre a pressão parcial de oxigênio (P_{O2}), P_{CO2} e pH plasmático, possibilitando que o mesmo promova os ajustes adequados (GUYENET; STORNETTA; BAYLISS, 2010), e adicionalmente, em situações onde ocorrem alterações drásticas desses parâmetros, como por exemplo, na hipóxia (redução na P_{O2}) ou na hipercapnia (redução na P_{CO2}), as células quimiossensíveis sofrem alterações equivalentes na atividade, produzindo vigorosas alterações na atividade respiratória, frequentemente acompanhados por alterações na pressão arterial (BASTING et al., 2015; BURKE et al., 2015; GUYENET; BAYLISS, 2015; MULKEY et al., 2004; PRABHAKAR, 2013; WENKER et al., 2012).

1.3.1 Quimiorrecepção periférica

Todas as células do organismo possuem uma capacidade intrínseca de detectar variações na concentração extracelular de O_2, CO_2 e pH e, de certo modo, responder a tais alterações (SEMENZA, 2011). Entretanto, um conjunto de células neuroepiteliais derivadas da crista neural localizadas principalmente nos corpúsculos carotídeos apresentam a peculiaridade de se despolarizar em condições de hipóxia, hipercapnia ou acidose (BISCOE; DUCHEN, 1990a, 1990b; FITZGERALD; SHIRAHATA; ISHIZAWA, 1996; LAHIRI et al., 2006), sensibilizando terminais nervosos aferentes que com elas fazem contato, os quais enviam potenciais de ação para o sistema nervoso central, promovendo, assim, a ativação de ajustes cardiovasculares e respiratórios com o objetivo de restaurar a P_{O2} para valores adequados. Esse processo constitui o quimirreflexo periférico, o qual consiste num dos principais elementos na manuntenção da homeostase cardiorrespiratória (MACHADO, 2001; PRABHAKAR, 2013). Os ajustes promovidos pela ativação desse reflexo se caracterizam por aumento da pressão arterial, decorrente de um incremento na atividade simpática vascular e o aumento da ventilação (BARROS et al., 2002; BRAGA et al., 2007; ZOCCAL et al., 2008), as quais ocorrem de forma sincronizada com o objetivo de otimizar os processos de troca gasosa pulmonar e do débito cardíaco, melhorando a eficiência da captação de O_2 e da perfusão tecidual. Em situações de hipóxia, as células glomus encontradas na bifurcação carotídea e na curvatura da aorta ativam as vias aferentes, e veiculam esta informação via nervos vago e glossofaríngeo até o SNC (FELDMAN; MITCHELL; NATTIE, 2003; KUMAR; PRABHAKAR, 2012; PRABHAKAR, 2013; TAKAKURA et al., 2006). Suas

fibras entram no tronco cerebral na fissura póstero-lateral do bulbo, incorporando-se ao trato solitário, terminando predominantemente nas porções comissural medial e lateral dos núcleos do trato solitário (NTScom) (FINLEY; KATZ, 1992; KUMAR; PRABHAKAR, 2012; TAKAKURA et al., 2006). Abordagens funcionais e neuroanatômicas revelaram que o NTS envia projeções à superfície ventrolateral do bulbo (AICHER et al., 1996; GRANATA, 1994; KOSHIYA; GUYENET, 1996b; URBANSKI; SAPRU, 1988), uma região que abriga populações neuronais fisiologicamente distintas, como o grupamento C1 mencionado acima e os complexos neuronais responsáveis pela geração e manutenção do padrão inspiratório e ritmo respiratório normal (FELDMAN; DEL NEGRO, 2006). Há evidências de que essas projeções do NTS para o bulbo ventrolateral rostral e adjacências representem o substrato neuroanatômico correspondente às vias funcionais simpato-excitatória e ventilatórias do quimiorreflexo periférico, as quais fazem sinapses com neurônios C1 ou ventilatórios, respectivamente (GUYENET; KOSHIYA, 1995; KOSHIYA; GUYENET, 1996a; MORAES et al., 2011).

1.3.2 Quimiorrecepção central

Quimiorreceptores centrais são altamente sensíveis ao aumento na P_{CO2} e à acidose. Embora seja crescente o número de trabalhos postulando que núcleos de células quimiossensíveis estejam distribuídos ao longo do encéfalo (NATTIE, 2011), é comumente aceito que o núcleo retrotrapezóide (RTN), localizado na porção ventral ao núcleo motor do núcleo facial e muito próximo da superfície ventrolateral do bulbo (CONNELLY; ELLENBERGER; FELDMAN, 1990; HUCKSTEPP et al., 2015; MULKEY et al., 2004; WANG et al., 2013a) concentra o principal grupamento de células quimiossensíveis, uma vez que este núcleo atente a todas as exigências para ser considerado um grupamento neural quimiossensível, como: (a) sensibilidade ao CO_2/H^+ *in vivo* e *in vitro*, (b) fenótipo neuroquímico excitatório e (c) projetar para o centro gerador do padrão respiratório (MOREIRA et al., 2015).

Dados da literatura têm oferecido várias evidências sobre os mecanismos moleculares responsáveis pela quimiorrecepção central no RTN (BURKE et al., 2015; GOURINE et al., 2010; GUYENET; BAYLISS, 2015; HUCKSTEPP et al., 2010a; KUMAR et al., 2015; MULKEY et al., 2004; WANG et al., 2013b) e dentre esses mecanismos, podemos destacar a participação de canais de K^+ (MULKEY et al., 2004, 2007). Como a corrente de sensibilidade ao pH, identificada nos neurônios do RTN, mostrou uma pequena retificação e persistiu

mesmo quando o cálcio intracelular foi neutralizado, sugeriu-se que a sensibilidade desses neurônios seja mediada pela inibição de canais de K^+ (MULKEY et al., 2004, 2007), muito provavelmente canais do tipo TASK da família K2P. Constatou-se, no entanto, que a sensibilidade não depende de canais do tipo TASK-1 ou TASK-3 (canais de K^+ da família K2P sensíveis a acidose) (MULKEY et al., 2007) mas que dependia parcialmente de canais TASK-2 (canais sensíveis a alcalose) (WANG et al., 2013b), já que o RTN de camundongos "knock-out" para canais TASK-1 e TASK-3 continuavam apresentando sensibilidade ao pH (MULKEY et al., 2007), enquanto que a depleção seletiva de TASK-2 em neurônios Phox2b promoveu a redução da sensibilidade ao pH em muitos neurônios do RTN *in vitro* e atenuação da resposta a alcalose *in situ* (WANG et al., 2013b).

Como sugerido acima, a sensibilidade ao CO_2/H^+ é apenas parcialmente alterada pela depleção de TASK-2, indicando que esta propriedade não é mediada apenas por estes canais. Sabemos agora no entanto, que a quimiossensibilidade desses neurônios depende fortemente de uma ação em conjunto entre canais TASK-2 e receptores ativados por prótons ligados a proteína G do tipo GPR4, onde a depleção deste último produz alterações muito mais expressivas (KUMAR et al., 2015). A depleção ou silenciamento de GPR4 reproduz padrões respiratórios caracteristicamente observados em animais com depleção de neurônios Phox2b , apresentando aumento no número de episódios de apneias espontâneas e redução da resposta a hipercapnia (KUMAR et al., 2015), além de reduzir expressivamente o número de neurônios ativados por CO_2/pH *in vivo* e *in vitro.* Finalmente, depleção simultânea de GPR4 e TASK2 no mesmo animal promoveu alta taxa de mortalidade e massiva redução da resposta a hipercapnia nos animais sobreviventes (~86%) (KUMAR et al., 2015).

Os mecanismos de quimiorrecepção central em outros grupamentos são menos conhecidos, e provavelmente por esta razão, mais controversos. Por exemplo, acidificação local do NTScom produz alterações respiratórias mais expressivas em níveis caudais deste núcleo, em comparação aos níveis mais rostrais do NTS (NATTIE; LI, 2002b). Neurônios quimiossensíveis da porção caudal do NTScom também foram identificados *in vitro* (NICHOLS et al., 2009; SOBRINHO et al., 2014), mas a inibição com muscimol desta região não alterou a resposta quimiorreceptora durante a hipercapnia *in vivo* (FAVERO et al., 2011). Por outro lado, a aplicação de muscimol em porções mais rostrais do NTS parece prejudicar a resposta quimiorreceptora central (NATTIE; LI, 2008).

Núcleos da rafe encontrados no bulbo (núcleos pálido, magno e obscuro da rafe, além de neurônios da região parapiramidal) também são postulados como quimiorreceptores centrais (RICHERSON, 2004), uma vez que em experimentos *in vitro* os neurônios

serotoninérgicos dessas regiões foram estimulados ou inibidos por alterações na concentração de CO_2/pH (RICHERSON, 2004; SOBRINHO et al., 2014). Adicionalmente, uma pequena porcentagem de neurônios serotoninérgicos mesencéfalicos (núcleo dorsal da rafe) também apresentaram alterações na atividade em resposta a acidose (SEVERSON et al., 2003; VEASEY et al., 1997). Experimentos *in vivo*, foi detectado aumento da atividade respiratória em resposta a acidose local induzida por microdiálise em grupamentos bulbares (NATTIE; LI, 2001), enquanto que a inibição com muscimol na rafe bulbar em porcos promove redução da resposta respiratória a hipercapnia (MESSIER; LI; NATTIE, 2002).

Como mencionado anteriormente, uma série de outros grupamentos apresentam características quimiossensíveis (BIANCARDI et al., 2008; DENG et al., 2007; MARTINO et al., 2006a; TAXINI et al., 2011; WILLIAMS et al., 2007), porém não serão retratados aqui por não constituírem parte do foco deste estudo.

1.3.3 Integração do quimiorreflexo central e periférico: participação do bulbo ventrolateral rostral

Ao longo dos últimos anos, diferentes estudos comprovaram a existência de uma importante interação entre os quimiorreceptores periféricos e os quimiorreceptores centrais localizados no RTN (MULKEY et al., 2004; SMITH et al., 2006; TAKAKURA et al., 2006). A estimulação dos quimiorreceptores carotídeos promove um aumento na frequência de disparos dos neurônios do RTN, sendo abolida pela desnervação periférica dos quimiorreceptores, sem afetar a propriedade intrínseca desses neurônios em resposta à hipercapnia (MULKEY et al., 2004). Ademais, projeções diretas, predominantemente excitatórias, do NTScom para os neurônios do RTN também já foram descritas (TAKAKURA et al., 2006). Além de receber densas projeções do NTScom, o RTN estabelece ainda conexões recíprocas com outras áreas cardiorrespiratórias como o complexo parabraquial/Kolliker-Fuse, a coluna respiratória ventral, o grupamento noradrenérgico A5, bem como áreas hipotalâmicas (ROSIN; CHANG; GUYENET, 2006).

Mais recentemente tem sido proposto que neurônios C1 também podem influenciar a respiração (BURKE et al., 2014; WENKER et al., 2013). Como já mencionado anteriormente, neurônios C1 recebem projeções do NTScom que veiculam informações de quimiorreceptores periféricos (KOSHIYA; GUYENET, 1996b) e além das projeções bulbo-espinais, os neurônios C1 também apresentam projeções para o locus coeruleus, região A5, núcleo motor

dorsal do vago e hipotálamo, ou seja, áreas em potencial para influenciar a ventilação (GUYENET et al., 2013).

Esses dados sugerem que a região RVL (RTN/C1) e o NTScom representam duas importantes regiões onde ocorrem integrações cardiorrespiratórias. Esta organização anátomo-funcional sugere uma integração entre o quimiorreflexo central, a regulação central da respiração e funções autônomas (MOREIRA et al., 2011; ROSIN; CHANG; GUYENET, 2006). Dado a importância desse processo, o presente trabalho tem como um dos seus objetivos ampliar o nosso conhecimento a respeito da integração entre o quimiorreflexo central e periférico na região do RVL.

1.4 Sinalização Purinérgica

O ATP, produzido durante a glicólise, é incontestavelmente mais estudado e mais popular devido sua atuação como fonte de energia celular. No entanto, após um consideravél número de evidências experimentais que datam desde o final dos anos 20 até os dias atuais, sua importância também é reconhecida como molécula sinalizadora intracelular e extracelular (paracrina e exócrina), inclusive atuando na comunicão central e periférica, na co-transmissão com outros neurotransmissores e mais recentemente, como molécula sinalizadora na interação glia-neurônio (ABBRACCHIO et al., 2009; BURNSTOCK, 2006a, 2007; FUNK, 2013; GOURINE; WOOD; BURNSTOCK, 2009). O sistema que integra a sinalização purinérgica, conta ainda com grande variedade de ectonucleotidases, enzimas que degradam o ATP, e de diferentes subtipos de receptores que podem ser responsivos ao ATP ou ao produto da sua degradação (FUNK, 2013).

Muitas teorias ainda são postuladas sobre o possível mecanimo pelo qual o ATP, presente no citosol, é liberado ao espaço extracelular. Neste sentido, já foi demonstrado que o ATP pode ser armazenado em vesiculas, por ação de transportadores vesiculares de nucleotídeos dependentes de Cl^- (SAWADA et al., 2008) e liberado via exocitose como um neurotransmissor clássico, via aumento nas concentrações de Ca^{2+} intracelular (ANGELOVA et al., 2015; GOURINE et al., 2010). Mais recentemente, tem sido proposto que o ATP, também pode ser liberado via hemicanais de gap-junctions, como por exemplo do subtipo denominado conexinas 26 (HUCKSTEPP et al., 2010a, 2010b; WENKER et al., 2012).

Uma vez na fenda sinaptica, o ATP pode atuar diretamente em receptores específicos, ou pode ser degradado, via ectonucleotidades, em difosfato de adenosina (ADP), monofosfato de adenosina (AMP) e por fim em adenosina (ADO). As ectonucleotidases são divididas em 4

famílias, algumas delas com diversos membros. A ectonucleotidase trifosfato de difosfohidrolase (E-NTPDases 1, 2 , 3 e 8), a fosfatase alcalina e a ectonucleotidase ecto-5', já foram bem descritas no SNC em diferentes tipos de células (glias e neurônios) (ABBRACCHIO et al., 2009; BURNSTOCK, 2006b, 2007; FUNK, 2013).

Os receptores purinérgicos são divididos em 3 grandes grupos. Os receptores P2X apresentam sete subtipos, sendo todos eles canais iônicos, e, portanto, receptores ionotrópicos sensiveis ao ATP. Os receptores P2Y respondem ao ATP e ao ADP, e são receptores metabotrópicos acoplados a proteina G. Oito subtipos são encontrados no SNC, sendo o P2Y1 o subtipo mais abundante. Os subtipos P2Y1, 2, 4, 6 e 11 são receptores ligados a proteina $G_{q/11}$ ou G_s, e portanto excitatórios, enquanto que os subtipos P2Y12 e 13 são ligados a proteina G_i, e portanto inibitórios. O terceiro grupo é formado por uma familia de receptores também acoplados a proteina G com 4 membros, os receptores P1, sendo eles P1 A1 e A3 acoplados a Gi e $G_{\alpha i}$, respectivamente, enquanto os receptores P1 A2a e A2b são acoplados a Gs. Receptores P1 são mais sensiveis a ADO (FUNK, 2013). É interessante mencionar que uma ectonucleotidase pode ter mais afinidade pelo ATP ou ser seletiva a um subproduto da via de degradação, pois desta forma, se houver maior expressão de um subtipo em determinada região, isto pode ser determinante na quantidade e do tipo de purina presente na fenda. Vinculado a distribuição de seus respectivos receptores, determinam o tipo, a potência e a duração da resposta pré e pós sinaptica exercida pela purina.

1.5 Glia, sinalização purinérgica e quimiorrecepção central

Adicionalmente a quimiorrecepção intrínseca dos neurônios do RTN, tem sido demonstrado experimentalmente que outras células do SNC (possivelmente células da glia, mais precisamente os astrócitos) poderiam estar atuando também como sensores de CO_2 (ERLICHMAN; LEITER; GOURINE, 2010; FUKUDA; LOESCHCKE, 1977; GOURINE et al., 2010; MULKEY; WENKER, 2011; WENKER et al., 2010). Estudos anteriores mostraram a existência na região do RTN, de um grupamento de células sensíveis ao CO_2 e com baixa atividade elétrica, sugerindo que astrócitos nessa região também poderiam apresentar quimiosenssíbilidade. Sendo assim, outra possível forma desses neurônios serem ativados pelo aumento na P_{CO2} seria uma forma indireta, ou seja, via astrócitos. As células da glia também são sensíveis ao CO_2 (WENKER et al., 2010), mas o entendimento a respeito do mecanismo pelo qual essa ativação gera a ativação dos neurônios do RTN ainda está

evoluindo. Dessa maneira, estamos propondo que tanto neurônios como as células da glia da região do RTN estariam envolvidos na detecção dos níveis de CO_2/H^+.

Originalmente, acreditava-se que a glia contribuía para o mecanismo de quimiorrecepção por potencializar uma acidose extracelular produzida pelo CO_2 (ERLICHMAN; LI; NATTIE, 1998; HOLLERAN; BABBIE; ERLICHMAN, 2001). Astrócitos e oligodendrócitos ajudam a regular o K^+ extracelular por captar o excesso de K^+ durante períodos de aumento de atividade neuronal. Esse processo neutraliza alterações no K^+ extracelular, mas pode também aumentar o co-transporte de Na^+/HCO_3^- para a glia, aumentando a queda no pH extracelular induzido pelo CO_2 e potencializar a ativação dos quimiorreceptores do RTN (CHESLER, 2003; ERLICHMAN et al., 2004).

Mais recentemente, evidências têm demonstrado uma segunda participação das células da glia na detecção do CO_2. Em outras regiões do SNC, já está bem estabelecido que a ativação de astrócitos é capaz de promover a liberação de neurotransmissores, entre eles o ATP (FELLIN, 2009). Além do mais, acredita-se que as células gliais sejam a principal fonte de liberação de ATP durante uma situação de hipercapnia (GOURINE et al., 2010; HUCKSTEPP et al., 2010a; MULKEY; WENKER, 2011). Níveis elevados de CO_2 promovem a liberação de ATP no RTN promovendo um aumento na atividade respiratória (GOURINE et al., 2005a). As alterações respiratórias promovidas pelo aumento dos níveis de CO2 são atenuados pelo uso do antagonista de receptores purinérgicos P2, PPADS, sugerindo que esses receptores estariam envolvidos nas respostas ventilatórias ao ATP (GOURINE et al., 2005a). Além disso, a aplicação de ATP no RTN promove um aumento na respiração, mimetizando os resultados observados pelos níveis elevados de CO_2 (GOURINE et al., 2005a; HUCKSTEPP et al., 2010a; WENKER et al., 2010, 2012). Dessa forma, fica claro que o ATP exerce um papel de importante mediador da quimiorrecepção central via células da glia (GOURINE et al., 2010).

Dessa maneira, a sinalização purinérgica emergiu como um sistema de grande relevância para a importância da atividade funcional integrativa entre neurônios, glia e células vasculares no SNC, facilitando um sistema de sinalização intracelular que permite uma funcionalidade ideal entre neurônio-glia (BURNSTOCK; FREDHOLM; VERKHRATSKY, 2011). Considerando que um dos pontos chave para o entendimento da quimiorrecepção central consiste no esclarecimento de mecanismos neuro-moleculares envolvidos nesse processo, o presente trabalho teve como um dos objetivos avaliar os possíveis mecanismos de interação glia-neurônio na região do RTN durante uma situação de hipercapnia, bem como a participação dos receptores purinérgicos neste processo.

2 OBJETIVOS

Diante das informações que foram expostas acima, tivemos como objetivo central do presente estudo ampliar o conhecimento sobre como o SNC, mais precisamente a regiões quimiorreceptoras processam e integram informações, gerando respostas cardiorrespiratórias adequadas durante a hipóxia e hipercapnia.

2.1 Objetivos específicos

1) Avaliar a contribuição da sinalização purinérgica no RTN, bem como de receptores purinérgicos P2 para a resposta cardiorrespiratória a ativação de quimiorreceptores centrais (hipercapnia).

2) Avaliar a contribuição da sinalização purinérgica na região RVL (RTN/C1) para a resposta cardiorrespiratória a ativação de quimiorreceptores periféricos (hipóxia).

3) Avaliar a contribuição da sinalização purinérgica para resposta cardiorrepiratória central a hipercapnia no NTScom e no RPa/PPy.

4) Avaliar se manipulação farmacológica de células da glia no RTN, no NTScom e no RPa/PPy promove alterações da atividade cardiorrespiratória e a contribuição da sinalização purinérgica local para estas respostas.

3 MATERIAIS E MÉTODOS

3.1 Animais

Foram utilizados um total de 98 ratos adultos (Rattus norvegicus, linhagem Wistar) provenientes do biotério central do Instituto de Ciências Biomédicas da Universidade de São Paulo (ICB - USP), com idade entre 60 e 90 dias e pesando entre 250 e 350 g. Os animais foram acondicionados no biotério com ciclo claro/escuro de 12h, temperatura de 23 °C e acesso livre a ração e água. Os procedimentos foram conduzidos com base no protocolo de ética em experimentação animal adotado pelo Instituto de Ciências Biomédicas da Universidade de São Paulo, (protocolo do Comitê de Ética no Uso de Animais: número 118/11- fl. 102).

3.2 Procedimentos cirúrgicos e anestesia

Inicialmente, os animais foram anestesiados com halotano 5% em 100% de oxigênio. Os animais foram posteriormente traqueostomizados e colocados em ventilação artificial com 1,4 - 1,5% de halotano em 100% de oxigênio para continuação dos procedimentos cirúrgicos. Em todos experimentos foram realizados os seguintes procedimentos cirúrgicos:

1) canulação da artéria femoral para registro de pressão arterial média (PAM) e canulação da veia femoral para administração de farmacos;

2) todos os animais foram vagotomizados bilateralmente a fim de prevenir uma influência da ventilação na atividade do nervo frênico.

4) colocados em um aparelho estereotáxico (modelo Kopf 1760, David Kopf Instruments, Tujunga, CA, USA);

3) localização e exposição do nervo frênico via posição dorsolateral (HAWRYLUK et al., 2012; MOREIRA et al., 2006; SOBRINHO et al., 2014; TAKAKURA et al., 2008; TAXINI et al., 2011; WENKER et al., 2012, 2013).

Em uma série de experimentos foi realizado também o registro da atividade simpática, obtida através do registro do ramo esplâcnico do plexo simpático via acesso retroperitonial (MOREIRA et al., 2006; TAXINI et al., 2011; TOTOLA et al., 2013; WENKER et al., 2013).

Após a finalização dos procedimentos cirúrgicos, o anestésico halotano foi substituído pelo anestésico endovenoso uretano (1,2 g/kg). Os animais foram ventilados com 100% de oxigênio durante todo o período experimental, exceto durante os testes de quimioreflexo central, quando CO_2 foi adicionado a ventilação. Os animais receberam uma sonda retal para monitorização da temperatura corpórea e sua temperatura foi mantida em 37 ºC, utilizando-se

um colchão com resistência interna para aquecimento. O índice de CO_2-expirado foi monitorado durante todo o experimento por meio de um capnômetro (Columbus Instruments, Ohio, USA). O nível da anestesia foi sempre monitorado testando-se a ausência de efeitos no reflexo de retirada, ausência de mudanças na pressão arterial e na atividade do nervo frênico após o pinçamento da pata do animal. Satisfeitos esses critérios, o relaxante muscular (pancurônio) foi administrado endovenosamente com uma dose inicial de 1 mg/kg.

3.3 Farmacos utilizados

Fluorocitrato (FCt) (sal de bário): toxina glial (Sigma-Aldrich, USA): 1 mM. O preparo do FCt foi realizado conforme descrito em trabalhos anteriores (COSTA; MORAES; MACHADO, 2013; ERLICHMAN; LI; NATTIE, 1998; HOLLERAN; BABBIE; ERLICHMAN, 2001). Primeiramente, o FCt foi dissolvido em 0,1 M HCl. Após essa dissolução, o sal de bário foi precipitado com a adição de 0,1 M Na_2SO_4. Essa solução foi tamponada com 0,1 M $NaPO_4$ e centrifugada a 800 rpm durante 10 minutos. O sobrenadante, contendo o sal de bário, foi removido e recebeu adição de solução salina até atingir a concentração final de 1 mM, então o pH foi corrigido para 7.4.

- PPADS (pyridoxal-phosphate-6-azophenyl-2',4'-disulfonic acid): antagonista de receptores purinérgicos P2 (Sigma-Aldrich, USA): 3 mM.
- MRS2179 (2′-deoxy-N6-methyladenosine-3′,5′-bisphosphate): antagonista de receptores purinérgicos P2Y1 (Tocris, USA): 100 μM.
- Ácido quinurênico: antagonista de receptores glutamatérgicos ionotrópicos (Sigma-Aldrich, USA): 100 mM
- Trifosfato de adenosina (ATP) (Sigma-Aldrich, USA): 10 mM, pH ajustado para 7.4
- Cianeto de potássio (KCN): 40 µg/0,1 ml por animal.Cianeto de potássio foi injetado i.v para testar a funcionalidade do quimiorreflexo periférico.
- Neurotoxina Saporina (anti-dopamina β-hidroxilase: anti-DβH-SAP) (Advanced Targeting Systems, San Diego, CA): toxina saporina seletiva para neurônios catecolaminérgicos: 2,4 ng em 100 nl.

As doses utilizadas foram baseadas em trabalhos anteriores que mostraram os efeitos dessas drogas no tronco encefálico ou perifericamente (ALVARES et al., 2014; GOURINE et al., 2010; TAKAKURA et al., 2008, 2011; TAXINI et al., 2011; WANG et al., 2001;

WENKER et al., 2012). Microsesferas de latex fluorescentes (microbeads 2%, Lumafluor) foram adicionados à diluição dos farmacos para facilitar a visualização dos sítios de injeção.

3.4 Injeções encefálicas

As injeções de farmacos no SNC foram realizadas sob pressão com nitrogênio, utilizando-se pipetas de vidro (diâmetro interno 25-30 μm, Sutter Instrument Co, CA) acopladas ao aparelho PicoSpritzer III (General Valve Corporation, NJ). O volume das injeções foi de 50 nl. Nos experimentos que realizamos injeções bilaterais, o volume de 50 nl foi injetado em cada lado.

Duas diferentes métodologias foram empregadas para realização de injeções no RTN:

1) Baseadas no potencial antidrômico do núcleo facial: Quando esta metodologia foi empregada, após a trepanação do osso occipital, foram realizadas incisões laterais na pele da face do animal e um eletrodo bipolar foi utilizado para estimular o ramo mandibular do nervo facial (100 μsec; 0.5-2 mA; 1 Hz). Um eletrodo foi inserido dentro da pipeta de vidro contendo o farmaco e conectado ao amplificador, enquanto o monitoramento era realizado pelooscilloscoópio. Como a origem dos neurônios deste nervo estão no núcleo facial, avisualização do sinal elétrico propagado desaparece com o distanciamento da pipeta núcleo facial. Injeções foram realizadas 200 μm rostrais à porção caudal do núcleo facial, 1,8 mm lateral à linha média e 250 μm ventral ao limite ventral do potencial antidrômico do núcleo facial.Os registros eletrofisiológicos foram realizados em um lado e a segunda injeção foi realizada simetricamente do outro lado baseado nas coordenadas estereotáxicas da primeira injeção.

2) O lambda e a sutura sagital também foram frequentemente utilizados para obtenção das coordenadas estereotáxicas. Quando utilizadas, as coordenadas estreotáxicas foram: Antero-posterior (AP) 1,8 mm caudal ao lambda; médio-lateral (ML) 2,3 mm lateral a linha média e dorso-ventral (DV) 8,4 mm abaixo da dura-máter.

Para as injeções na região do núcleo palido da rafe/parapiramidal (RPa/PPy), as coordenadas utilizadas também foram baseadas a partir do potencial antidrômico do núcleo facial ou lambda. As injeções foram realizadas 200 μm rostral à porção caudal do núcleo facial, 0-1,0 mm lateral à linha média e 250 μm ventral ao limite ventral do potencial antidrômico do núcleo facial ou AP 1,8 mm caudal ao lambda; ML 0-1,0 mm lateral à linha média e DV 8,3 mm ventral a dura-máter.

As coordenadas utilizadas para atingir o núcleo do trato solitário comissural (NTScom) foram baseadas em relação ao calamus scriptorius (MOREIRA et al., 2005; TOTOLA et al., 2013). As injeções foram realizadas 300-400 μm caudal ao calamus scriptorius, na linha média e 500 μm ventral em relação à superfície do bulbo.

3.5 Registro da pressão arterial

Para o registro das variáveis cardiovasculares, os animais foram submetidos à canulação da artéria femoral com tubo de polietileno (PE-10 conectado a um PE-50) para registro da pressão arterial pulsátil (PAP) e pressão arterial média (PAM). A cânula da artéria femoral foi conectada a um transdutor de pressão (Physiological Pressure Transducer mod. MLT844, ADInstruments) acoplado a um pré-amplificador (Bridge Bio Amplifier mod. ML221, ADInstruments) e ao sistema de registro computadorizado Cambridge Electronic Design (CED-1401) de 8 canais. Foram registradas, simultaneamente, a PAP e a PAM.

Adicionalmente, os animais tiveram a veia femoral canulada com tubo de polietileno (PE-10 conectado a um PE-50) para a infusão de farmacos de forma sistêmica.

3.6 Medida da atividade do nervo frênico

O nervo frênico direito foi exposto e isolado da divisão ventral do quinto ramo doplexo cervical via acesso dorsolateral da região do pescoço. O nervo foi cortado distalmente e colocado num eletrodo bipolar em forma de gancho. A atividade do nervo frênico foi filtrada de 100 a 3000 Hz.O nervo e o eletrodo de registro foram cobertos com uma pasta de moldagem dental (Kwik-Cast^{TM}). O eletrodo bipolar em que o nervo foi colocado estava conectado a um conversor analógico-digital (modelo CED-1401) da Cambridge Electronics Design (CED, Cambridge, UK) de 8canais. Este aparelho possui filtro passa-baixo, ligação AC-DC (corrente direta-alternada), filtro de corte, permite variação do ganho e possibilita correção da linha de base. A partir deste aparelho, o sinal foi copiado para um sistema de aquisição de dados versão 6 do Spike2 software (CED). Os resultados foram gravados em DVD e posteriormente analisados.

3.7 Medida da atividade da atividade simpática

Através de um acesso retroperitonial na lateral do abdomem o ramo esplâcnico do plexo simpático foi dissecado e o segmento distal do nervo esplâncnico foi colocado sobre um eletrodo bipolar em forma de gancho, conforme descrito préviamente (FAVERO et al., 2011; MOREIRA et al., 2005; TAKAKURA et al., 2011; TOTOLA et al., 2013). Os passos seguintes da preparação (isolamento, filtragem, registro e equipamentos utilizados) seguiram os mesmos parâmetros utilizados para registro do nervo frênico.

3.8 Lesão de neurônios catecolaminérgicos do grupamento C1

Após a cirurgia cerebral, os ratos receberam uma injeção intramuscular (0,2 ml/rato) de Pentabiótico Veterinário - Pequeno Porte (Fort Dodge Saúde Animal Ltda.) e do analgésico/anti-inflamatório Ketoflex (cetoprofeno 1%, 0,03 ml/rato). Os animais receberam água e ração *ad libitum* e foram mantidos no biotério de experimentação com temperatura e umidade controladas. Os animais permaneceram por um período de 1 semana antes do início dos experimentos.

As cirurgias para lesão dos neurônios catecalaminérgicos do grupamento C1 foram realizadas 15 dias antes da realização dos experimentos. Os animais foram inicialmente anestesiados intraperitonealmente com coquetel composto por cetamina (80 mg/kg) e xilazina (7 mg/kg), e foram posicionados em aparelho estereotáxico (Kopf 1760, Davi Kopf Instruments, Tujunga, CA, USA). Após uma incisão longitudinal na pele e no tecido subcutâneo para a exposição da calota craniana, o lambda e o bregma foram utilizados como referência para nivelar as cabeças dos animais. Injeções bilaterais (volume de 100 nl) de saporina conjugada anti-dopamina β-hidroxilase (anti-DβH-SAP) (Advanced Targeting Systems, San Diego, CA) ou salina (controle) na região C1 foram realizadas bilateralmente sob pressão com nitrogênio, utilizando-se pipetas de vidro (diâmetro interno 25-30 µm, Sutter Instrument Co, CA) acopladas ao aparelho PicoSpritzer III (General Valve Corporation, NJ). As coordenas estereotaxicas utilizadas foram: 2,7 mm caudais ao lambda; 1,8 mm laterais a sutura sagital e 8,2 mm ventral a dura-mater. Durante a cirurgia foram tomadas todas as precauções para assepsia para reduzir o risco a infecções e após término, os ratos receberam uma injeção intramuscular (0,2 ml/rato) de Pentabiótico Veterinário para animais de pequeno porte (Fort Dodge Saúde Animal Ltda.) e de subcultânea de analgésico/anti-inflamatório Ketoflex (cetoprofeno 1%, 0,03 ml/rato).

3.9 Injeção de traçador anterógrado no NTScom

A injeçãodo traçador anterógrado Biotina Dextrana Amina (BDA, MW 10000; 10% w/v in 10 mM tampão fosfato, pH 7.4; Molecular Probes, Eugene, OR, USA) foi realizada em animais anestesiados intraperitonealmente com uma mistura anestésica de cetamina (80 mg/kg) e xilasina (7 mg/kg). Posteriormente, os animais foram adaptados a um aparelho estereotáxico e recebeu injeções, sob pressão com nitrogênio, utilizando-se pipetas de vidro (diâmetro interno 25-30 µm, Sutter Instrument Co, CA) acopladas ao aparelho PicoSpritzer III (General Valve Corporation, NJ) do traçador anterógrado BDA. As coordenas estereotaxicas utilizadas foram: 0,4 mm caudal ao *calamus scriptorius*, na linha média e 0,3-0,5 mm abaixo da superfie dorsal do tronco encefálico. Durante a cirurgia foram tomadas todas as precauções para assepsia para reduzir o risco a infecções e após término, os ratos receberam uma injeção intramuscular (0,2 ml/rato) de Pentabiótico Veterinário para animais de pequeno porte (Fort Dodge Saúde Animal Ltda.) e do analgésico/anti-inflamatório Ketoflex via subcutânea (cetoprofeno 1%, 0,03 ml/rato). Sete a dez dias após a cirurgia, os animais foram profundamente anestesiados com pentobarbital de sódio (60 mg/kg) e perfundidos por via intracardíaca. Os encéfalos foram removidos e processados para análise imunoistoquímica para identificação de BDA, da vesicula transportadora para glutamato (VGLUT-2) e da transportador vesicular de nucleotídeos (VNUT).

3.10 Perfusão, histologia e imunoistoquímica

Ao término dos experimentos, os animais ainda sob efeito de anestesia foram perfundidos através do ventrículo cardíaco esquerdo com solução salina 0,9 % seguido de formaldeído (4% em 0,1 M de fosfato, pH 7,4). Os encéfalos foram removidos e armazenados durante 2 h nesse fixador a 4 °C para pós-fixação, e, então, transferidos para solução de sacarose a 20% diluída em tampão fosfato de potássio (0,2M), onde permaneceram durante aproximadamente 12h também a 4 °C.

Em seguida, cortes coronais de 40 µm foram obtidos através de micrótomo de congelamento e armazenados em solução anti-congelante(crioprotetora: 20% de glicerol, 30% de etileno glicol em 50 mM de fostato, pH 7.4) que preserva as qualidades do tecido encefálico para posterior tratamento imunoistoquímico. Uma série de cortes histológicos foi

montada em lâminas gelatinizadas, corada pela técnica de Níssl e utilizada como controle citoarquitetônico.

A imunorreatividade para BDA foi revelada utilizando estreptavidina Alexa 488 (1:2000, Jackson Immunoresearch, USA). Transportador vesicular de nucleotídeo (VNUT) foi detectado utilizando-se um anticorpo primário coelho anti-VNUT (1:200, MBL, Japan) seguido de Cy3 burro anti-coelho IgG (1:200; Invitrogen Carlsbad, CA, USA). Transportador vesicular de glutamato (VGLUT2) foi detectado utilizando-se um anticorpo primário porco-da-india anti-VGLUT2 (AB 5907; 1:2000; Chemicon International, Temecula, CA, USA). Todas as reações foram realizadas pelo método de fluorescência.

Para avaliar a extensão da lesão pela injeção da toxina anti-DβH-saporina, os cortes encefálicos foram processados para visualização do marcador tirosina hidroxilase (TH). A detecção da TH foi realizada utilizando-se o anticorpo primário camundongo anti-TH (1:1000; Millipore, USA), seguido de cabra anti-camundongo biotinilado (1:500; Jackson, West Grove, PA, USA). Para observar os neurônios Phox2b intactos do RTN foi utilizado o anticorpo primário coelho anti-Phox2b (1:800), seguido de burro anti-coelho biotinilado (1:500; Jackson, West Grove, PA, USA). Ambas reações foram realizadas pelo método de peroxidase (BARNA; TAKAKURA; MOREIRA, 2012, 2014).

Finalmente, os cortes encefálicos foram montados em lâminas em sequência rostro-caudal. Quando processadas por peroxidase, as lâminas foram desidratadas com álcool e xilol e cobertas com Krystalon (EMD Chemicals Inc, NJ).

Depois de finalizados os tratamentos imunoistoquímicos, os cortes encefálicos foram analisados num microscópio de fluorescência ou campo claro (Zeiss Axioskop 2), conforme tratamento, para conferir a localização dos grupamentos neuronais marcados. Toda a nomenclatura anatômica foi baseada no Atlas de Paxinos e Watson (Paxinos e Watson, 1998) e em trabalhos anteriores (BARNA; TAKAKURA; MOREIRA, 2012, 2014; NATTIE; GDOVIN; LI, 1993; TAKAKURA et al., 2008, 2006; WENKER et al., 2012, 2013).

3.11 Análise dos resultados

Os resultados foram tabelados. A média e o erro padrão da média foram representados em gráficos. Teste T de student não pareado ou análise de variância de uma via seguido do teste de Newman Keuls foram utilizados para as comparações entre diferentes tratamentos. Diferenças foram consideradas significantes para $p<0,05$.

4 RESULTADOS

4.1 Alterações cardiorrespiratórias frente ao estímulo hipercapnico são moduladas pela pela sinalização purinérgica na região do RTN

4.1.1 Receptores purinérgicos P2 no RTN participam nas alterações cardiorrespiratórias promovidas pela ativação dos quimiorreceptores centrais

Para avaliar o envolvimento da sinalização purinérgica nas respostas cardiorrespiratórias promovidas pela ativação dos quimiorreceptores centrais, o protocolo experimental a seguir foi delineado para promover uma situação de hipercapnia antes e após o bloqueio bilateral de receptores purinérgicos na região do RTN (Figs. 2B-C).

Como esperado, a hipercapnia produzida pelo aumento nos de níveis de CO_2 de 5% para 10% produziu alterações imediatas nas atividades cardiorrespiratórias em animais que receberam injeções controle (salina). O rapido aumento nos níveis de CO_2 produziu queda da pressão arterial (-11 ± 7 mmHg, $p<0,05$), seguida por um aumento gradual da pressão arterial para valores basais. Imediatamente após o retorno dos níveis de CO_2 para os valores basais (5%), ocorreu um aumento da pressão arterial (21 ± 8 mmHg, $p<0,05$) retornando ao valor basal após 5 minutos (Figs. 2A e 2D). A atividade do nervo frênico aumentou em amplitude (100 ± 2%) e frequência (99 ± 2%) (Figs. 2A, D e E).

Injeções bilaterais de PPADS (3 mM - 50 nl) na região do RTN não promoveram alterações na pressão arterial basal (124 ± 6 mmHg, vs. salina: 126 ± 5 mmHg, $p>0,05$) e na amplitude e frequência do nervo frênico basal (99 ± 7% em relação ao controle, $p>0,05$) e não alteraram a resposta hipotensora produzida pela hipercapnia (Fig. 2D). No entanto, o bloqueio dos receptores purinérgicos P2 do RTN aboliu a resposta pressora produzida no final da hipercapnia de 21 ± 6 para 8 ± 4 mmHg ($p<0,05$) (Figs. 2A e 2D) e reduziu o aumento da amplitude (62 ± 9%, $p<0,05$) e da frequência (77 ± 3%, $p<0,05$) do nervo frênico produzido pela hipercapnia (Figs. 2A, 2E-F).

As injeções bilaterais de PPADS estavam localizadas na região que contém a grande população de neurônios quimiossensíveis na região do RTN (Fig. 2B-C). A figura esquematica representada em 2C ilustra injeções de PPADS centradas no RTN ou em regiões próximas, mais especificamente localizadas na região da rafe parapiramidal (n= 4), no núcleo motor do facial (n= 3). O bloqueio dos receptores purinérgicos fora do RTN não foi capaz de reduzir as respostas cardiorrespiratórias promovidas pela ativação dos quimiorreceptores centrais.

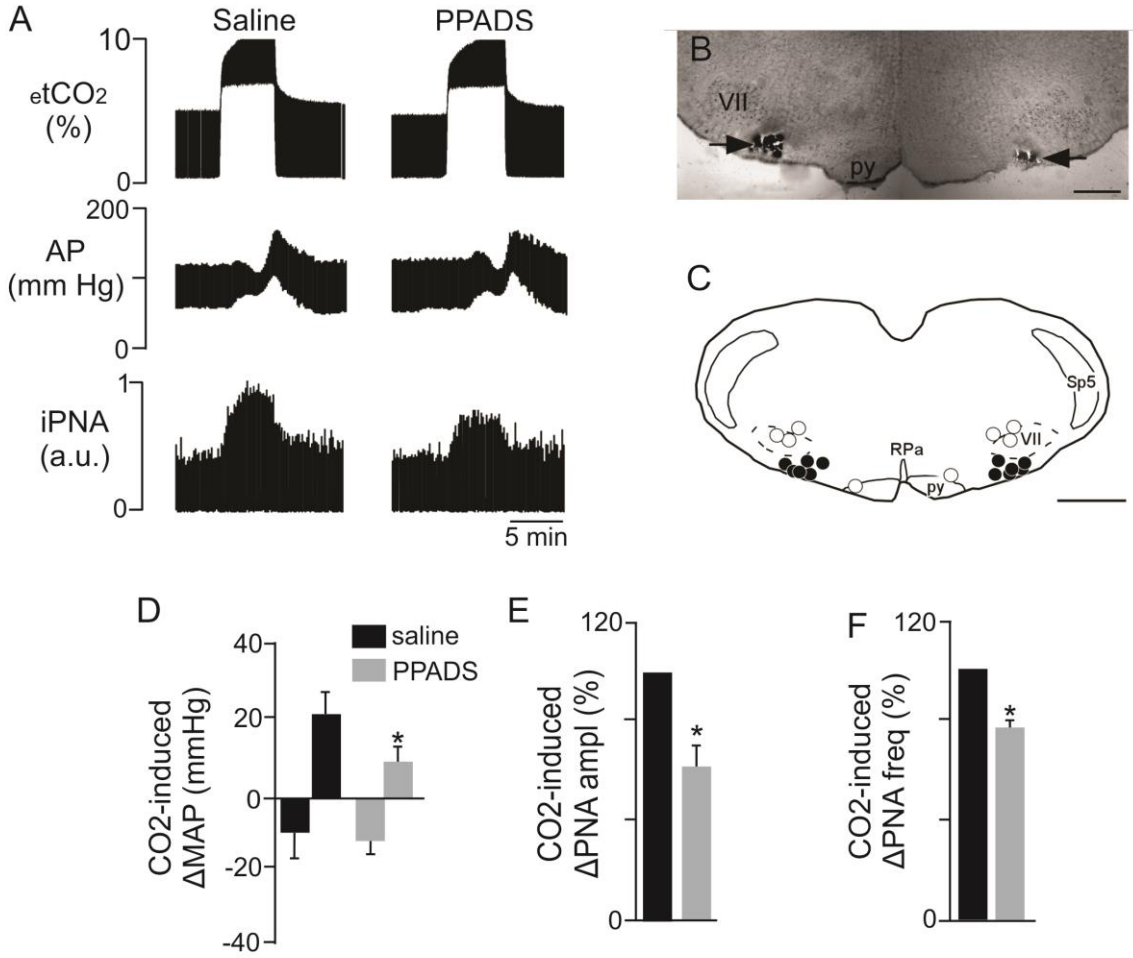

Figura 2: Injeção bilateral de PPADS no núcleo retrotrapezóide atenua a resposta cardiorrespiratória produzida pela estimulação dos quimiorreceptores centrais.

A) Traçado representativo demonstrando os efeitos produzidos pelo aumento nos níveis de CO_2 de 5 para 10% na pressão arterial, e na atividade do nervo frênico 10 minutos após a aplicação bilateral de salina ou PPADS (3 mM - 50 nl) no núcleo retrotrapezóide. **B-C)** Local das injeções no núcleo retrotrapezóide. O corte coronal representa Bregma = -11.6 mm, (Paxinos e Watson, 2007). Escala = 1 mm. **D)** Efeitos produzidos pelo aumento nos níveis de CO_2 de 5 to 10% na amplitude do nervo frênico após a injeção de salina ou PPADS no RTN. **E)** Efeitos produzidos pelo aumento nos níveis de CO_2 de 5 to 10% na frequência do nervo frênico após a injeção de salina ou PPADS no RTN. **F)** Efeitos produzidos pelo aumento nos níveis de CO_2 de 5 to 10% na pressão arterial média após a injeção de salina ou PPADS no RTN. *diferente da injeção de de salina. Abreviações: py, trato pirâmidal; VII, núcleo motor do nervo facial; RPa, núcleo pálido da rafe, Sp5, trato espinal do trigêmeo ($p<0,05$); n = 6 ratos.

4.1.2 Receptores purinérgicos P2Y1 no RTN não contribuem para as alterações cardiorrespiratórias promovidas pela ativação dos quimiorreceptores centrais

Para avaliar o envolvimento de receptores purinérgicos P2Y1 nas respostas cardiorrespiratórias promovidas pela ativação dos quimiorreceptores centrais, avaliamos as alterações cardiorrespiratórias promovidas pela hipercapnia antes e após o bloqueio de receptores purinérgicos P2Y1 na região do RTN mediante a aplicação bilateral de antagonista MRS 2179. Afim de confirmar os resultados encontrados, realizamos também injeções de agonista P2Y1 MRS 2365 unilateral na região do RTN (Figs. 3 e 4)

Como descrito anteriormente, em animais do grupo controle (salina), a hipercapnia (de 5% para 10% nos níveis de CO_2) produziu uma imediata queda da pressão arterial (-16 ± 6 mmHg, $p<0,05$) seguida por um aumento gradual da pressão arterial para valores basais. Imediatamente após o retorno dos níveis de CO_2 para os valores basais (5%), ocorreu um aumento da pressão arterial (24 ± 5 mmHg, $p>0,05$), retornando ao valor basal após 5 minutos (Figs. 3A e 3C). A atividade do nervo frênico apresentou aumento em amplitude (98 ± 3%) e frequência (97 ± 2%) (Figs. 3A, 3D-E).

Injeções bilaterais de MRS 2179 (100 μM - 50 nl) na região do RTN não promoveram alterações na pressão arterial basal (119 ± 7 mmHg vs. salina: 121 ± 3 mmHg, $p>0.05$) ou atividade basal do nervo frênico (102 ± 5% do controle, $p>0,05$). As injeções bilaterais de MRS 2179 no RTN foram inefetivas em promover alterações na resposta pressora produzida no final da hipercapnia (24±5 mmHg vs salina: 19±4, $p>0,05$) (Figs. 3A e C). MRS 2179 no RTN também não alterou o aumento da amplitude (86 ± 15% do controle, $p>0,05$) e da frequência (108 ± 5%, do controle, $p>0,05$) do nervo frênico produzido pela hipercapnia (Figs. 3A, 3D-E). A figura esquematica 2B ilustra a distibuição das injeções de MRS 2179 localizadas no RTN.

Injeções unilaterais de agonista P2Y1, MRS 2365 (100 μM -50 nl) na região do RTN promoveu aumento da PAM (23 ± 1 mmHg vs controle 4 ± 1 mmHg, $p<0,05$) e na atividade do nervo frênico em amplitude e frequência (59 ± 6% e 53 ± 5%, respectivamente). Adicionalmente, a aplicação prévia de antagonista P2Y1 MRS 2179 bloqueou a resposta ao agonista, sugerindo que a dose que estávamos utilizando do antagonista era efetiva em bloquear os efeitos do agonista P2Y1 (Figs. 4A-E).

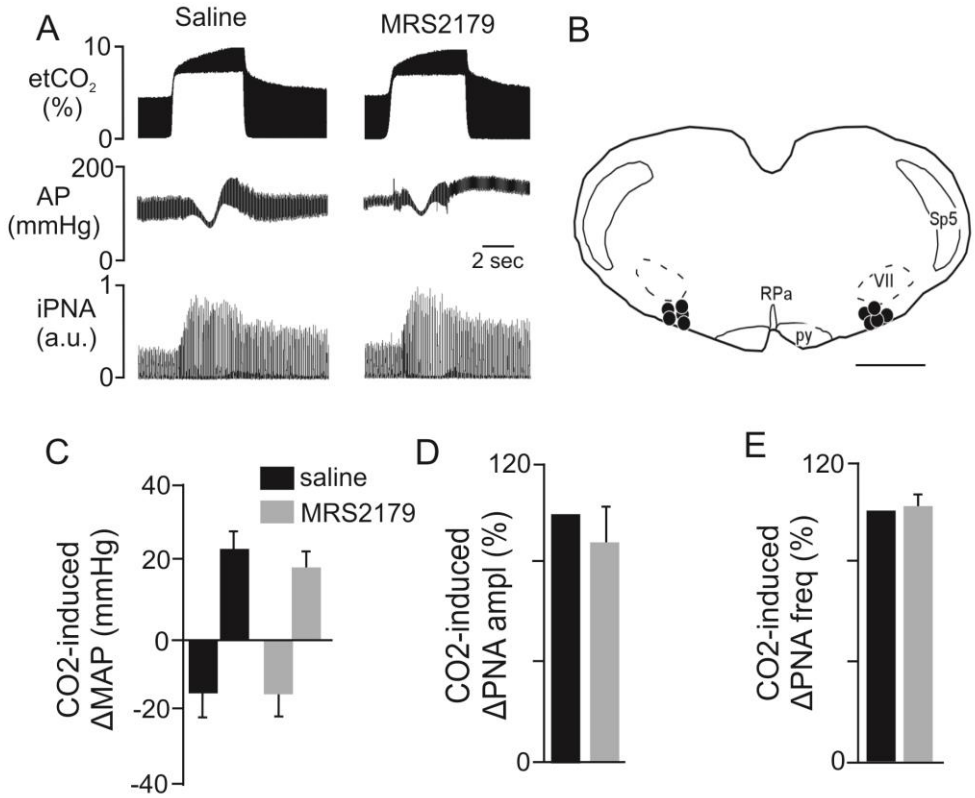

Figura 3: Injeção bilateral de MRS 2179 no núcleo retrotrapezóide não altera a resposta cardiorrespiratória produzida pela estimulação dos quimiorreceptores centrais.

A) Traçado representativo demonstrando os efeitos produzidos pelo aumento nos níveis de CO_2 de 5 to 10% na pressão arterial, e atividade do nervo frênico 10 minutos após a aplicação de injeções bilaterais de salina ou MRS 2179 (100 μM - 50 nl) no núcleo retrotrapezóide (RTN). **B)** Local das injeções no RTN. O corte coronal representa Bregma = -11.6 mm (Paxinos e Watson, 2007). Efeitos produzidos pelo aumento nos níveis de CO_2 de 5 to 10% na **C)** pressão arterial média, **D)** amplitude do nervo frênico e **E)** frequência do nervo frênicoapós a injeção de salina ou MRS 2179 no RTN. Abreviações: py, trato piramidal; VII, núcleo motor do nervo facial; RPa, núcleo pálido da rafe, Sp5, trato espinal do trigemeo ($p<0,05$); *diferente da injeção de de salina. n = 5 ratos.

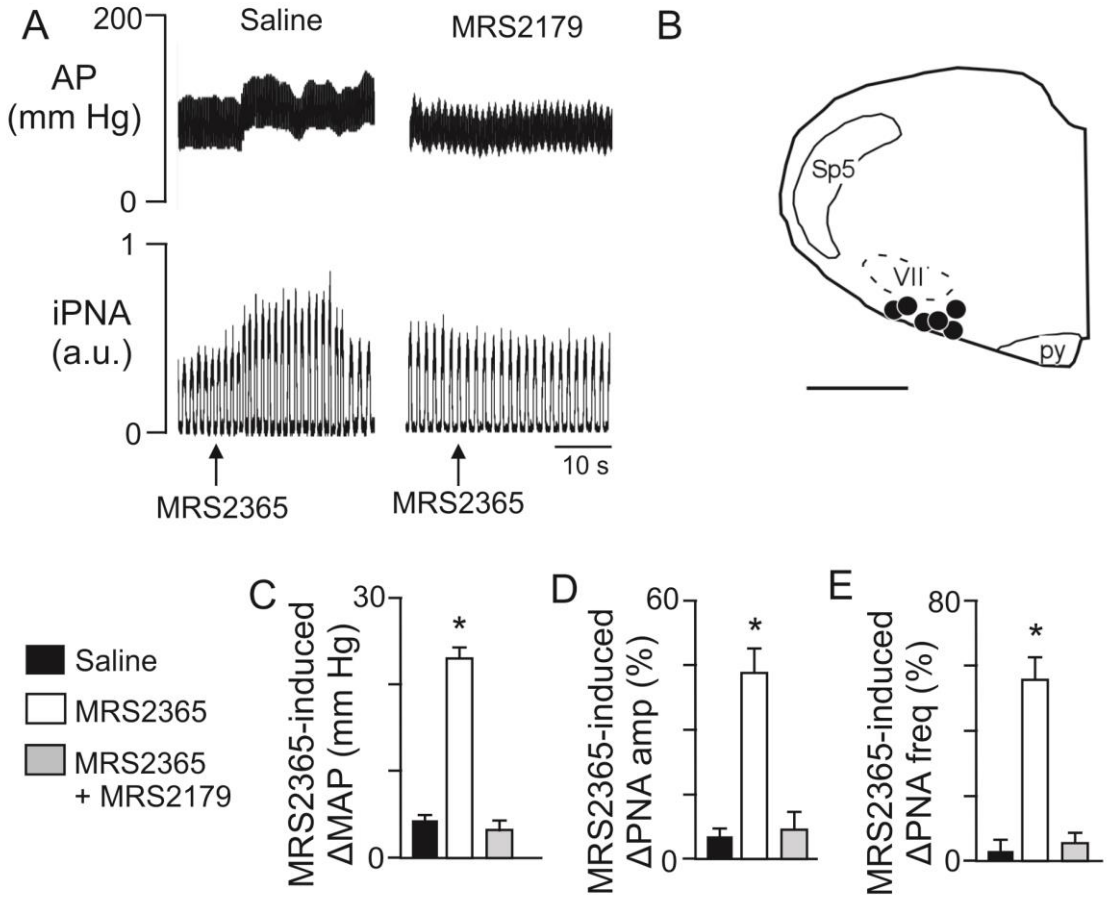

Figura 4: Efeitos cardiorrespiratórios promovidos pela injeção unilateral do agonista purinérgico P2Y1 na região do núcleo retrotrapezóide.

A) Traçado representativo demonstrando os efeitos cardiorespiratórios produzidos pela injeção unilateral do agonista purinérgico P2Y1 MRS2365 (100 μM - 50 nl) antes e após o bloqueio dos receptores P2Y1 com MRS 2179 (100 μM - 50 nl) na região do RTN. **B)** Local das injeções unilaterais de MRS2365 no RTN. O corte coronal representa Bregma = -11.6 mm (Paxinos e Watson, 2007). Escala = 1 mm.Alterações na **C)** pressão arterial média, **D)** amplitude do nervo frênico e **E)** frequência do nervo frênicoproduzidos pela injeção unilateral do agonista purinérgico P2Y1 MRS2365 antes e após o bloqueio bilateral dos receptores P2Y1 com MRS 2179 na região do RTN. * Diferente da injeção de salina + MRS2365. Abreviações: py, trato piramidal; VII, núcleo motor do nervo facial; RPa, núcleo pálido da rafe, Sp5, trato espinal do trigemeo ($p<0,05$); n = 6 ratos.

4.2 Receptores purinérgicos P2Y1 em neurônios C1 modulam a resposta respiratória, simpática e pressora à ativação dos quimiorreceptores periféricos

4.2.1 Receptores purinérgicos P2 na região do bulbo ventrolateral rostral participam das alterações cardiorrespiratórias promovidas pela ativação dos quimiorreceptores periféricos

Para avaliar o envolvimento da sinalização purinérgica na região RVL nas respostas cardiorrespiratórias e simpática promovidas pela ativação dos quimiorreceptores periféricos, o próximo protocolo experimental foi delineado para promover a exposição a uma hipóxia citotóxica, promovido pela injeção de KCN, antes e após o bloqueio de receptores purinérgicos na região do RTN.

A injeção intravenosa de KCN (40 μg/0,1 ml) promoveu um aumento na PAM (25 ± 6 mmHg, vs salina: 2 ± 2 mmHg, $p<0,01$), bem como aumento na atividade simpática (228 ± 13%, $p<0,001$) e na respiração, observados na amplitude (121 ± 4%, vs salina: 5 ± 4 %, $p<0,001$) e na frequência (118 ± 8%, vs salina: 4 ± 4%, $p<0,001$) da atividade do nervo frênico (Figs. 5A, 5C-F). Injeções bilaterais de PPADS (3 mM - 50 nl) na região do bulbo ventrolateral rostral (Fig. 4B) não promoveram alterações na pressão arterial basal (119 ± 5 mmHg, vs. salina: 121 ± 6 mmHg, $p>0,05$), na atividade simpática (101 ± 11% do controle, $p>0,05$) ou na atividade do nervo frênico basal (97 ± 4% em relação ao controle, $p>0,05$) (Fig. 5A).

Tal como a resposta a ativação de quimiorreceptores centrais, a resposta a ativação de quimiorreceptores periféricos também foi atenuada pela administração prévia de antagonista purinérgico PPADS. Assim, a administração bilateral de PPADS no bulbo ventrolateral rostral de animais anestesiados, reduziu o aumento da PAM (12 ± 3 mmHg, vs salina: 25 ± 6 mmHg, $p<0,05$), atenuou o aumento da atividade simpática (78 ± 4% vs salina 211 ± 13%, $p<0,05$) e o aumento da amplitude (84 ± 4%, vs salina: 121 ± 14%, $p<0,05$) e frequência (45 ± 7%, vs salina: 118 ± 8%, $p<0,05$) do nervo frênico em resposta a injeção endovenosa de KCN (Figs. 5A e 5C-F).

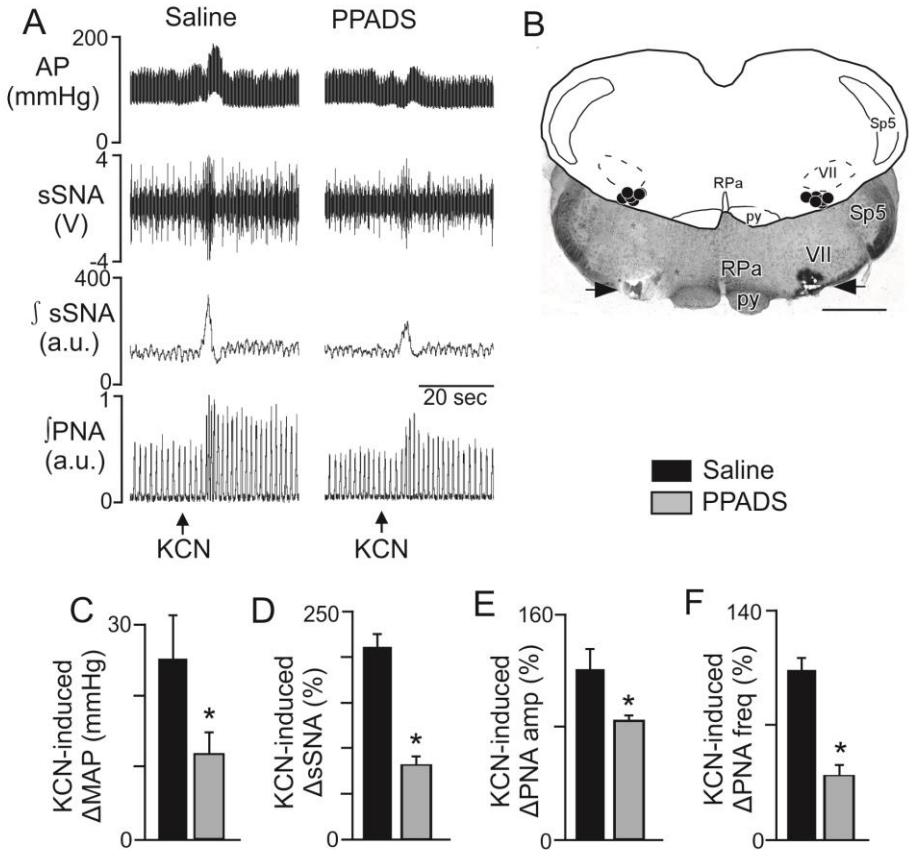

Figura 5: Efeitos cardiorrespiratórios produzidos pelas injeções bilaterais de PPADS no bulbo ventrolateral rostral nas respostas cardiovasculares e respiratórias produzidas pela estimulação dos quimiorreceptores periféricos.

A) Traçado representativo demonstrando os efeitos produzidos pela injeção endovenosa de cianeto de potássio (KCN - 40 μg/0,1 ml) sobre a pressão arterial, atividade simpática (sSNA) e atividade do nervo frênico (PNA) após 10 minutos das injeções bilaterais de salina ou PPADS (3 mM - 50 nl) no bulbo ventrolateral rostral, próximo ao núcleo retrotrapezóide. **B)** local das injeções no bulbo ventrolateral rostral. O corte coronal representa Bregma = -11.6 a -11.8 mm (Paxinos e Watson, 2007). Escala = 1 mm. Efeitos produzidos pela injeção endovenosa de KCN na **C)** pressão arterial média, **D)** atividade do ramo esplâcnico do nervo simpático, **E)** amplitude do nervo frênico e **F)** frequência do nervo frênico após a injeção de salina ou PPADS no RTN. Abreviações: py, trato piramidal; VII, núcleo motor do nervo facial; RPa, núcleo pálido da rafe, Sp5, trato espinal do trigemeo ($p<0,05$); * Diferente da injeção de salina. n = 6.

4.2.2 Participação de receptores purinérgicos P2Y1 e glutamatérgicos inotrópicos do bulbo ventrolateral rostral nas alterações cardiorrespiratórias promovidas pela ativação dos quimiorreceptores periféricos

Neste experimento testamos inicialmente se a aplicação bilateral de antagonista seletivo P2Y1 MRS 2179 no bulbo ventrolateral rostral seria capaz de atenuar as respostas cardiorrespiratórias à ativação de quimiorreceptores periféricos pela injeção endovenosa de KCN. Adicionalmente, avaliamos a resposta quimiorreceptora periférica após o bloqueio da neurotrasmissão glutamatérgica na mesma região pela administração do antagonista de receptores glutamatérgicos ionotrópicos, ácido quinurênico (Kyn). Finalmente, testamos a resposta a hipóxia citotóxica induzida na presença de ambos os antagonistas (MRS 21798 e Kyn).

A injeção bilateral do antagonista purinérgico seletivo para receptores P2Y1 (MRS 2179: 100 μM - 50 nl) no bulbo ventrolateral rostral reduziu as respostas cardiovasculares, simpáticas e respiratórias ao KCN (Fig. 6). O antagonista foi efetivo em atenuar o aumento da PAM (22 ± 3 mmHg, vs salina: 15 ± 2 mmHg, $p<0,05$), a resposta simpatoexcitatória (218 ± 17% vs salina: 126 ± 8%, $p<0,05$) e o aumento da amplitude (25 ± 6%, $p>0,05$) e frequência (77 ± 6%, $p<0,05$) do nervo frênico em resposta a injeção de KCN (Figs. 6A e 5C-F). Apenas a injeção de MRS 2179 no bulbo ventrolateral rostral não foi capaz de promover alterações na pressão arterial basal (123 ± 6 mmHg, vs. basal: 122 ± 8 mmHg, $p>0,05$) e na amplitude e frequência do nervo frênico basal (102 ± 5% em relação ao basal, $p>0,05$) (Fig. 6A-B).

Consistente com resultados prévios da literatura, a injeção bilateral de ácido quinurênico na região do bulbo ventrolateral rostral foi capaz de promover uma redução da amplitude (59 ± 5% em relação ao basal, $p<0,05$) e um aumento da frequência basal (46 ± 4% em relação ao basal, $p<0,05$) do nervo frênico, mas não alterou atividade simpática ou PAM basal. A injeção de ácido quinurênico no RTN foi capaz de reduzir as respostas cardiorrespiratórias promovidas pela ativação dos quimiorreceptores periféricos com injeções endovenosas de KCN (Fig. 6).

A combinação das injeções de MRS 2179 e kyn no bulbo ventrolateral rostral reduziu ainda mais o aumento da PAM (8 ± 2 mmHg, vs salina: 22 ± 3 mmHg, $p<0,01$), da atividade simpática (75 ± 9 mmHg, vs salina: 218 ± 17 mmHg, $p<0,001$) e da amplitude (39 ± 6%, vs salina: 124 ± 11%, $p<0,05$) do nervo frênico após a injeção de KCN. O aumento da frequência do nervo frênico mediado pela injeção de kyn no bulbo ventrolateral rostral não foi alterado

após a combinação com a injeção do MRS2179 (137 ± 6 vs: Kyn: 169 ± 169 ± 4%, p>0,05) (Fig. 6F).

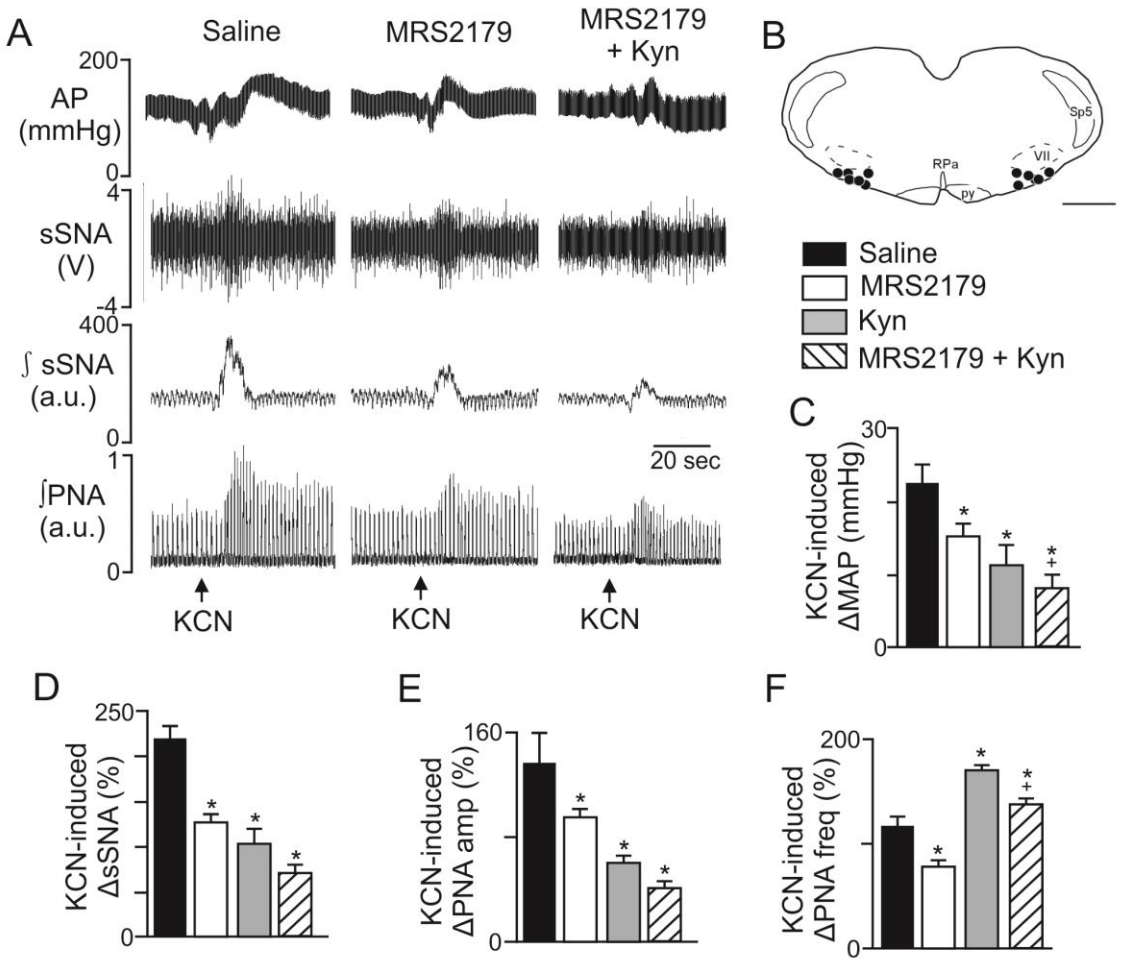

Figura 6: Efeitos produzidos pelas injeções bilaterais de MRS2179 e ácido quinurênico no bulbo venrolateral rostral nas respostas cardiovasculares, simpáticas e respiratórias produzidas pela estimulação dos quimiorreceptores periféricos.

A) Traçado representativo demonstrando os efeitos produzidos pela injeção endovenosa de cianeto de potássio (KCN - 40 μg/0,1 ml) sobre a pressão arterial, atividade simpática do ramo esplâcnico (sSNA) e atividade do nervo frênico (PNA) após 10 minutos das injeções bilaterais de salina ou MRS2179 (100 μM - 50 nl) ou ácido quinurênico (Kyn - 100 mM - 50 nl) ou a combinação de MRS2179 + ácido quinurênico na proção mais rostral do bulbo ventrolateral rostral, próximo aonúcleo retrotrapezóide. **B)** Local das injeções no bulbo ventrolateral rostral. O corte coronal representa Bregma = -11.6 a -11.8 mm (Paxinos e Watson, 2007). Escala = 1 mm. Efeitos produzidos pela injeção intravenosa de KCN na**C)** pressão arterial média, **D)** atividade simpática, **E)** amplitude do nervo frênico e **F)** frequência do nervo frênico após a injeção de salina ou MRS2179 ou Kyn ou Kyn+MRS2179 no bulbo ventrolateral rostral. Abreviações: py, trato piramidal; VII, núcleo motor do nervo facial; NA, núcleo ambíguo; RPa, núcleo pálido da rafe, Sp5, trato espinal do trigêmeo. p>0,05. Diferente da injeção de salina. + Diferente da injeção de MRS2179. n = 6.

4.2.3 Alterações cardiorrespiratórias promovidas pela ativação dosreceptores P2Y1 no bulbo ventrolateral rostral são mediadas por neurônios catecolaminérgicos C1

O registro de neurônios em fatias de tronco encefálico contendo a região do bulbo ventrolateral rostral revelou que os neurônios que respondem ao agonista MRS 2365 com aumento da frequência de potenciais de ação não são os neurônios quimiosenssíveis localizados na porção mais rostral do bulbo ventrolateral rostral, mais precisamente na região do núcleo retrotrapezóide (RTN), mas sim os neurônios pré-motores simpáticos do grupamento catecolaminérgico C1 (WENKER et al., 2013). Para confirmar se contribuição de receptores P2Y1 na região do bulbo ventrolateral rostral, nas respostas cardiorrespiratórias é mediada por neurônios do grupamento C1, realizamos injeções intravenosas de KCN (KCN - 40 μg/0,1 ml) e injeções no bulbo ventrolateral rostral de agonista P2Y1 MRS 2365 de animais que préviamente (15 dias) foram submetidos a lesão seletiva de neurônios catecoláminérgicos pela toxina saporina conjulgada com Dopamina-β-Hidroxilase (anti-DβH-SAP) ou lesão fictícia (salina).

A avaliação imunoistoquímica revelou que a aplicação de anti-DβH-SAP no bulbo ventrolateral rostral foi efetiva em promover uma redução no número dos neurônios catecolaminérgicos (imunorreativos para tirosina hidroxilase - TH) na região do bulbo ventrolateral rostral (redução de 86 ± 4%, $p<0,001$) (Figs.7A-B; 7E-F). Os animais que receberam injeções de anti-DβH-SAP não tiveram uma redução dos neurônios catecolaminérgicos em níveis mais caudais do bulbo ventrolateral (Fig. 7F) ou em outras regiões catecolaminérgicas do tronco encefálico (grupamento A2 no NTS ou grupamento A5 na ponte ventrolateral) (Figs. 7C-D), indicando que a lesão dos neurônios C1, neste modelo, foi específica para o bulbo ventrolateral rostral. Não houve diferença no número de neurônios quimiossensíveis do RTN ($Phox2b^{+}/TH^{-}$) nos animais controle ou com injeção da anti-DβH-SAP (486 ± 45, vs. controle: 472 ± 34; $p>0,05$) (Figs. 7G).

Animais com lesão de neurônios C1 exibiram redução da atividade simpatica basal (72 ± 6% do grupo controle, $p<0,05$), mas não apresentaram alterações na PAM (116 ± 4 mmHg vs controle: 119 ± 8 mmHg, $p>0,05$) ou da atividade do nervo frênico (98 ± 2 do grupo controle, $p>0,05$) (Fig. 8A).

O aumento da atividade simpática (2 ± 3% vs controle: 131 ± 7%, $p<0,001$) e da PAM (1 ± 3 mmHg vs. controle: 25 ± 2 mmHg, $p<0,01$) produzidos pela injeção unilateral de MRS 2365 no bulbo ventrolateral rostral (100 μm - 50 nl) foi bloqueado nos animais que tiveram a

lesão do grupamento C1 (Figs. 8H-I). As respostas de aumento da atividade respiratória (amplitude e frequência do nervo frênico) produzidas pela injeção do MRS 2365 foram atenuadas nos animais com lesão do grupamento C1 (Figs. 8J-K). As injeções de MRS2365 foram localizadas na região rostral do bulbo ventrolateral, região que contem a grande população de neurônios pré-motores simpáticos (Figs. 8B-C)

Nesses mesmos animais, lesão do grupamento C1, observamos também uma redução do aumento da atividade simpática (53 ± 6% vs controle: 299 ± 11%, $p<0,01$) e da PAM (2 ± 2 mmHg vs. controle:18 ± 6 mmHg, $p<0,05$) produzidos pela injeção unilateral de MRS 2365 no bulbo ventrolateral rostral (Figs. 8D-E). O aumento da amplitude e frequência do nervo frênico produzido pela injeção endovenosa de KCN não foi alterada nos animais com lesão do grupamento C1 (Figs. 8F-G).

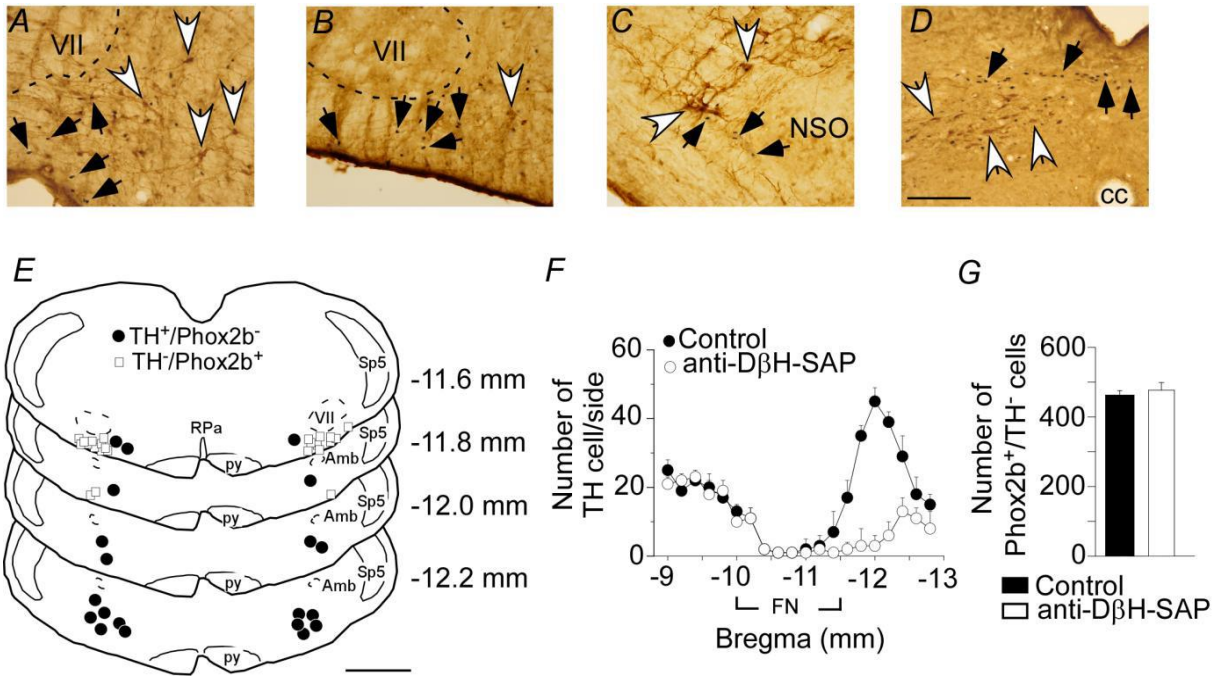

Figura 7: Lesão seletiva de neurônios catecoláminérgicos C1 promovida pela injeção da toxina saporina conjulgada com Dopamina-β-Hidroxilase

A-B) Fotomicrografias ao nível da região catecolaminérgica C1 (bregma -11.80 mm) ilustrando a imunorreatividade para tirosina hidroxilase (TH) a integridade dos neurônios catecolaminérgicos em animais do grupo controle (A) e a depleção de neurônios catecoláminérgicos do grupamento C1 em animais que receberam injeções de anti-DβH-SAP (B). **C-D)** Fotomicrográfias da região A5 (C) e da região A2 (D) ilustrando a imunorreatividade para TH a integridade dos neurônios catecolaminérgicos em outros grupamentos catecoláminérgicos do tronco encefálico em animais que receberam injeções de anti-DβH-SAP no grupamento C1. **E)** Figura esquemática ilustrando a distribuição de neurônios TH^+ (●) e de neurônios $phox2b^+$(□) na região do bulbo ventrolateral rostral em diversos planos coronais (bregma de -11.6 a 12.2 mm). **F)** número de neurônios TH^+ por secção ao longo do bregma em animais controle e lesados. A contagem foi feita em secções de 40 µm e intervalo de 240 µm entre elas. **G)** Número neurônios $Phox2b^+$ em animais submetidos a lesão com anti-DβH-SAPou controle. Abreviações: VII, núcleo motor do facial; Amb, núcleo ambiguo; cc, canal central; IO, núcleo olivar inferior; NSO, núcleo olivar superior; py, trato piramidal; Sp5, trato espinal do trigêmeo; setas pretas representam neurônios $Phox2b^+$ e setas brancas representam neurônios TH^+.

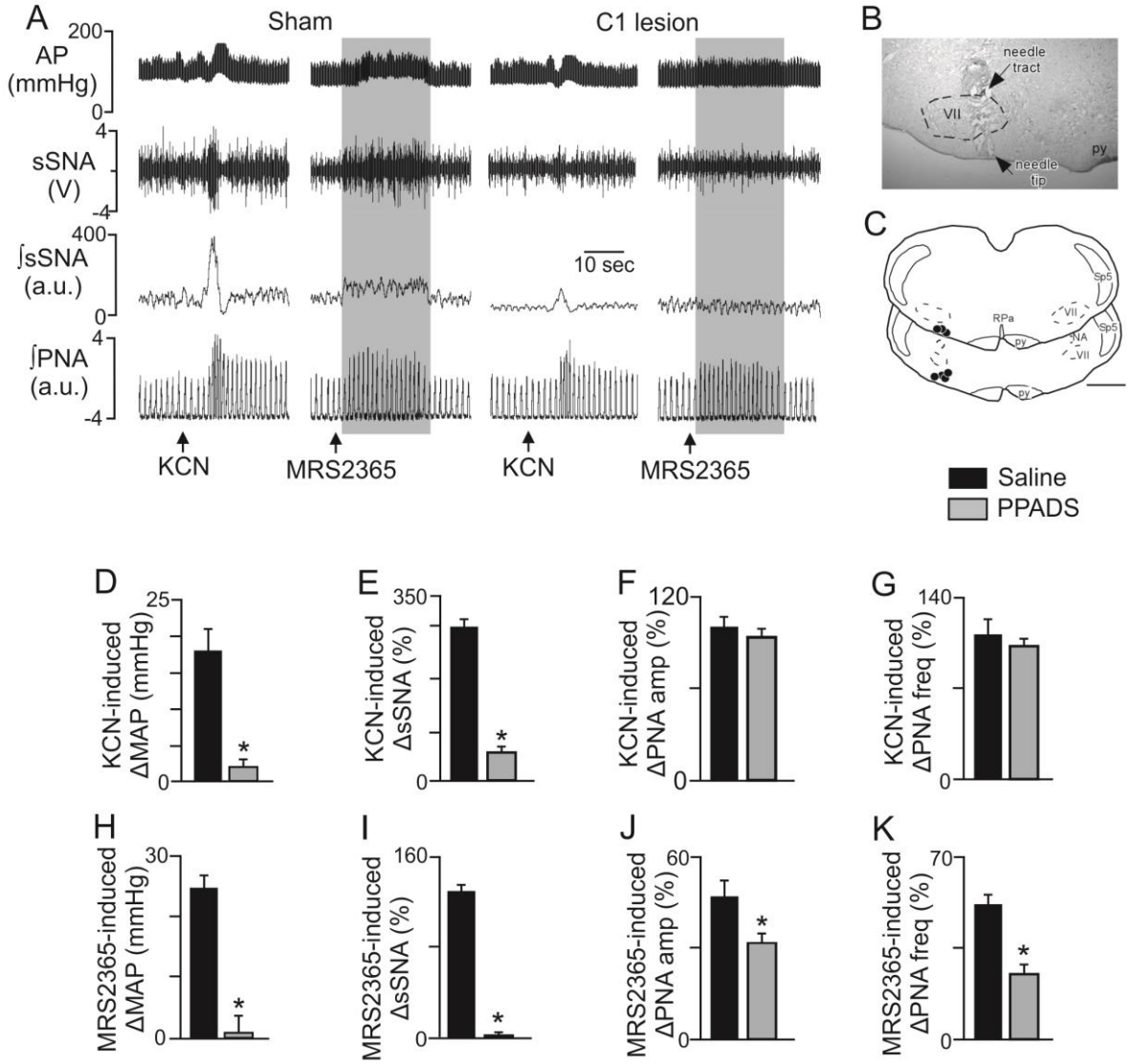

Figura 8: Alterações cardiorrespiratórias promovidas pela ativaçãodos receptores P2Y1 no bulbo ventrolateral rostral em animais submetidos a lesão do grupamento catecolaminérgicos C1

A) Traçado representativo demonstrando os efeitos produzidos pela lesão fictícia (controle) ou seletiva dos neurônios C1 na região do bulbo ventrolateral rostral sobre a PAM, atividade simpática (sSNA) e atividade do nervo frênico (PNA) em uma situação basal, em resposta a estimulação dos quimiorreceptores periféricosoua injeção unilateral do agonista P2Y1 MRS 2365 **B)** Fotomicrografia e **C)** Figura esquemática demonstrando o local de injeções de MRS 2365 na região do bulbo ventrolateral rostral. **D-G)** Efeitos produzidos pela injeção endovenosa de cianeto de potássio (KCN - 40 μg/0,1 ml) sobre a pressão arterial, atividade simpática (sSNA) e atividade do nervo frênico (PNA) em animais controle ou com depleção de neurônios C1. **H-K)** Efeitos produzidos pela injeçãode agonista P2Y1 MRS2365 no bulbo ventrolateral rostralsobre a pressão arterial, atividade simpática (sSNA) e atividade do nervo frênico (PNA) em animais controle ou com depleção de neurônios C1.Abreviações: py, pirâmides; VII, núcleo motor do nervo facial; NA, núcleo ambíguo; RPa, núcleo pálido da rafe, Sp5, trato espinal do trigêmeo, n = 6/grupo.*diferente do grupo controle.

4.2.4 Varicosidades presentes no bulbo ventroalteral rostral oriundos do NTS comissural expressam VNUT e VGLUT2

O NTScom é o primeiro núcleo no SNC que recebe informações provenientes dos quimiorreceptores periféricos (FINLEY; KATZ, 1992), e envia projeções excitatórias (glutamatérgicas) para o bulbo ventrolateral rostral (AICHER et al., 1996; TAKAKURA et al., 2006). Nossos achados sugerem que a sinalização purinérgica poderia ser co-liberada com glutamato no bulbo ventrolateral rostral durante a ativação de quimiorreceptores periféricos, como já sugerido em outras áreas (LARSSON et al., 2012). Na tentativa de confirmar nossa hipótese, realizamos injeções do traçador anterogrado Biotina Dextrana Amina (BDA) no NTScom (Figs. 9A-B) combinado a avaliação imunoistoquímica para determinar se os terminais imunorreativos para BDA no bulbo ventrolateral rostral (portanto, provenientes do NTScom) expressam o transportador vesicular de nucleotídeos (VNUT) e se são co-localizados com vesiculas glutamatérgicas (VGLUT2) (Figs. 9C-G).

Nossas injeções de BDA foram centradas no NTScom, como ilustrado na fotomicrografia 9A e pela figura esquemática 9B, representando a distribuição do centro das injeções (n = 4). Como esperado, varicosidades imunorreativas para BDA (Alexa488 - verde) foram encontradas na região do bulbo ventrolateral rostral (Fig. 9C), e foram colocalizadas para VGLUT-2 (Fig. 8D, CY-3 - vermelho) e VNUT (Fig. 9E, azul). Em um total de 126 terminais contados em 31 secções contendo o bulbo ventrolateral rostral (n = 4), 72 delas (representando 57%) foram possitivas também para VGLUT2 e VNUT (Figs. 9F e G). Também foram encontrados 25 terminais colocalizados apenas com VGLUT2 (20%) e 8 apenas com VNUT (6%), além de 21 terminais (17%) onde não foi possivel identificar a expressão de ambos (Fig.9G).

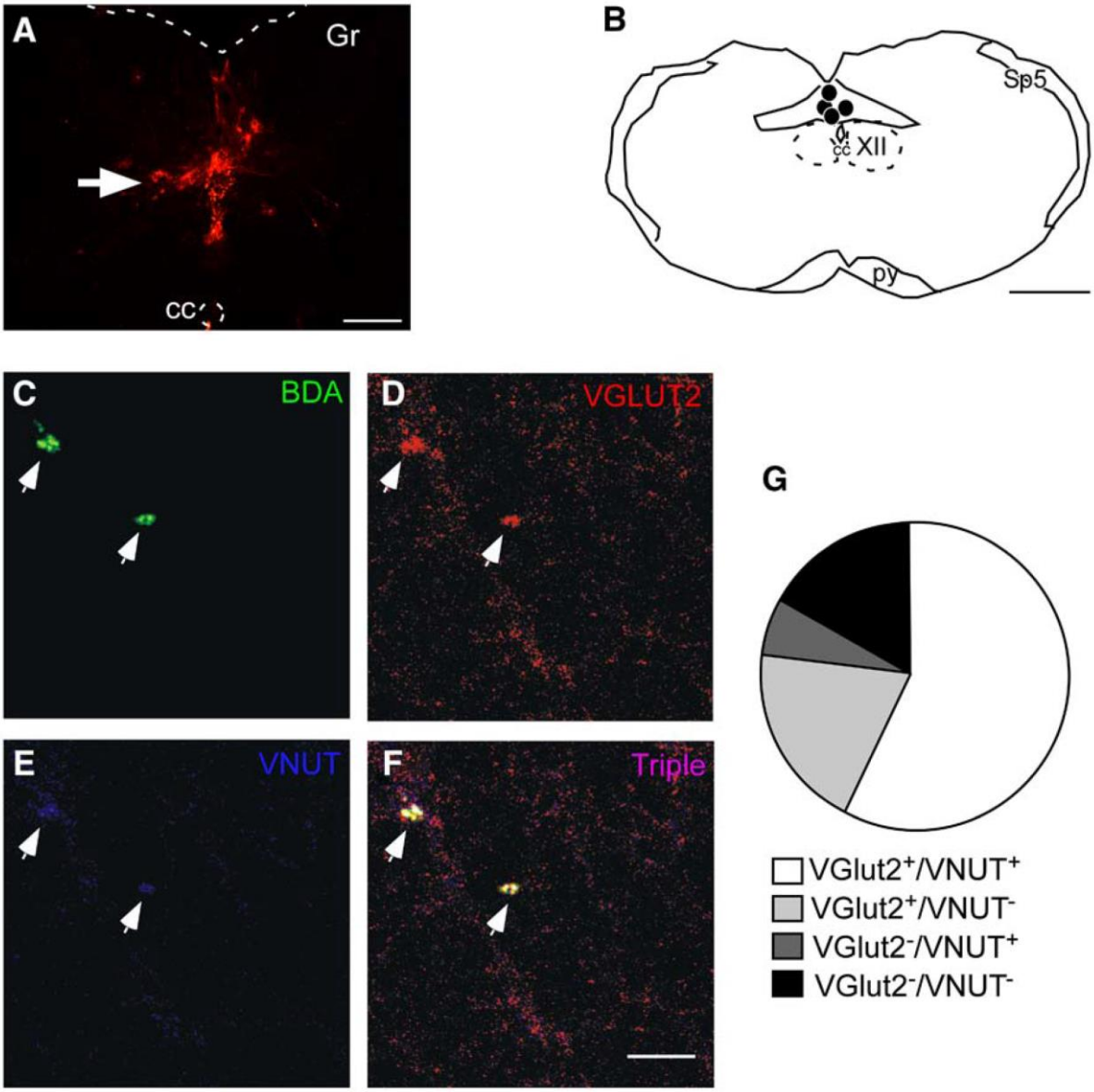

Figura 9: Projeções do NTScom para o bulbo ventrolateral rostral são imunorreativas para VGLUT2 e VNUT

A) Fotomicrografia ilustrando a injeção de traçador anterógrado Biotina Dextran Amina (BDA - 10 KD) na região do NTScom. **B)** Figura esquemática ilustrando a distribuição das injeções de BDA no NTScom. **C)** Terminais positivos para BDA na região RVL na região do RTN (Alexa488 - verde). **D)** Fotomicrografia demonstrando a imunorreatividade de VGLUT2 (CY-3 - vermelho). **E)** Fotomicrografia demonstrando a imunorreatividade de VNUT (azul). **F)** Sobrepossição das 3 imagens anteriores demonstrando que VGLUT e VNUT são expressos em varicosidades imunorreativas para BDA. **G)** Sumarização dos dados demonstrando a porcentagem de terminais imunorreativos para BDA que apresentaram colocalização para VGLUT E VNUT (branco), que expressam VGLUT2 (cinza claro), que expressam VNUT (cinza escuro) ou que não apresentaram imunorreatividade para VGLUT ou VNUT (preto). Abreviaçoes: XII, núcleo hipoglosso; cc, canal central; Gr, Núcleo Grácil; py, Trato piramidal; Sp5, trato espinal do trigêmeo.

4.3 Participação da sinalização purinérgica no controle da atividade cardiorrespiratória no núcleo retrotrapezóide, núcleo do trato solitário e região do núcleo pálido da rafe /região parapiramidal: possível envolvimento na quimiossensíbilidade central

4.3.1 Aplicação de ATP no NTScom promove alterações cardiorrespiratórias, mas o bloqueio de receptores P2 não altera as respostas cardiorrespiratórias promovidas pela ativação do quimiorreflexo central

Experimentos *in vitro* revelaram que embora os neurônios quimiossensíveis da porção comissural do núcleo do trato solitário (NTScom) sejam altamente responsivos para aplicação local de ATP, o bloqueio de receptores P2 não foi efetivo em alterar a responsividade desses neurônios ao CO_2/H^+ (SOBRINHO et al., 2014). Para avaliar se a sinalização purinérgica no NTScom participa das respostas cardiorrespiratórias devido a ativação do quimiorreflexo central e confirmar se ausência de respostas *in vitro* é devido a resposta funcional e não devido a perturbações provocadas pela preparação, realizamos injeções de ATP (10 mM - 50 nl) no NTScom de animais anestesiados antes e após a injeção de antagonistas P2, PPADS (3 mM -50 nl). Avaliamos também se a injeção de PPADS seria capaz de atenuar as alterações cardiorrespiratórias promovidas pela ativação do quimiorreflexo central.

A injeção de ATP no NTScom de animais anestesiados, vagotomizados, e ventilados artificialmente produziu um aumento da PAM (21 ± 4 mmHg vs salina: 3 ± 1 mmHg, $p<0,05$ e um aumento da atividade do nervo frênico em amplitude (46 ± 6% vs salina: 6 ± 4%; $p<0,01$) e frequência (103 ± 11% vs salina: 5 ± 5%, $p<0,0001$) (Figs. 10A; 10C-E).

A injeção do antagonista de receptores purinérgicos P2 PPADS no NTScom não produziu alterações mensuraveis nos parâmetros cardiorrespiratórios basais avaliados, mas foi efetiva em bloquear a resposta para aplicação de ATP (Figs 10A, C-E). Apesar da responsividade do NTScom, mediada por P2, para aplicação exógena de ATP (Fig.10), a injeção do antagonista purinérgico PPADS no NTScom não foi efetiva em alterar as respostas cardiorrespiratórias ao CO_2. O aumento da atividade do nervo frênico em amplitude (103 ± 9% vs. salina: 99 ± 13%, $p>0,05$), frequência (107 ± 6% vs salina: 105 ± 4%, $p>0,05$) e o aumento da PAM (18 ± 7 mmHg vs salina: 21 ± 4 mmHg, $p> 0,05$) não foram alterados após a injeção de PPADS no NTScom durante a ativação dos quimiorreceptores centrais (aumento dos níveis de CO_2 de 3-4% para 9-10%) (Figs. 10A, 10C-E).

As injeções no NTScom foram realizadas na linha média, 400 µm caudais ao calamus scriptorius e 500 µm ventrais a supericie dorsal, região descrita na literatura que contém a grande população de neurônios quimiossensíveis localizados no NTScom (Figs. 10B e 11B).

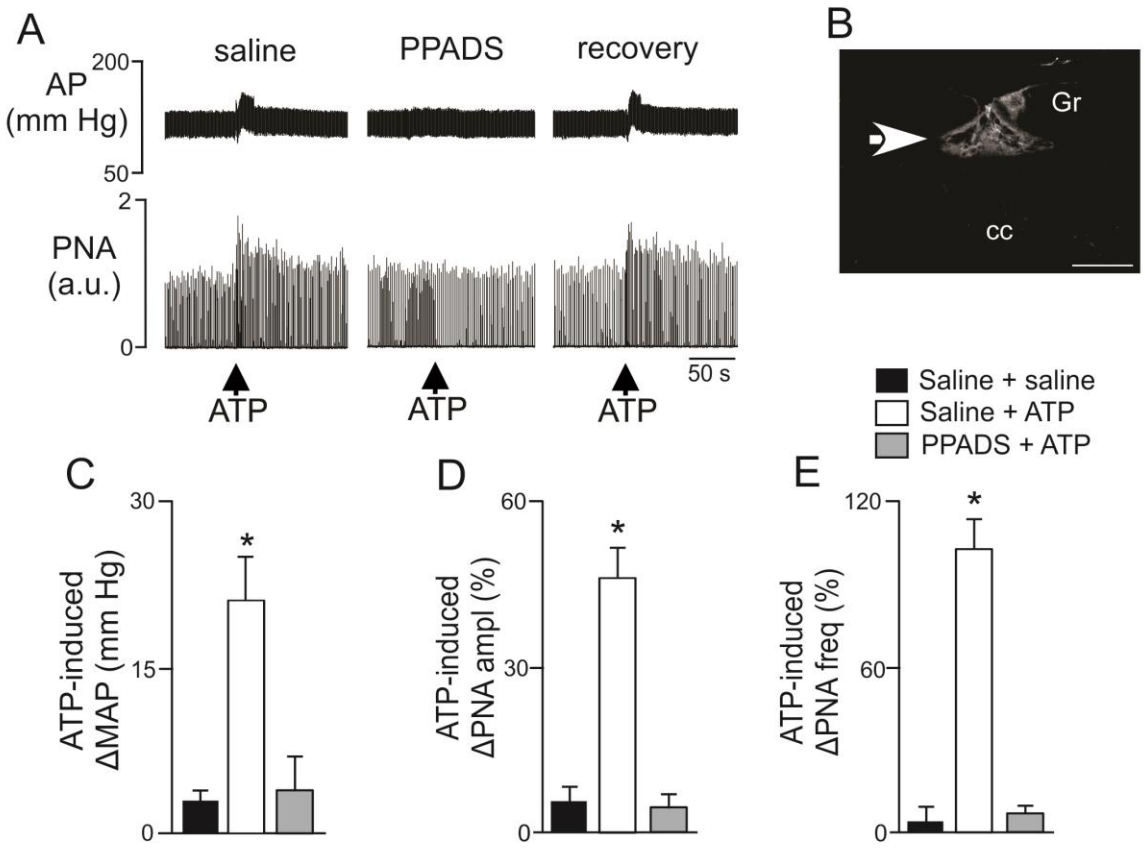

Figura 10: Aplicação de ATP no NTScomissural promove alteraçõescardiorrespiratórias mediadas por receptores P2

A) Traçado representativo demonstrando os efeitos produzidos pela injeção de ATP (10 mM - 50 nl) na porção comissural do núcleo do trato solitário sobre a pressão arterial média (PAM) e atividade do nervo frênico (PNA) antes e após a aplicação de antagonista P2 PPADS (3 mM - 50 nl) no NTScom. **B)** Fotomicrografia ilustrando o local tipico de injeções de ATP ou PPADS no NTScom. Efeitos produzidos pela aplicação de ATP antes e após a aplicação de PPADS no NTScom sobre a **C)** PAM; **D)** amplitude de PNA e **E)** frequência de PNA. Abreviações: cc, canal central; Gr, núcleo grácil; n = 5 ratos. *diferente de salina ($p<0,05$).

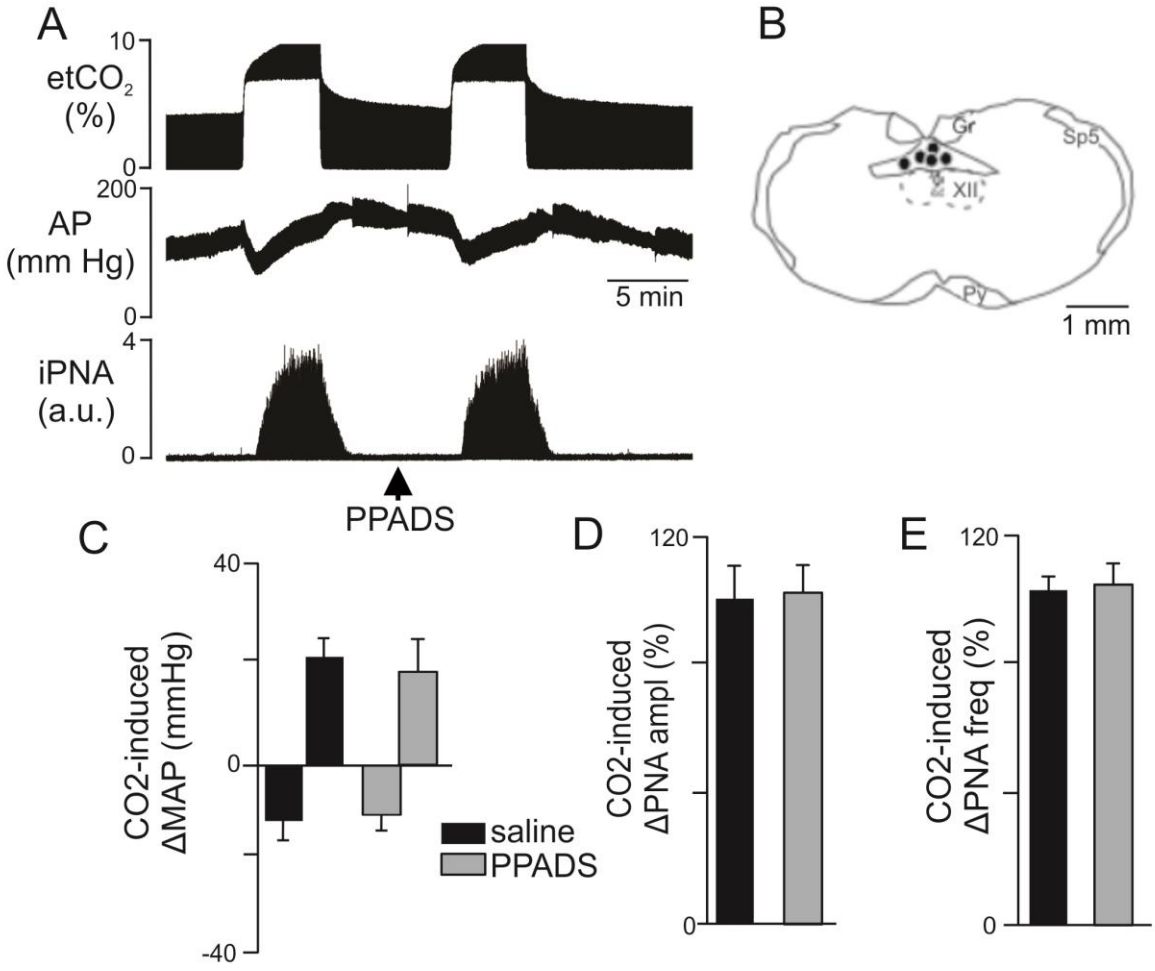

Figura 11: Injeção de PPADS na porção comissural do núcleo do trato solitárionão altera a resposta cardiorrespiratória produzida pela estimulação dos quimiorreceptores centrais.

A) Traçado Representativo demonstrando os efeitos produzidos pelo aumento nos níveis de CO_2 de 3-4 para 9-10% na pressão arterial, e na atividade do nervo frênico (PNA) 10 minutos após a aplicação de salina ou PPADS (3 mM - 50 nl) no NTScom. **B)** figura esquemática ilustrando o local das injeções no NTScom. Escala = 1 mm. Efeitos produzidos pelo aumento nos níveis de CO_2 de 3-4 para 9-10% na **C)** pressão arterial média (PAM), **D)** amplitude do nervo frênico e **E)** frequência do nervo frênico após a injeção de salina ou PPADS no NTScom.Abreviações: XII, núcleo hipoglosso; cc, canal central; Gr, núcleo grácil; py, trato piramidal; Sp5, núcleo espinal do trigêmeo. n = 6 ratos.

4.3.2 Aplicação de ATP na RPa/PPy não promove alterações cardiorrespiratórias e o bloqueio de receptores P2 também não foi efetivo em alterar as respostas cardiorrespiratórias promovidas pela ativação do quimiorreflexo central

Bem como o NTScom, a literatura delimita que a região que engloba o núcleo pálido da rafe (RPa) e a região parapiramidal (PPy), localizada na linha média do bulbo, no mesmo bregma onde encontramos os neurônios quimiossensíveis do RTN, também possui neurônios que atuam como quimiorreceptores centrais (NATTIE; LI, 2001; RICHERSON, 2004). Através de experimentos realizados *in vitro*, demonstramos que os neurônios serotoninérgicos da região da RPa/PPy não são sensíveis a aplicação exógena de ATP (SOBRINHO et al., 2014). Assim, na tentativa de elucidar se a sinalização purinérgica no RPa/PPy participa na resposta quimiorreceptora central e confirmar se ausência de respostas *in vitro* seja devido a resposta funcional e não devido a perturbações provocadas pela preparação, realizamos injeções de ATP (10 mM - 50 nl) na região da RPa/PPy de animais anestesiados, e avaliamos a resposta para hipercapnia antes e após a aplicação local de antagonistas P2, PPADS (3 mM - 50 nl).

Injeções de ATP (10 mM - 50 nl) direcionadas a região RPa/PPy (n = 7) foram centradas 1mm da linha média, no mesmo nível coronal do RTN (Fig. 11B). Injeções de ATP nesta região não produziram alterações nos parâmetros cardiorrespiratórios avaliados (Figs. 12A, 12C-E).

A injeção bilateral do antagonista PPADS na região RPa/PPy (n = 5) não afetou a atividade cardiorrespiratória basal e foi ineficaz em atenuar as respostas cardiorrespiratórias promovidas pela hipercapnia (aumento dos níveis de CO_2 de 3-4% para 9-10%). Mais especificamente, o aumento da atividade do nervo frênico em amplitude (106 ± 11% vs salina: 105 ± 14%, $p>0,05$) e frequência (102 ± 8% vs salina: 101 ± 5%, $p>0,05$) e o aumento da PAM (22 ± 4 mmHg vs salina: 22 ± 6 mmHg, $p>0,05$) promovido pela hipercapnia não foi diferente após o bloqueio dos receptores purinérgicos na região da RPa/PPy (Figs. 13A, 13 C-E).

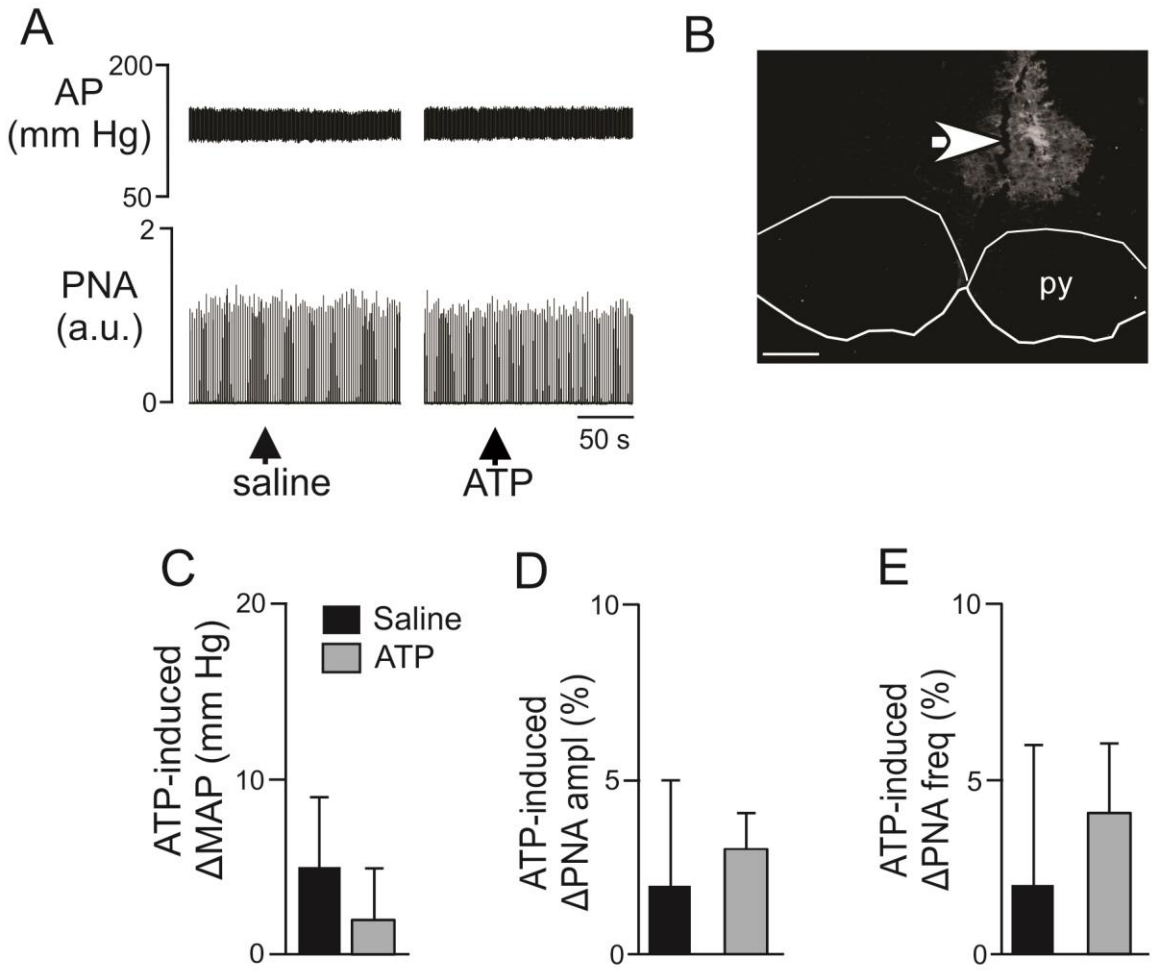

Figura 12: Aplicação de ATP na região da RPa/PPy não produz alterações cardiorrespiratórias

A) Traçado representativo demonstrando que a injeção de ATP (10 mM - 50 nl) na região do núcleo pálido da rafe (RPa)/região parapiramidal (PPy) não produz alterações sobre a pressão arterial média (PAM) e na atividade do nervo frênico (PNA). **B)** Fotomicrografia ilustrando o local típico de injeções de ATP no RPa/PPy. **C-E)** Sumarização dos dados demonstrando que a aplicação de ATP no RPA/PPy não altera a PAM ou PNA. Abreviação: py, trato piramidal. n = 7 ratos.

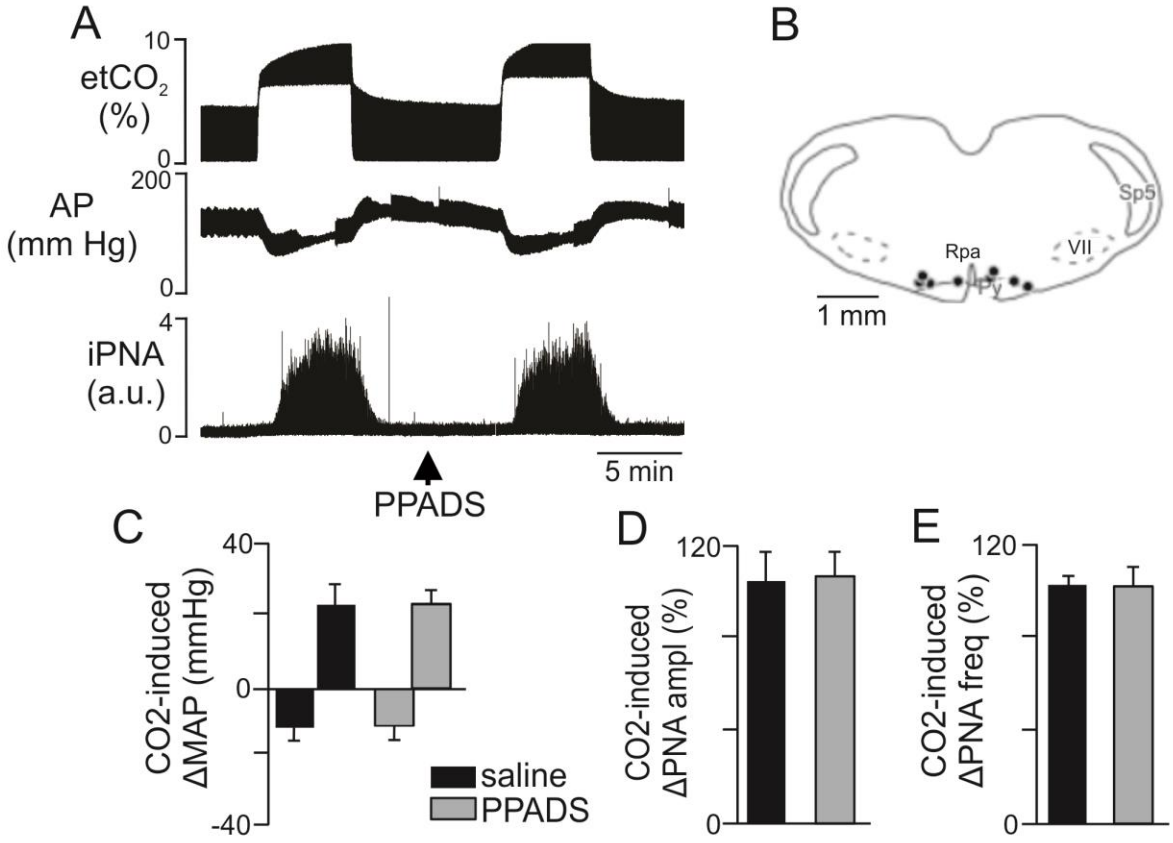

Figura 13: Injeção bilteral de PPADS na região RPa/PPy não altera a resposta cardiorrespiratória produzida pela estimulação dos quimiorreceptores centrais.

A) Traçado representativo demonstrando os efeitos produzidos pelo aumento nos níveis de CO_2 de 3-4 para 9-10% na pressão arterial, e na atividade do nervo frênico (PNA) 10 minutos após a aplicação bilateral de salina ou PPADS (3 mM - 50 nl) no RPa/PPy. **B)** figura esquemática ilustrando o local das injeções no RPa/PPy. Escala = 1 mm. Efeitos produzidos pelo aumento nos níveis de CO_2 de 3-4 para 9-10% na **C)** pressão arterial média (PAM), **D)** na amplitude do nervo frênico e **E)** na frequência do nervo frênico após a injeção de salina ou PPADS no Rpa/PPy. Abreviações: py, pirâmides; VII, núcleo motor do nervo facial; RPa, núcleo pálido da rafe. n = 5 ratos.

4.3.3 Aplicação de ATP no RTN promove alterações cardiorrespiratórias mediadas pelos de receptores P2

Injeções unilaterais de ATP (10 mM -50 nl, n = 6) no RTN foram realizadas como um controle positivo dos demias dados apresentados acima. Como esperado, a aplicação de ATP no RTN de animais anestesiados, vagotomizados e ventilados artificialmente produziu aumento na PAM (14 ± 3 mmHg vs salina: 2 ± 3 mmHg, $p<0,05$) e da atividade do nervo frênico, observada pelo aumento da amplitude (26 ± 5%, $p<0,05$) e frequência (21 ± 4%, $p<0,05$) (Figs. 14A, 14C-E). As alterações cardiorrespiratórias promovidas pela injeção de ATP no RTN foram bloqueadas pela aplicação prévia do antagonista de receptores purinérgicos P2, PPADS (3 mM - 50 nl) no RTN (Figs 14A, 14C-E). As injeções de ATP e PPADS no RTN estavam localizadas na região que contém a maior população de neurônios quimiossensíveis do bulbo ventrolateral rostral e já descrito em resultados anteriores nessa tese (Figs. 14B).

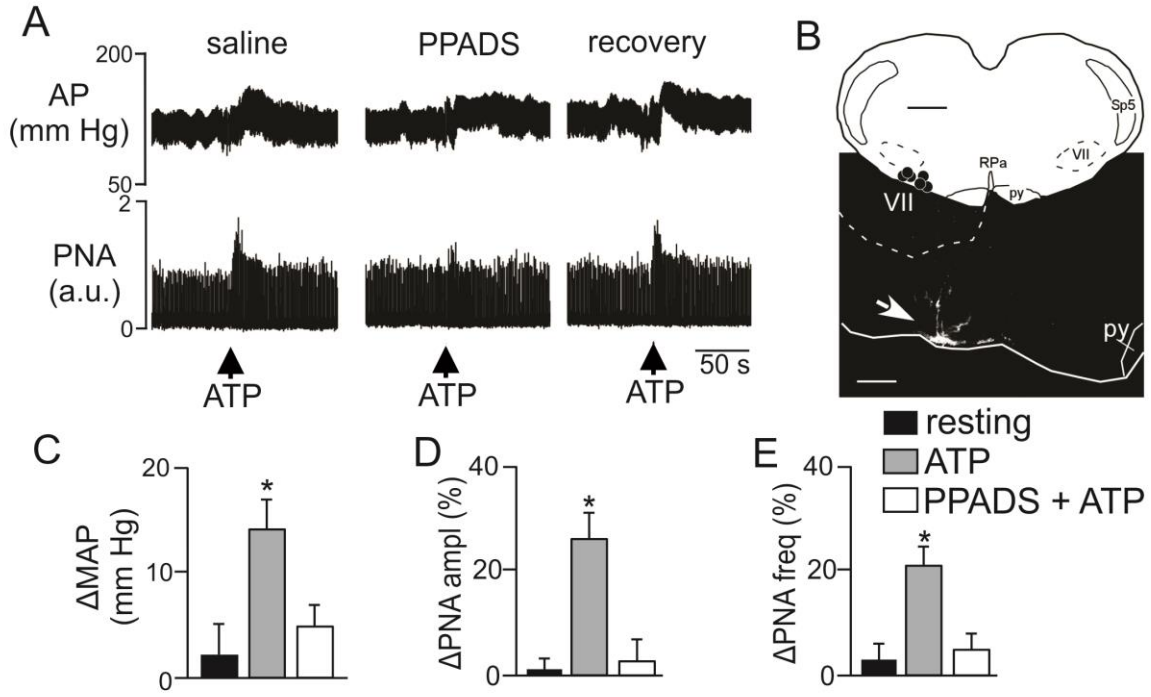

Figure 14: Aplicação de ATP no RTN promove alterações cardiorrespiratórias

A) Traçado representativo demonstrando os efeitos produzidos pela injeção de ATP (10 mM - 50 nl) no núcleo retrotrapezoide sobre a pressão arterial média (PAM) e atividade do nervo frênico (PNA) antes e após a aplicação de antagonista P2 PPADS (3 mM - 50 nl) no RTN. **B)** Fotomicrografia ilustrando o local tipico de injeções de ATP ou PPADS no RTN. Efeitos produzidos pela aplicação de ATP antes e após a aplicação de PPADS no RTN sobre a **C)** PAM, **D)** a amplitude do PNA e **E)** a frequência do PNA. Abreviações: py, pirâmides; VII, núcleo motor do nervo facial. Escala = 1 mm., n= 6 ratos. * diferente de salina ($p<0,05$).

4.4 Efeitos cardiorrespiratórios produzidos pela injeção de fluorocitrato em diferentes áreas encefálicas com caracteristicas quimiossensíveis: interação entre as células gliais e neurônios e a possível participação da sinalização purinérgica

4.4.1 Injeção unilateral de fluorocitrato no núcleo retrotrapezóide promove alterações respiratórias

Os resultados acima corroboram com trabalhos prévios da literatura que sugerem que a sinalização purinérgica na região do bulbo ventrolateral rostral (RTN ou grupamento C1) contribui para respostas de ativação dos quimiorreceptores centrais e periféricos (GOURINE et al., 2005a, 2010; HUCKSTEPP et al., 2010a; SOBRINHO et al., 2014; WENKER et al., 2012, 2013). Os mesmos trabalhos que oferecem suporte ao presente estudo, oferecem ainda evidências que os astrócitos localizados na região do RTN podem atuar como células quimiossensíveis e promover aumento na ventilação durante a ativação do quimioreflexo central, seja pela liberação ATP na região do RTN (GOURINE et al., 2010; HUCKSTEPP et al., 2010; WENKER et al., 2010, 2012) ou pela regulação do equilíbrio dos íons K^+ou H^+ entre os meios extracelular e intracelular (ERLICHMAN; LI; NATTIE, 1998; HOLLERAN; BABBIE; ERLICHMAN, 2001).

Assim, o protocolo experimental a seguir foi realizado para avaliar as alterações cardiorrespiratórias mediadas pela ativação das células da glia (astrócitos) na região do RTN em animais anestesiados. Para este fim, utilizamos a gliotoxina fluorocitrato (1 mM - 50 nl) que foi injetada unilateralmente no RTN de animais sob duas condições experimentais: a) em uma situação de hiperóxia (quando os animais estão respirando 100% de O_2 e apresentam nível de CO_2 expirado < 5%) e b) durante uma situação de hipercapnia (através da adição de CO_2 a ventilação e os animais apresentam nível de CO_2 no ar expirado > 5%).

A figura 15 está ilustrando o local típico das injeções unilaterais de FCt no núcleo retrotrapezóide (RTN). O centro das nossas injeções estavam localizadas abaixo do núcleo motor do facial, região que contém grande concentração de neurônios quimiossensíveis do RTN como já demonstrado anteriormente (ilustrado pela imunorreatividade para o fator de transcrição Phox2b - núcleos marcados em vermelho - Cy3).

Em uma situação em que os animais anestesiados são mantidos abaixo do limiar apneico (< 5% CO_2 expirado), o nervo frênico apresenta baixa atividade e frequentemente é possível observar seu silenciamento (Fig. 16A). A injeção unilateral de FCt no RTN, sob essas condições (abaixo do limiar apneico), promove em alguns minutos (Δ = 5-7 min) a

geração de atividade respiratória, mais precisamente observamos um aumento da amplitude do nervo frênico (42 ± 5%, $p<0,01$) (Fig. 16A). Sob as mesmas condições experimentais, mas em animais que já apresentam atividade de nervo frênico (CO_2 expirado > 5%, acima do limiar apneico), a injeção unilateral de FCt no RTN promove em alguns minutos (Δ = 5-7 min) o aumento na amplitude (54±5% do controle, $p<0,05$) e na frequência respiratória (38 ± 2% do controle, $p<0,05$), (Figs. 16A-C). A injeção de FCt na região do RTN não promoveu alterações significantes na PAM (115 ± 6, vs. salina: 118 ± 7 mmHg, $p>0,05$) (Fig. 16A).

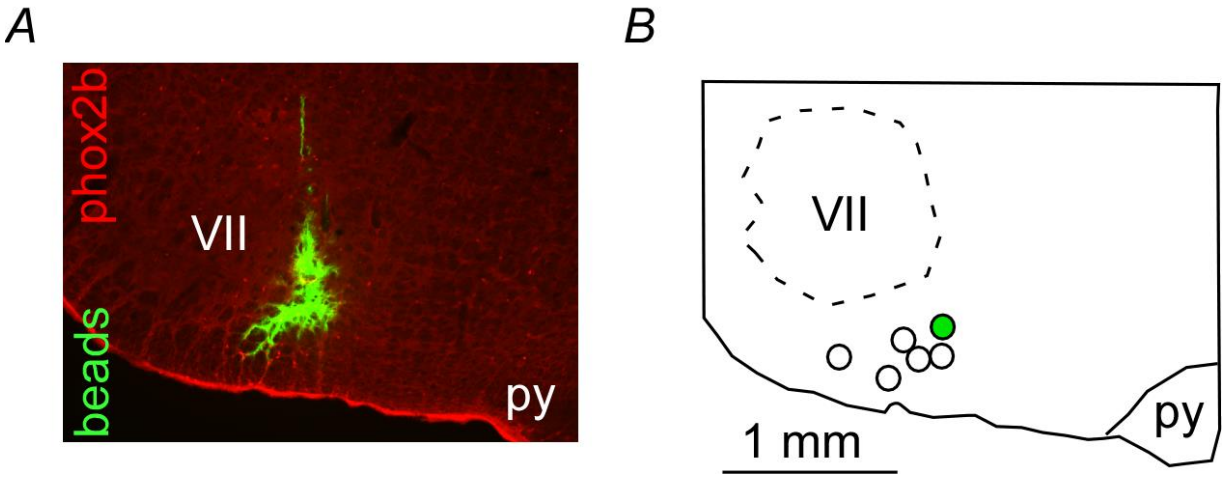

Figura 15: Local das injeções de fluorocitrato no núcleo retrotrapezóide.

A) Fotomicrografia de uma injeção típica na região do núcleo retrotrapezóide (RTN). Em vermelho estão representados os neurônios Phox2b^{+} (núcleos marcados - Cy3) na região do RTN e em verde (Alexa 488) as microesferas de látex, ilustrando o local da injeção. **B)** Ilustração mostrando o local das injeções no RTN (n = 6). Abreviações: py, pirâmides; VII, núcleo motor do nervo facial. Escala = 1 mm

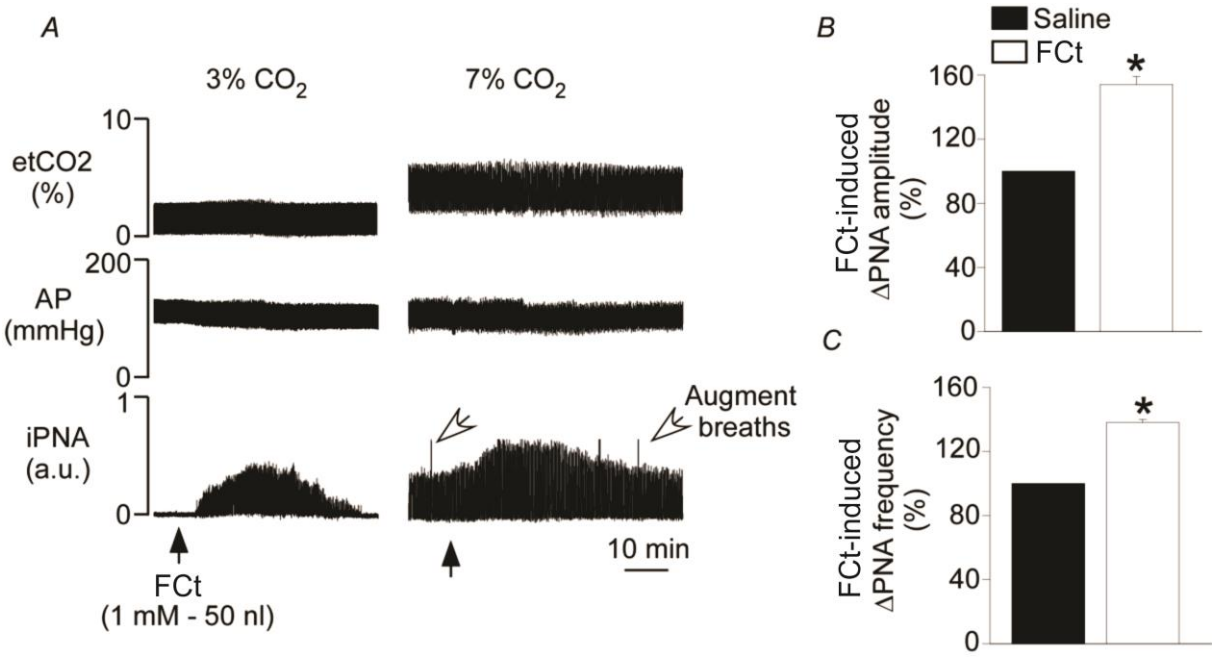

Figura 16: Efeitos cardiorrespiratórios promovidos pela injeção unilateral de fluorocitrato na região do núcleo retrotrapezóide.

A) Traçado representativo demonstrando os efeitos cardiorespiratórios produzidos pela injeção unilateral de fluorocitrato (1 mM - 50 nl) na região do RTN em animais mantidos abaixo do limiar apneico (CO_2 < 5%) e acima do limiar apneico (CO_2 > 5%), respectivamente. **B)** alterações na amplitude do nervo frênico produzidos pela injeção unilateral de fluorocitrato na região do RTN. **C)** alterações na frequência do nervo frênico produzidos pela injeção unilateral de fluorocitrato na região do RTN. * Diferente da injeção de salina ($p<0,05$); n = 6 ratos. As setas no traçado representam respirações com amplitude aumentadaapresentados pelo animal. Esses eventos são comuns em preparações de animais anestesiados com uretano.

4.4.2 Efeitos cardiorrespiratórios promovidos pela injeção unilateral de fluorocitrato no núcleo do trato solitário comissural ou na região do núcleo pálido da rafe/região parapiramidal

Para avaliar se astrócitos de outras regiões do SNC descritas com características quimiossensiveis podem influenciar a atividade cardiorrespiratória, injeções unilaterais de FCt (1 mM - 50 nl) foram realizadas no NTScom ou na região RPa/PPy de animais anestesiados (Figs. 17D e H).

Não foram observadas alterações significantes na PAM, na amplitude e frequência do nervo frênico após injeções de FCt no NTScom ou na RPa/PPy (Figs. 17A-C e 17E-G).

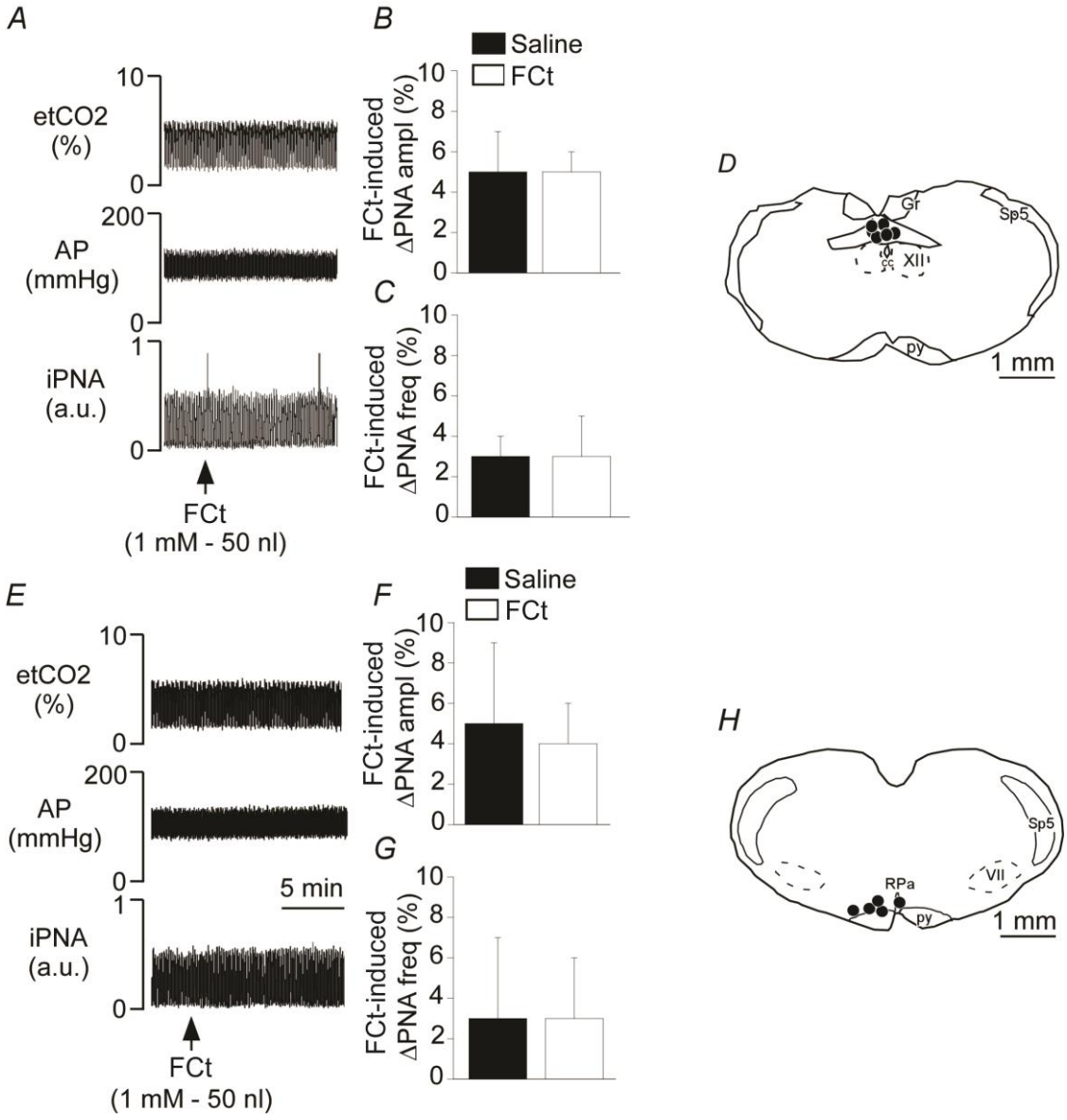

Figura 17: Efeitos cardiorrespiratórios promovidos pela injeção de fluorocitrato na região do núcleo do trato solitário comissural ou no núcleo pálido da rafe/região parapiramidal.

A) Traçado representativo demonstrando que a injeção unilateral de fluorocitrato (FCt) na região do NTScomem animais anestesiados não produz alterações cardiorrespiratórias. **B-C)** Sumarização dos dados demonstrando que a aplicação de FCt no NTScom não altera amplitude e frequência do nervo frênico (PNA). **D)** Figura esquemática ilustrando o local das injeções de FCt no NTScom. O corte coronal representa Bregma = -14.3 mm (Paxinos e Watson, 2007). n = 6. Escala = 1 mm. **E)** Traçado representativo demonstrando que a injeção unilateral de FCt na região RPa/PPy em animais anestesiados não produz alterações cardiorrespiratórias. **F-G)** Sumarização dos dados demonstrando que a aplicação de FCt no RPa/PPy não altera amplitude e frequência do PNA. **H)** Figura esquemática ilustrando o local das injeções de FCt no RPa/PPy.O corte coronal representa Bregma = -11.6 mm (Paxinos e Watson, 2007). n = 5. Escala = 1 mm.

4.4.2 Participação de receptores purinérgicos P2 nas alterações respiratórias promovidos pela injeção unilateral de fluorocitrato no núcleo retrotrapezóide

O aumento na ventilação promovido pela injeção unilateral de FCt (1 mM - 50 nl) na região do RTN já havia sido demonstrado préviamente (ERLICHMAN; LI; NATTIE, 1998; HOLLERAN; BABBIE; ERLICHMAN, 2001). Os autores dos referidos trabalhos demonstraram que a injeção de FCt em doses sub-tóxicas na região do RTN promove alterações no pH extracelular mediado por seus efeitos em astrócitos. No entanto, Wenker e colaboradores (WENKER et al., 2010) demonstraram em preparações *in vitro* que o FCt é capaz de promover despolarização da membrana de astrócitos do RTN e aumento da atividade de neurônios quimiossensíveis vizinhos por um mecanismo dependende da sinalização purinérgica. Diante do exposto acima, o próximo protocolo experimental foi realizado para tentar avaliar a participação de receptores purinérgicos P2 na região do RTN nas respostas respiratórias promovidas pela manipulação farmacológica das células da glia.

Injeções bilaterais de PPADS (3 mM - 50 nl) no RTN não promoveram alterações na PAM (113 ± 7, vs. salina: 115 ± 4 mmHg, $p>0,05$) e na amplitude (55 ± 3 vs. salina: 58 ± 6%, $p>0,05$) e frequência do nervo frênico (37 ± 4 vs. salina: 42 ± 4 %, $p>0,05$) basais (Fig. 18A). A injeção bilateral de PPADS no RTN foi efetiva em reduzir as respostas de aumento de amplitude (37 ± 3%, vs FCt: 54 ± 5%, $p<0,05$) e frequência (30 ± 1%, vs FCt: 38 ± 2%, $p<0,05$) do nervo frênico induzido pela injeção unilateral de FCt no RTN (Figs. 18A-C). Importante retratar que a injeção bilateral de PPADS no RTN não modificou o tempo (Δ = 4,2 ± 3, vs FCt: 5,5 ± 2 min, $p>0,05$) que o FCt é capaz de aumentar a atividade respiratória.

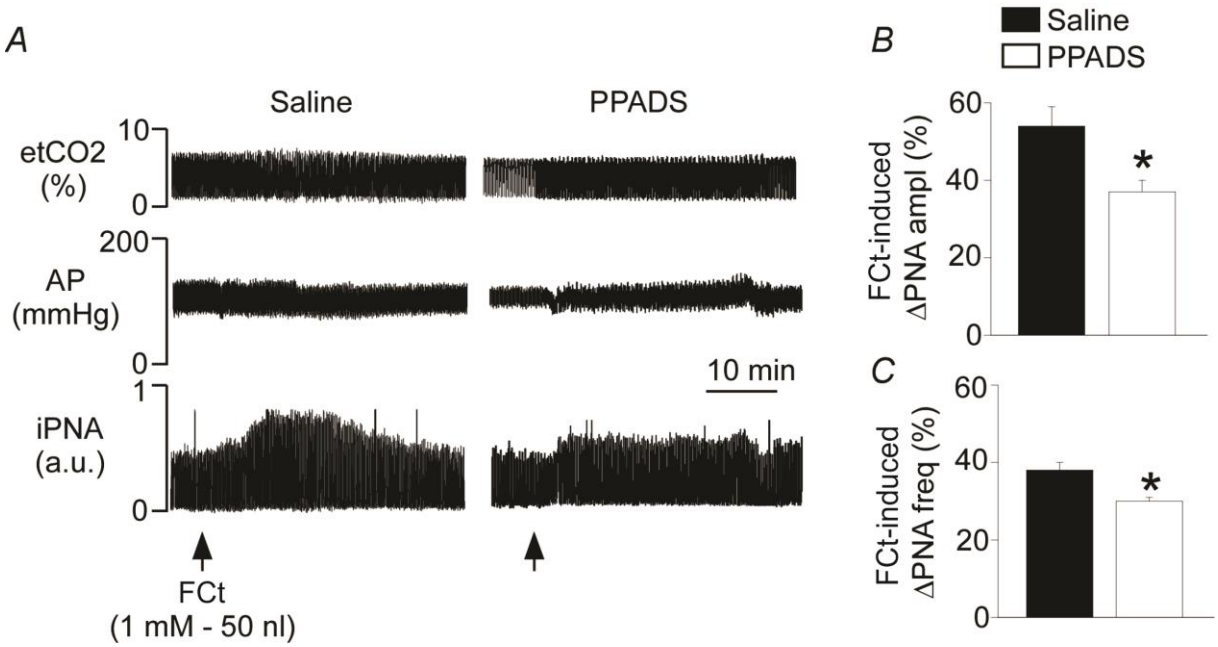

Figura 18: Injeção bilateral de PPADS no núcleo retrotrapezóide atenua a resposta respiratória produzida pela injeção unilateral de fluorocitrato no núcleo retrotrapezóide

A) Traçado representativo demonstrando efeitos respiratórios produzidos pela injeção unilateral de fluorocitrato (FCt: 1 mM - 50 nl) na região do RTN antes e após a injeção bilateral do antagonista de receptores P2 PPADS (3 mM - 50 nl). Alterações na **B)** amplitude e **C)** frequência do nervo frênico produzidos pela injeção unilateral de FCt na região do RTN antes e após a injeção bilateral de PPADS no RTN. * Diferente da injeção de salina ($p<0,05$); n = 6 ratos.

4.4.3 Receptores purinérgicos P2Y1 não estão envolvidos nas alterações respiratórias promovidos pela injeção unilateral de fluorocitrato no núcleo retrotrapezóide

Injeções bilaterais de MRS2179 (100 µM - 50 nl) no RTN não foi capaz de promover alterações na PAM (118 ± 4, vs. salina: 117 ± 5 mmHg, p>0,05) e na amplitude (58 ± 5 vs. salina: 54 ± 6 %, p>0,05) e frequência do nervo frênico (42 ± 2 vs. salina: 43 ± 5 %, p>0,05) basais (Fig. 19A). A injeção bilateral de MRS 2179 no RTN também não foi eficaz em atenuar as respostas de aumento de amplitude (58 ± 6%, vs FCt: 55 ± 5%, p>0,05) e frequência (41 ± 3%, vs FCt: 39 ± 4%, p>0,05) do nervo frênico induzido pela injeção unilateral de FCt no RTN (Figs. 19A-C).

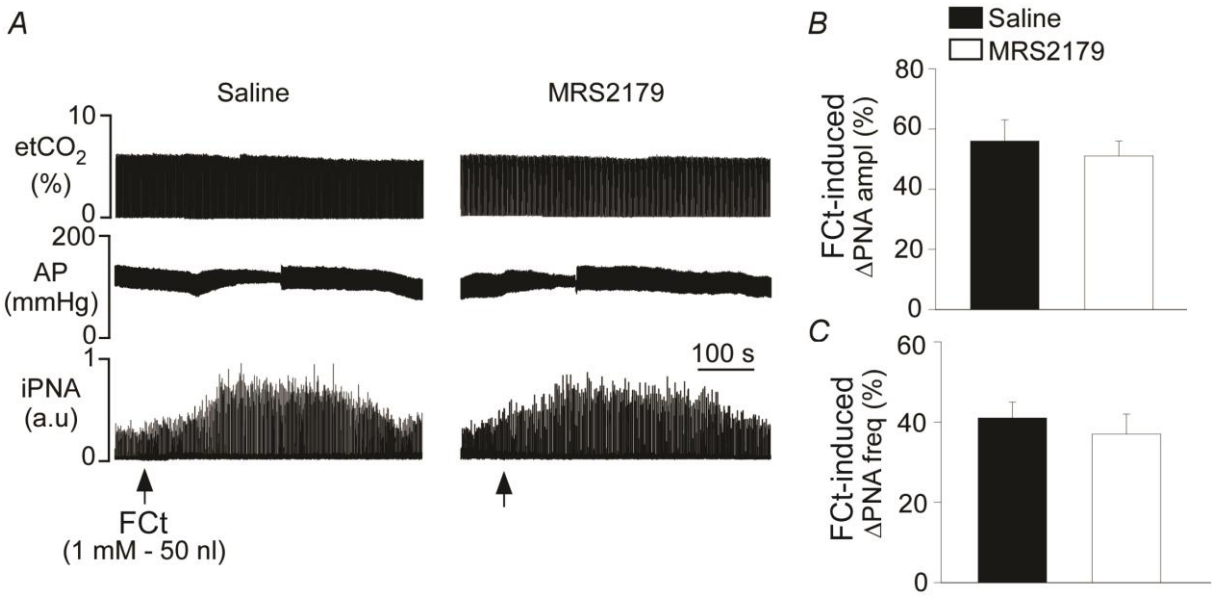

Figura 19: Injeção bilateral de MRS 2179 no núcleo retrotrapezóide não altera a resposta respiratória produzida pela injeção unilateral de fluorocitrato no núcleo retrotrapezóide.

A) Traçado representativo demonstrando que a injeção bilateral de antagonista P2Y1 MRS 2179 (100 μM - 50 nl) não altera a resposta respiratória promovida injeção unilateral de fluorocitrato (FCt: 1 mM - 50 nl) na região do RTN. Sumário dos dados demonstrando alterações na **B)** amplitude e **C)** frequência do nervo frênico produzidos pela injeção unilateral de FCt na região do RTN antes e após a injeção bilateral de MRS 2179 no RTN. n = 6 ratos.

5 DISCUSSÃO

“Falhar não é uma opção” (FELDMAN; DEL NEGRO; GRAY, 2012). Esta frase retrata muito bem a importância crucial do sistema respiratório na manutenção da homeostase, cuja função primordial é a manutenção dos níveis de gases no organismo com o objetivo de evitar alterações no equilíbrio ácido-base. Neste sentido, os quimiorreceptores (centrais e periféricos) frequentemente são os principais alvos de grupos de pesquisa que buscam desvendar os mecanismos dessa complexa organização que controla o sistema cardiorrespiratório.

O presente trabalho consiste em uma série de 4 estudos, onde buscamos elucidar os mecanismos de participação da sinalização purinérgica no bulbo ventrolateral rostral (o RTN e o grupamento catecolaminérgico C1) na geração ou modulação de respostas cardiorrespiratórias durante a ativação de quimiorreceptores centrais e periféricos, bem como a participação de células da glia neste processo. Os resultados obtidos são embasados por evidências prévias da literatura e a nossa contribuição demonstrou que:

1- Alterações cardiorrespiratórias induzidas pela ativação de quimiorreceptores centrais (hipercapnia) são atenuadas pelo bloqueio de receptores purinérgicos P2 com PPADS no RTN. Possivelmente, essas respostas devem ser mediadas via receptores purinérgicos P2X, uma vez que o bloqueio seletivo de receptores purinérgicos P2Y1 no RTN não foi capaz de alterar as respostas cardiorrespiratórias promovidas pela ativação dos quimiorreceptores centrais. No entanto, a aplicação de agonista P2Y1 nesta região promoveu aumento da atividade cardiorrespiratória (WENKER et al., 2012);

2: Por outro lado, observamos que ambos antagonistas purinérgicos, não seletivo (PPADS) e seletivo para receptores P2Y1 (MRS2179) injetados bilateralmente no bulbo ventrolateral rostral, foram efetivos em atenuar as respostas cardiorrespiratórias e simpato-excitatórias produzidas pela estimulação dos quimiorreceptores periféricos (hipóxia citotóxica). O bloqueio associado entre os receptores purinérgicos e os receptores glutamatérgicos ionotrópicos foi capaz de reduzir ainda mais o aumento da PAM, atividade simpática e respiratória promovidas pela estimulação dos quimiorreceptores periféricos, sugerindo uma possível interação glutamatérgica e purinérgica na região do bulbo ventrolateral rostral durante a ativação dos quimiorreceptores periféricos. Tratando-se de sinalização purinérgica no bulbo ventrolateral rostral, experimentos realizados em animais com depleção de neurônios catecoláminérgicos do grupamento C1 foi possível demontrar que a resposta simpato-excitatória e pressórica parece ser mediada pelos receptores P2Y1, localizados exclusivamente nos neurônios C1. Além disso, observamos que terminais axonais no bulbo vetrolateral rostral provenientes do NTScom expressam VGLUT2 e VNUT,

indicando que estes terminais liberam glutamato e, possivelmente purinas (WENKER et al., 2013);

3- Muito embora o NTScom e o RTN, mas não a região RPa/PPy, responda a aplicação exógena de ATP com aumento na atividade cardiorrespiratória, a aplicação de antagonista purinérgico P2 não seletivo no NTScom ou RPa/PPy não foi capaz de alterar a resposta de ativação dos quimiorreceptores centrais. Essas respostas foram diferentes do observado previamente com injeções dos antagonistas P2 no RTN, sugerindo que a sinalização purinérgica parece não modular a quimiossensibilidade ao CO_2/pH nestes grupamentos neurais (SOBRINHO et al., 2014).

4- A injeção da gliotoxina FCt no RTN, de animais anestesiados e vagotomizados, foi capaz de gerar ou aumentar o padrão inspiratório na atividade do nervo frênico, mediada em grande parte pela sinalização purinérgica nos receptores P2X, uma vez que a aplicação de antagonista não seletivo P2, PPADS, mas não o antagonista seletivo P2Y1, MRS2179, atenuou as respostas respiratórias promovidas pela aplicação de FCt. A injeção de FCt no NTScom ou na RPa/PPy não foi capaz de promover alterações cardiorrespiratórias (SOBRINHO et al., 2015, manuscrito em preparação).

5.1 Contribuição da sinalização purinérgica na região do RTN no controle cardiorrespiratório promovido pela ativação dos quimiorreceptores centrais

O RTN representa o principal grupamento neural com propriedades quimiossensíveis (CONNELLY; ELLENBERGER; FELDMAN, 1990; GUYENET; BAYLISS, 2015; GUYENET; STORNETTA; BAYLISS, 2010; MULKEY et al., 2004; NATTIE; GDOVIN; LI, 1993). Este reconhecimento pode ser atribuído ao fato que, dentre os núcleos encefálicos que apresentam propriedades quimiossensíveis, este é o grupamento que melhor preenche todos os critérios para ser considerado uma área quimiorreceptora. As unidades funcionais presentes na região do RTN apresentam: a) sensibilidade intrínseca ao CO_2/H^+ *in vivo* e *in vitro*, b) fenótipo neuroquímico excitatório (VGLUT2$^+$), c) os neurônios dessa região projetam-se para o centro gerador do padrão respiratório, d) recebem influências dos quimiorreeptores periféricos e dos receptores de distensão pulmonar, e) geneticamente expressam o fator de transcrição Phox2b e mutações seletivas desse fator de transcrição, na região do RTN, promove uma síndrome extremamente rara chamada de Síndrome da Hipoventilação Congênita Central, f) as células gliais da região do RTN parecem apresentar também uma propriedade quimiossensível, e essa propriedade parece ser exclusiva dessa

região do encéfalo (GOURINE et al., 2005a; GUYENET; BAYLISS, 2015; HUCKSTEPP et al., 2010a; MOREIRA et al., 2015; MULKEY et al., 2004; SOBRINHO et al., 2014; WENKER et al., 2012).

Variações nas concentrações de CO_2/H^+ promovem oscilações proporcionais na atividade de neurônios do RTN *in vitro* (MULKEY et al., 2004; KUMAR et al., 2015) e *in vivo* (MULKEY et al., 2004; STORNETTA et al., 2006; TAKAKURA et al., 2006; WENKER et al., 2012), nesta última, acompanhada pelo reflexo de aumento da atividade ventilatória. Por exemplo, em animais anestesiados e ventilados artificialmente, tanto os neurônios do RTN quanto a atividade do nervo frênico foram silentes abaixo de 4.1% de CO_2 (também observado no presente estudo, Fig. 15), apresentaram aumento gradual na frequência de disparos e da ventilação de acordo com o aumento da $P_{CO2,}$ e atingem em média 14 potenciais de ação por segundo quando estes animais exibem 10% de CO_2 no ar expirado (MULKEY et al., 2004). Lesões ou inativação seletiva do RTN resultam em redução na ventilação basal, aumento na P_{CO2} em animais não anestesiados (NATTIE; LI, 2002b; TAKAKURA et al., 2013, 2014) e atenuação da resposta ventilatória durante a hipercapnia em animais anestesiados e não anestesiados (NATTIE; LI, 2002b; TAKAKURA et al., 2008, 2014).

O mecanismo pelo qual os neurônios do RTN detectam as variações de CO_2/H^+ finalmente começa a ser elucidado. Recentemente, foi identificado que receptores de membrana do tipo GPR4, ligados a proteína G, atuando em sinergia com um canal de K^+ sensível a alcalinização (TASK2), são expressos em neurônios do RTN e parecem ser os mecanismos principais pelo qual os neurônios do RTN percebem variações de CO_2 e pH (KUMAR et al., 2015). Resumidamente, camundongos com depleção ou silenciamento genético das proteínas GPR4 exibem aumentos consideráveis no número de episódios espontâneos de apneia e redução da resposta ventilatória para hipercapnia. Esses animais apresentam ainda redução no número de neurônios que expressam Fos após exposição ao CO_2, indicando redução no número de neurônios ativados pela hipercapnia, bem como redução em número de neurônios que respondem a acidificação *in vitro,* e da sensibilidade ao pH dos neurônios que ainda apresentam resposta residual à acidose. As alterações neurais e respiratórias são revertidas após a reinserção do receptor GPR4, enquanto que a depleção simultânea de GPR4 e TASK2 no mesmo animal promoveu alta taxa de mortalidade e a eliminação quase completa das respostas ventilatórias e neuronais a hipercapnia nos animais sobreviventes (KUMAR et al., 2015).

Somando-se a propriedade intrínseca, os neurônios do RTN respondem a múltiplos neurotransmissores e neuromoduladores. Muitos estudos tem demonstrado que o RTN é altamente responsivo a substância P, glutamato, GABA, ATP, orexina, serotonina (5-HT), acetilcolina, noradrenalina e outros (ERLICHMAN; LEITER; GOURINE, 2010; FUKUDA; LOESCHCKE, 1979; GOURINE et al., 2010; LAZARENKO et al., 2011; MULKEY et al., 2004; TAKAKURA et al., 2008; WENKER et al., 2012). Apesar da importante influência desses neurotransmissores/neuromoduladores na atividade dos neurônios do RTN, a sensibilidade ao CO_2 dessas células quimiossensíveis tende a não ser alterada na presença de seus antagonistas.

No presente estudo, demonstramos que alterações cardiorrespiratórias induzidas pela hipercapnia são atenuadas pela aplicação prévia de antagonista de receptores purinérgicos P2, PPADS, no RTN. Em nossos experimentos, a hipercapnia produziu inicialmente uma hipotensão, que seria resultado de um efeito direto do CO_2 nos vasos sanguíneos e também, possivelmente, na contratilidade cardíaca. Após a hipotensão, a pressão arterial começa a voltar aos valores normais e, no fim do episódio da hipercapnia, a pressão arterial apresenta uma tendência de aumento. Assume-se que esse aumento seria resultante de um aumento na atividade simpática eferente, quando o efeito local do CO_2 nos vasos sanguíneos já tenha sido removido. A hipercapnia induziu também um aumento na PAM e na atividade respiratória (MOREIRA et al., 2006; MULKEY et al., 2004; WENKER et al., 2012). Em nossos resultados, constatamos que o bloqueio de receptores purinérgicos P2 pela injeção bilateral de PPADS na região RTN reduziu o aumento na PAM e na atividade respiratória (amplitude e frequência) em animais anestesiados submetidos à hipercapnia, sem alterar os valores basais (WENKER et al., 2012).

Embora não tenhamos testado toda a diversidade de receptores P2Y, ou mesmo utilizado um antagonista seletivo para receptores P2X, sugerimos que o receptor atuante neste contexto pertence ao grupo de receptores purinérgicos ionotrópicos (P2X1-7), uma vez que a aplicação de antagonista seletivo de receptores purinérgicos P2Y1 no RTN não foi capaz de alterar as respostas cardiorrespiratórias promovidas pela ativação dos quimiorreceptores centrais. Não podemos descartar a participação de outros receptores P2Y, pois resultados recentes mostraram que os receptores P2Y são os receptores purinérgicos mais abundantes na região do Complexo de Pré-Botzinger e medeiam as respostas respiratórias produzidas pelo ATP (FUNK, 2013; LORIER et al., 2007, 2008).

O aumento nos níveis de CO_2 promove a liberação de ATP na superfície ventral do bulbo, promovendo um aumento na atividade respiratória (GOURINE et al., 2005a). Através

de micro-sensores colocados na superfície ventral do bulbo, foi observado que as áreas onde ocorre maior concentração de ATP situam-se próximas as áreas classicamente descritas com propriedades quimiossensíveis, como o RTN e os neurônios serotoninérgicos da Rafe (GOURINE et al., 2005a). O nosso trabalho confirma e extende os dados da literatura mostrando que a injeção de ATP diretamente na região do RTN é capaz de promover aumento da atividade cardiorrespiratória (GOURINE et al., 2005a; SOBRINHO et al., 2014). Ademias mostramos que tanto o efeito da aplicação exógena de ATP quanto as alterações promovidas pela hipercapnia podem ser atenuadas pela aplicação prévia de PPADS (GOURINE et al., 2005a; WENKER et al., 2012; SOBRINHO et al., 2014).

O uso de diferentes modelos experimentais pode ser importante para a completa elucidação dos processos fisiológicos, uma vez que os resultados obtidos entre eles podem apresentar algumas divergências. Por exemplo, em trabalhos prévios realizados *in vitro*, nossos colaboradores já se contrapuseram em relação a participação da sinalização purinérgica na resposta a ativação dos quimiorreceptores centrais localizados no RTN (MULKEY et al., 2006), enquanto que Gourine e colaboradores utilizam tanto modelos de animais anestesiados quanto experimentos *in vitro*, defendiam que a que a sinalização purinérgica, dependente de receptors P2Y na superfície ventrolateral do bulbo, seria indispensável para as resposta de ativação dos quimiorreceptores centrais e periféricos (GOURINE et al., 2005a, 2005b, 2010). Onimaru e colaboradores, por outro lado, usaram um modelo de isolamento do tronco encefálicos de ratos com idade de 1 a 4 dias de vida e foram taxativos na afirmação que os receptores purinérgicos não são capazes de alterar as respostas de atividade elétrica dos neurônios da região do RTN promovidas pela hipercapnia (ONIMARU; IKEDA; KAWAKAMI, 2012). Dessa maneira, parece existir algumas controvérsias na literatura em um passado muito recente no que diz respeito à atuação da sinalização purinérgica no controle respiratório.

Nossos dados foram obtidos em animais anestesiados, vagotomizados e ventilados artificialmente, e nossos achados foram fortemente corroborados pelos resultados obtidos em outros modelos experimentais utilizados por nosso grupo. *In vitro,* através do registro eletrofisiológico da atividade elétrica de neurônios em fatias coronais do bulbo contendo o RTN (patch-clamp em fatias de encéfalo na configuração “loose-patch”), encontramos que na presença de antagonistas P2 não seletivos, PPADS ou suramim, mas não os receptores P2Y1 (MRS 2179), o aumento na taxa de disparos dos neurônios do RTN promovido pelo CO_2/H^+ é atenuado (WENKER et al., 2012). Da mesma forma, em animais não anestesiados e submetidos a experimentos de pletismografia de corpo inteiro, a aplicação de PPADS, mas

não MRS 2179 no RTN, promoveu redução da resposta ventilatória a hipercapnia (BARNA et al., 2015, submetido). É interessante mencionar que as porcentagens de inibição obtidas entre os modelos foram muito semelhantes (~30%).

5.2 Contribuição dos mecanismos purinérgicos na região do bulbo ventrolateral rostral no controle cardiorrespiratório promovido pela ativação dos quimiorreceptores periféricos

Hipercapnia induz liberação de ATP na superfície ventral do bulbo (GOURINE et al., 2005a, 2010), e contribui para a resposta quimiorreceptora através de receptores P2 na região do RTN (BARNA et al., 2015, submetido; GOURINE et al., 2005a; SOBRINHO et al., 2014; WENKER et al., 2012). Níveis elevados de ATP nesta região também foram detectados em resposta a hipóxia (redução na P_{O2}), mesmo em animais com os quimiorreceptores periféricos desnervados (GOURINE et al., 2005b). Em nossos experimentos, encontramos que o bloqueio dos receptores P2Y1 com a injeção do antagonista MRS 2179, o aumento na frequência de disparos de neurônios do RTN *in vitro*, bem como a resposta cardiorrespiratória *in vivo* para CO_2/H^+ permanecem inalteradas, sugerindo que esta classe de receptores não está envolvida na quimiorrecepção central, pelo menos na região do RTN (BARNA et al., 2015, submetido; WENKER et al., 2012).

Na tentativa de reforçar nossa hipótese de que a participação da sinalização purinérgica nas respostas quimiorreceptoras centrais no RTN são inteiramente mediadas por receptores P2X e que não há participação de receptores P2Y1, realizamos a injeção do agonista P2Y1, MRS 2365 no RTN, enquanto registrávamos a atividade de neurônios do RTN *in vitro* ou a atividade cardiorrespiratória de animais anestesiados (WENKER et al., 2012, 2013). Como esperado, a injeção do antagonista de receptores P2Y1, MRS 2179, no RTN, não produziu efeitos na frequência de potenciais de ação de neurônios quimiossensíveis. No entanto, a aplicação do agonista P2Y1, MRS2365, no RTN de animais anestesiados promoveu aumento da PAM e da atividade do nervo frênico, sugerindo que receptores P2Y parecem não participar das respostas cardiorrespiratórias promovidas pela ativação do quimiorreflexo central, mas os receptores P2Y1 estão presentes no bulbo ventrolateral e estariam modulando a atividade cardiorrespiratória em algum outro contexto fisiológico, como o quimioreflexo periférico (WENKER et al., 2012, 2013). Outra possibilidade que não podemos descartar é que nossas injeções de agonistas e antagonistas purinérgicos tenham atingido os neurônios catecolaminérgicos do grupamento C1. Evidências do nosso laboratório apontam para tal

evidência, pois experimentos realizados em animais não anestesiados e com lesão do grupamento catecolaminérgico C1, a injeção de fármacos que atuam em receptores P2Y na região do RTN não foram eficazes em produzir efeitos cardiorrespiratórios (BARNA et al., 2015, submetido). Em nossos estudos direcionados ao RTN, frequentemente focamos na região que circunda o terço caudal do núcleo motor do facial (ventral e caudal), pois nesta região ocorre a maior densidade de células quimiossensíveis (MULKEY et al., 2004). No entanto, os neurônios quimiossensíveis do RTN encontrados mais caudais estão localizados muito próximos de neurônios catecolaminérgicos do grupamento C1 ou mesmo distribuídos entre os neurônios da porção mais rostral desse grupamento (STORNETTA et al., 2006).

Está bem estabelecido na literatura que os neurônios localizados na região do bulbo ventrolateral (neurônios C1 bulbo-espinais e neurônios quimiossensíveis do RTN) são determinantes no controle e na integração de reflexos cardiorrespiratórios, regularmente atribuindo-se as respostas respiratórias aos neurônios do RTN e a resposta pressora e simpática aos neurônios C1 (ABBOTT et al., 2012, 2009a; BURKE et al., 2014, 2015; GUYENET, 2006; ROSIN; CHANG; GUYENET, 2006; STORNETTA et al., 2006; TAKAKURA et al., 2006). A destruição seletiva dos neurônios bulbo-espinais da região C1 ou dos neurônios quimiosenssíveis do RTN promovem a diminuição das respostas de ativação dos quimiorreceptores periféricos e centrais, respectivamente (SCHREIHOFER; GUYENET, 2000; TAKAKURA et al., 2014). Ademais, a ativação seletiva dos neurônios catecolaminérgicos da região C1 (bulbo-espinais) ou dos neurônios Phox2b^{+} (quimiosenssíveis) com a técnica da optogenética promove aumento da pressão arterial e da respiração (ABBOTT et al., 2009a, 2009b; BURKE et al., 2015).

No presente trabalho mostramos que a sinalização purinérgica na região do bulbo ventrolateral rostral tem importante papel para as respostas cardiorrespiratórias produzidas pela hipóxia. Os nossos achados também são consistentes com o fato de que o ATP é co-liberado com o glutamato em algumas sinapses, em especial nas sinapses da via do quimiorreflexo periférico, uma vez que o bloqueio simultâneo de receptores glutamatérgicos ionotrópicos e receptores P2Y1 produziram alterações mais acentuadas. Além do mais, foram detectadas varicosidades no bulbo ventrolateral rostral provenientes do NTScom expressam VGLUT e VNUT, corroborando com trabalhos anteriores que demonstram que a co-transmissão purinas/glutamato na região do bulbo ventrolateral rostral estaria envolvida na neurotransmissão do quimiorreflexo periférico (BRAGA et al., 2007; MORAES et al., 2011).

Os neurônios bulbo-espinais da região bulbo ventrolateral rostral são ativados pela aplicação de ATP (SUN; WAHLESTEDT; REIS, 1992) e a injeção de agonistas purinérgicos nessa região do encéfalo é capaz de promover alterações cardiorrespiratórias em animais anestesiados (GOURINE et al., 2005a; WENKER et al., 2012; SOBRINHO et al., 2014). Adicionalmente, a inibição de receptores purinérgicos P2 em regiões mais caudais do bulbo ventrolateral, mais especificamente nas regiões que englobam o complexo de Bötzinger e pré-Bötzinger, foram capazes de diminuir a resposta ventilatória, mas não a resposta pressora, da ativação dos quimiorreceptores periféricos em animais não anestesiados (MORAES et al., 2011). Em situações de hipóxia, as células glomus do corpúsculo carotídeo liberam ATP para promover a ativação de receptores purinérgicos P2X2 e P2X3 nas vias aferentes para o SNC, nas quais fazem a primeira sinapse na região do NTScom (FORD et al., 2015; MACHADO, 2001; PATON et al., 2001). Na região do NTScom, a injeção de ATP reproduz as respostas cardiorrespiratórias (hipertensão, bradicardia e taquipnéia) encontradas durante a ativação do quimiorreflexo periférico (DE PAULA et al., 2004). Consistente com a importância da neurotransmissão purinérgica na via do quimiorreflexo, a injeção do antagonista de receptores glutamatérgicos, ácido quinurênico, na região do NTS não foi capaz de alterar as respostas cardiovasculares promovidas pela ativação do quimiorreflexo periférico em animais acordados ou na preparação *in situ* (BRAGA et al., 2007), no entanto, o bloqueio simultâneo de receptores glutamatérgicos e purinérgicos no NTScom foi capaz de reduzir as respostas cardiorrespiratórias promovidas pela ativação dos quimiorreceptores periféricos (BRAGA et al., 2007).

Os resultados apresentados sugerem que as respostas cardiorrespiratórias promovidas pela ativação dos quimiorreceptores periféricos são mediadas em parte na região do bulbo ventrolateral rostral, por mecanismos dependentes de receptores purinérgicos P2Y1 presentes nos neurônios C1. Esta hipótese é fundamentada pelos experimentos realizados *in vivo* em animais que foram submetidos a lesão seletiva de neurônios C1 com a toxina saporina conjugada com dopamina-beta-hidroxilase (anti-DβH-SAP), em que o aumento da PAM, da atividade simpática e do nervo frênico produzido pela aplicação de agonista P2Y1 no bulbo ventrolateral rostral foram bloqueadas. Resultados obtidos *in vitro* também suportam esta hipótese, uma vez que neurônios imunorreativos para TH (possivelmente neurônios do grupamento C1) e insensíveis as variações de CO_2/H^+, são capazes de despolarizar após a aplicação do agonista P2Y1. Por outro lado, neurônios não catecolaminérgicos e imunorreativos para Phox2b ($TH^-/Phox2b^+$) e sensíveis as variações de CO_2/H^+ não alteram sua atividade após a aplicação do agonista purinérgico P2Y1 (WENKER et al., 2012). A

resposta pressora e simpato-excitatória ao KCN também foram atenuadas em animais com lesão de neurônios C1, mas a resposta respiratória foi inalterada (WENKER et al., 2013).

As observações descritas acima parecem bem consistentes, pois sabe-se que o NTScom não se projeta exclusivamente para o grupamento C1. Varicosidades oriundas do NTScom também são encontrados no RTN (TAKAKURA et al., 2006; WENKER et al., 2013) ou em grupamentos da coluna respiratória ventral (ALHEID; JIAO; MCCRIMMON, 2011). Dessa maneira, acreditamos que os neurônios bulbo-espinais do grupamento C1 e os neurônios quimiossensíveis do RTN apresentam comunicações recíprocas como parte da rede neural que integra informações respiratórias e simpáticas (BURKE et al., 2014; TAKAKURA; MOREIRA, 2011; TAKAKURA et al., 2011; WENKER et al., 2013).

5.3 Sinalização purinérgica não contribui para quimiorrecepção central na região comissural do núcleo do trato solitário e na região do núcleo pálido da rafe/região parapiramidal

Esta bem estabelecido que além do RTN, outras regiões do SNC podem atuar como quimiorreceptores centrais, como por exemplo o NTScom (NATTIE; LI, 2002b), locus coeruleus (BIANCARDI et al., 2008) e os núcleos da rafe bulbar (núcleos Magno, Obscuro, e a região que engloba o núcleo pálido da rafe/região parapiramidal (CORCORAN et al., 2009; MESSIER; LI; NATTIE, 2002; RICHERSON, 2004).

Como já descrito acima, os receptores purinérgicos do tipo P2 são amplamente distribuídos no SNC (FUNK, 2013; YAO et al., 2000). Neste sentido, já está bem estabelecido que a sinalização purinérgica pode atuar ao longo de toda a cadeia neural da via do quimioreflexo periférico (ALVARES et al., 2014; ANTUNES; BRAGA; MACHADO, 2006; BRAGA et al., 2007; DE PAULA et al., 2004; FUNK, 2013; MORAES et al., 2011; MOREIRA et al., 2015; PATON et al., 2001; WENKER et al., 2012, 2013), incluindo o NTScom, onde injeções de agonistas purinérgicos produz alterações cardiorrespiratórias que mimetizam a ativação dos quimiorreceptores periféricos (ANTUNES; BRAGA; MACHADO, 2006; DE PAULA et al., 2004). As alterações cardiorrespiratórias promovidas pela injeção de ATP no NTS parecem ser dependentes da ativação de receptores P2 (ANTUNES et al., 2001; SOBRINHO et al., 2014). No entanto, a aplicação do antagonista P2 PPADS no NTScom não foi capaz de alterar as respostas cardiorrespiratórias promovidas pela ativação do quimiorreflexo central (SOBRINHO et al., 2014). Em concordância com nossos achados *in vivo*, os neurônios quimiossensíveis do NTScom respondem a aplicação de ATP com aumento na frequência de potenciais de ação, mas a aplicação de antagonistas P2, PPADS ou

suramim, não produziram alterações na resposta ao CO_2/H^+, sugerindo que a sinalização purinérgica, na região do NTS, parece não participar da mobilização do quimiorreflexo central (SOBRINHO et al., 2014).

A sinalização purinérgica pode atuar na região do bulbo ventrolateral rostral como moduladora de respostas quimiorreceptoras centrais e periféricas (GOURINE et al., 2005a, 2010; WENKER et al., 2012, 2013). Neste contexto, já foi sugerido que a sinalização purinérgica também pode atuar em outros núcleos quimiossensíveis modulando respostas ventilatórias. Por exemplo, já foi demonstrado em trabalhos anteriores que antagonistas purinérgicos atenuam a resposta quimiorreceptora central em porções rostrais dos núcleos da rafe encontrados no bulbo, mas não nos grupamentos caudais (como o núcleo pálido da rafe) (DA SILVA et al., 2012). Além disso, injeções de ATP no núcleo pálido da rafe aumentam a atividade respiratória, enquanto que aplicações no núcleo magno da rafe levaram a redução (CAO; SONG, 2007).

Utilizando-se uma abordagem muito semelhante, nós não encontramos evidências em nossos experimentos *in vivo* e *in vitro* que a sinalização purinérgica na região da RPa/PPy possa atuar como moduladora da resposta quimiorreceptora central, como já demonstrado na região do RTN (SOBRINHO et al., 2014).

A região que engloba o núcleo pálido da rafe e a região parapiramidal, previamente reportada com propriedades quimiossensíveis está localizada ao mesmo nível rostro-caudal do RTN (DA SILVA et al., 2012; DEPUY et al., 2011; TAKAKURA; MOREIRA, 2013). Uma vez que esta estrutura encefálica esta localizada ventralmente no bulbo, é plausível que o ATP liberado durante a hipercapnia também promova alterações neste sítio. Apesar disso, injeções de ATP localizadas aproximadamente 1 mm mediais ao RTN não foram capazes de produzir alterações cardiorrespiratórias. Da mesma maneira, injeções bilaterais de PPADS na RPa/PPy não alteram as respostas a hipercapnia (SOBRINHO et al., 2014). Experimentos *in vitro*, mostraram que neurônios serotoninérgicos da RPa/PPy podem responder de forma distinta a variações de CO_2/H^+ (inibidos ou estimulados), no entanto, a atividade de ambas populações neuronais se mantiveram inalteradas na presença de ATP ou de antagonista purinérgico P2 (SOBRINHO et al., 2014).

5.4 Interação glia-neurônio na região do RTN no controle cardiorrespiratório

Neurônios do RTN possuem propriedades intrínsecas para detectar variações de CO_2/H^+, as quais envolvem receptores GPR4 e canais do tipo TASK-2 (GUYENET; BAYLISS, 2015; KUMAR et al., 2015; MULKEY et al., 2004; WANG et al., 2013a, 2013b). No entanto, como demonstrado no presente estudo, existem várias evidências que a sinalização purinérgica pode modular parcialmente a quimiorrecepção central nesta região (GOURINE et al., 2005a, 2010; MOREIRA et al., 2015; SOBRINHO et al., 2014; WENKER et al., 2012).

Evidências experimentais apontam que o aumento da concentração de ATP observado na superfície ventral em resposta hipercapnia é mediado por astrócitos (GOURINE et al., 2005a, 2010; HUCKSTEPP et al., 2010a). Gourine e colaboradores relataram que durante a hipercapnia, astrócitos da superfície ventral do bulbo apresentam aumento nas concentrações de Ca^{2+} intracelular, previamente a liberação de ATP (GOURINE et al., 2010).

O envolvimento de astrócitos com a sinalização purinérgica na região do RTN também já foi demonstrado em experimentos *in vitro*, através da adição de CO_2 ao líquido cérebro-espinal artificial (HUCKSTEPP et al., 2010a, 2010b; WENKER et al., 2010). Nesse sentido, demonstrou-se que 20% das células da glia, localizadas na região do RTN, são capazes de perceber alterações de pH mediante mecanismos de inibição de canais do tipo Kir 4.1-Kir 5.1 (WENKER et al., 2010). Adicionalmente, evidências experimentais mostraram que a liberação de ATP, em resposta ao aumento das concentrações de CO_2, possivelmente envolve a ativação de hemicanais de conexinas 26, e esse seria um mecanismo independente da mobilização de Ca^{2+} (HUCKSTEPP et al., 2010a). Corroborando com estes resultados, o bloqueio de hemicanais de conexinas através da aplicação de carbonexolona na região do RTN foi capaz de reduzir a atividade de neurônios quimiossensíveis nessa região (WENKER et al., 2012). Adicionalmente, trabalhos recentes reforçaram dados prévios demonstrando que aumento nas concentrações de ATP na superfície ventral do bulbo não ocorre apenas durante a hipercapnia, mas também durante hipóxia, e sugerem que os astrócitos dessa região podem atuar como células quimiossensíveis e estimular a atividade neuronal de neurônios C1, mediante liberação de ATP e lactato (ANGELOVA et al., 2015; MARINA et al., 2015).

O fluorocitratro é uma toxina que atua seletivamente e reversivelmente em células da glia. Esta toxina atua inibindo a aconitase, e bloqueia em até 95% a formação de glutamina, sem afetar a síntese de neurotransmissores como glutamato ou acetilcolina, o que reforça a hipótese de glio-seletividade (FONNUM; JOHNSEN; HASSEL, 1997; HASSEL et al., 1992).

Aqui nós reportamos que a injeção da gliotoxina FCt no RTN de animais anestesiados e vagotomizados, foi capaz de gerar ou aumentar o padrão inspiratório na atividade do nervo frênico. Além disso, demonstramos que o aumento na amplitude e frequência respiratória devido a injeção de FCt no RTN foi significativamente atenuada em animais que receberam injeções prévias do antagonista de receptores purinérgico P2 no RTN.

Um processo de acidificação encefálica é capaz de promover a despolarização de células identificadas como astrócitos localizados na surpefície ventrolateral do bulbo (FUKUDA; HONDA, 1975; WENKER et al., 2010). Erlichman e colaboradores (1998) observaram, através da microdiálise, que a infusão de FCt na superfície ventrolateral do bulbo promove queda no pH do parênquima, sugerindo que esta acidose tecidual poderia despolarizar neurônios quimiossensíveis na região, o que promoveria um aumento da atividade respiratória. Os autores também acreditam que a infusão de FCt estaria promovendo um desequilíbrio de íons K^+ mediante uma inativação de astrócitos.

Até o presente momento, não podemos propor nenhum mecanismo molecular de como a injeção de FCt, na região ventrolateral do bulbo, mais especificamente na região do RTN, estaria promovendo o aumento da atividade respiratória. A acidose local promovida pela injeção de FCt poderia ser a responsável pelo aumento na atividade neuronal ou mesmo pela liberação de ATP por astrócitos. No entanto, encontramos que o bloqueio de receptores P2 foi capaz de atenuar a resposta respiratória ao FCt, corroborando com resultados *in vitro* e eliminando que o efeito observado fosse apenas mediado pelo efeito da acidose em neurônios (WENKER et al., 2010). Também já foi demonstrado que a liberação de ATP por astrócitos é dependente de CO_2 e não de H^+, eliminando assim que o efeito observado seja devido aos efeitos da acidose promovida pelo FCt sobre os astrócitos (HUCKSTEPP et al., 2010a, 2010b). Propomos então que os efeitos observados em resposta a aplicação de FCt no RTN é, ao menos em parte, devido sua ação direta sobre astrócitos, que leva a despolarização da membrana dessas células gliais e consequente liberação de ATP.

Em nossos experimentos, a injeção de fluorocitrato em áreas relatadas por exibir propriedades quimiorreceptoras como o NTScom ou a região RPa/PPy falharam em promover alterações cardiorrespiratórias, enfatizando a hipótese que mecanismos intrínsecos de quimiorrecepção central envolvendo células da glia possam ser restritos a superfície ventrolateral do bulbo, em especial no RTN (SOBRINHO et al., 2015, em preparação). Suportando estes resultados, diferentemente da supericie ventral do bulbo, a acidificação no líquido cerebroespinal, utilizada em experimentos *in vitro*, não promoveu oscilações de Ca^{2+} em astrócitos de outras áreas do SNC, como por exemplo o córtex cerebral ou de fatias de

regiões mais dorsais do tronco encefálico (GOURINE et al., 2010). Recentemente, foi demonstrado através da técnica conhecida como preparação coração-tronco cerebral isolado (preparação *in situ*), que permite o registro simultâneo da atividade de diferentes nervos em animais livres de anestesia, que aplicação de FCt em diferentes regiões do NTS não promoveram alterações na atividade do nervo frênico (COSTA; MORAES; MACHADO, 2013). No entanto, a aplicação de FCt em sítios da coluna respiratória ventral localizados próximos ao RTN (PreBötzinger/Bötzinger) promoveram aumento da frequência, o qual os autores correlacionaram com a redução no tempo da fase expiratória (COSTA; MORAES; MACHADO, 2013). Em ambos os casos, não foram observadas alterações na atividade simpática (COSTA; MORAES; MACHADO, 2013), que auxiliam a entender a ausência de alterações nos registros de PAM de nossos experimentos.

6 CONCLUSÃO

A região do bulbo ventrolateral rostral contém duas populações distintas de neurônios que são importantes na modulação de reflexos cardiorrespiratórios. Neurônios com propriedades quimiossensíveis do RTN, os quais possuem propriedades intrínsecas para detecção de CO_2/H^+ e neurônios bulbo-espinais catecolaminérgicos do grupamento C1 que modulam o tônus simpático. Esses grupamentos apresentam projeções reciprocas e integram reflexos diversos, pois recebem informação de quimiorreceptores periféricos via projeções do NTScom (MOREIRA et al., 2015).

Essa mesma região contém uma população de células gliais (astrócitos) que também são sensíveis as variações de CO_2 e possivelmente de $O_{2,}$ e ao que tudo indica podem modular a atividade neuronal de neurônios vizinhos através da liberação de ATP que ativaria os receptores P2 expressos em neurônios do RTN. Por outro lado, a via glutamatérgica clássica entre o NTScom e o bulbo ventrolateral rostral, responsável por grande parte da interação de respostas reflexas durante a ativação de quimiorreceptores periféricos e centrais, também utiliza de purinas como molécula sinalizadora, promovendo co-transmissão com o glutamato através de receptores P2Y1 expressos em neurônios C1 e receptores P2 no RTN. Este processo aparentemente se restringe a estes grupamentos localizados próximos a superfície ventral do bulbo (Figs. 20A-B).

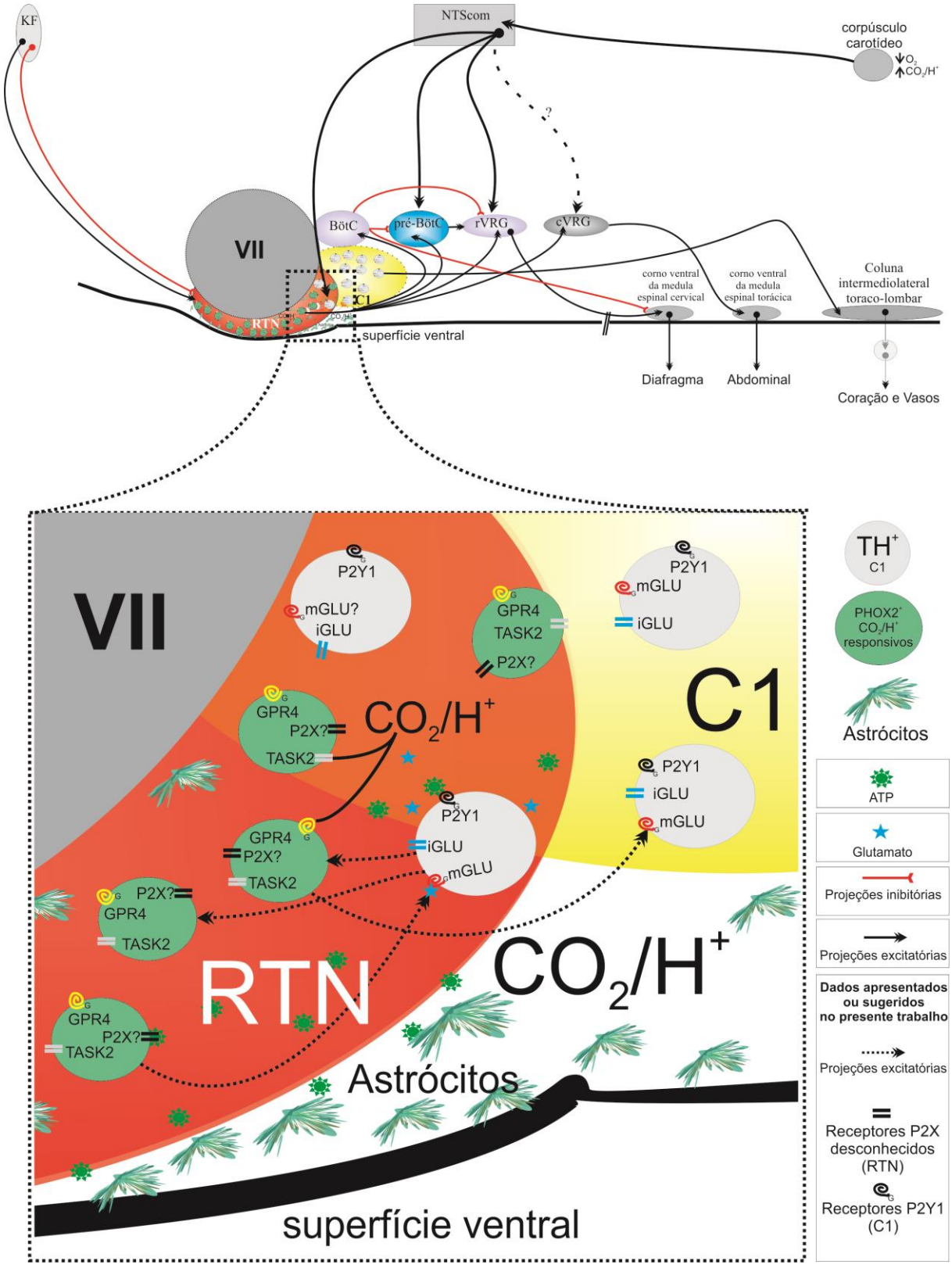
KF
NTScom
corpúsculo carotídeo
O_2
CO_2/H^+
?
VII
BötC
pré-BötC
rVRG
cVRG
C1
RTN
superfície ventral
corno ventral da medula espinal cervical
corno ventral da medula espinal torácica
Coluna intermediolateral toraco-lombar
Diafragma
Abdominal
Coração e Vasos
VII
P2Y1
mGLU?
iGLU
GPR4
TASK2
P2X?
CO_2/H^+
C1
mGLU
RTN
Astrócitos
superfície ventral
TH^+
C1
$PHOX2^+$
CO_2/H^+
responsivos
Astrócitos
ATP
Glutamato
Projeções inibitórias
Projeções excitatórias
Dados apresentados ou sugeridos no presente trabalho
Projeções excitatórias
Receptores P2X desconhecidos (RTN)
Receptores P2Y1 (C1)

Figura 20: Conclusão

A: Figura esquemática ilustrando a organização dos grupamentos respiratórios, suas conexões e os quimiorreceptores centrais e periféricos. A ativação de quimiorreceptores centrais ou periféricos promove massiva ativação da coluna respiratória ventral, tendo como consequência o aumento na ventilação e da atividade simpática causado pela estimulação do grupamento C1. **B**: Figura esquemática de uma ampliação da área delimitada pelo quadrado na figura A, ilustrando conexões reciprocas entre os neurônios do RTN e do grupamento C1. Note que a porção caudal do RTN (em vermelho) se sobrepõe parcialmente (em laranja) com o grupamento C1 (em amarelo). Os neurônios Phox2b do RTN (representados em verde) possuem mecanismos intrínsecos para detecção de ao CO_2/H^+ (GPR4 e TASK-2), mas também são estimulados por ATP, liberado por astrócitos durante a hipercapnia, provavelmente via receptores P2X. A ativação dos neurônios do RTN leva a ativação de neurônios catecolaminérgicos do grupamento C1 (representados em cinza), possivelmente através da ação de receptores glutamatérgicos metabotrópicos. Por outro lado, a ativação de quimiorreceptores periféricos promove a ativação de neurônios do RTN via projeções oriundas de neurônios do NTScom, mas também via projeções de neurônios C1, o qual é ativado pela ação do glutamato e do ATP, liberado pelos terminais axonais provenientes do NTScom em receptores iGLU e P2Y1, respectivamente.

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APÊNDICES

APÊNDICE A - Lista de artigos publicados referentes a tese de doutorado

1- Regulation of ventral surface CO2/H+-sensitive neurons by purinergic signalling. WENKER, I. C.; SOBRINHO, C. R.; TAKAKURA, A. C.; MOREIRA, T. S.; MULKEY, D. K.; **The Journal of physiology**, v. 590, n. Pt 9, p. 2137–50, 2012.

2- P2Y1 receptors expressed by C1 neurons determine peripheral chemoreceptor modulation of breathing, sympathetic activity, and blood pressure. WENKER, I. C*.; SOBRINHO, C. R*.; TAKAKURA, A. C.; MULKEY, D. K.; MOREIRA, T. S.; **Hypertension**, v. 62, n. 2, p. 263–273, 2013.

*ambos autores contribuiram igualmente com a primeira autoria

3- Purinergic signalling contributes to chemoreception in the retrotrapezoid nucleus but not the nucleus of the solitary tract or medullary raphe. SOBRINHO, C. R.*; WENKER, I. C.*; POSS, E. M.; TAKAKURA, A. C., MOREIRA, T. S., MULKEY, D. K.; **The Journal of physiology**, v. 592, n. Pt 6, p. 1309–23, 2014.

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4- Independent purinergic mechanisms of central and peripheral chemoreception in the rostral ventrolateral medulla. MOREIRA, T. S.; WENKER, I. C.; SOBRINHO, C. R.; BARNA BF, TAKAKURA AC, MULKEY DK. **The Journal of Physiology**, v. 1, n. 593 August 2014, p. 1067–74, 2015.

APÊNDICE B- Lista de artigos submetidos e em preparação

1- Cleyton R Sobrinho, Bárbara Falquetto Barna, Ana C Takakura, Thiago S Moreira, and Daniel K. Mulkey**-** Cholinergic control of ventral surface chemoreceptors involves Gq/IP3-mediated inhibition of KCNQ channels. Submetido para **Journal of Physiology**. 2015 (artigo referente ao periodo de estágio no exterior - submetido).

2- Cleyton R. Sobrinho, Ana C. Takakura, Thiago S. Moreria - Fluorocitrate injections on RTN, but not in NTScom or RPa/PPy increased breathing by a purinergic mechanism: Glia to neurons interactions. 2015 (artigo referente a tese - em preparação).

APÊNDICE C- Artigos publicados

J Physiol 590.9 (2012) pp 2137–2150

The Journal of Physiology

Regulation of ventral surface CO_2/H^+-sensitive neurons by purinergic signalling

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Key points

- The retrotrapezoid nucleus (RTN) is an important site of chemoreception, i.e. the mechanism by which the brain regulates breathing in response to changes in tissue CO_2/H^+.
- Mechanisms underlying RTN chemoreception appear to involve direct CO_2/H^+-mediated neuronal activation and indirect neuronal activation by CO_2-evoked ATP release (i.e. purinergic signalling) from astrocytes.
- Here, we show *in vitro* and *in vivo* that purinergic signalling in the RTN contributes to ∼30% of RTN chemoreception.
- Purinergic drive in the RTN involves gap junction hemichannels but not P2Y1 receptors.
- These results clearly indicate that purinergic signalling contributes to integrated output of the RTN during hypercapnia and thus is an important determinant of respiratory drive.

Abstract Central chemoreception is the mechanism by which the brain regulates breathing in response to changes in tissue CO_2/H^+. A brainstem region called the retrotrapezoid nucleus (RTN) contains a population of CO_2/H^+-sensitive neurons that appears to function as an important chemoreceptor. Evidence also indicates that CO_2-evoked ATP release from RTN astrocytes modulates activity of CO_2/H^+-sensitive neurons; however, the extent to which purinergic signalling contributes to chemoreception by RTN neurons is not clear and the mechanism(s) underlying CO_2/H^+-evoked ATP release is not fully elucidated. The goals of this study are to determine the extent to which ATP contributes to RTN chemoreception both *in vivo* and *in vitro*, and whether purinergic drive to chemoreceptors relies on extracellular Ca^{2+} or gap junction hemichannels. We also examine the possible contribution of P2Y1 receptors expressed in the RTN to the purinergic drive to breathe. We show that purinergic signalling contributes, in part, to the CO_2/H^+ sensitivity of RTN neurons. *In vivo*, phrenic nerve recordings of respiratory activity in adult rats show that bilateral injections of pyridoxal-phosphate-6-azophenyl-2′,4′-disulfonate (PPADS, a P2 receptor blocker) decreased the ventilatory response to CO_2 by 30%. *In vitro*, loose-patch recordings from RTN neurons show that P2 receptor blockers decreased responsiveness to both 10% and 15% CO_2 also by 30%. In the slice, the contribution of purinergic signalling to RTN chemoreception did not increase with temperature (22–35°C) and was retained in low extracellular Ca^{2+} medium. Conversely, the gap junction blockers carbenoxolone and cobalt decreased neuronal CO_2/H^+ sensitivity by an amount similar to P2 receptor antagonists. Inhibition of the P2Y1 receptor in the RTN had no effect on CO_2 responsivness *in vitro* or *in vivo*; thus, the identity of P2 receptors underlying the purinergic component of RTN chemoreception

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remains unknown. These results support the possibility that CO_2/H^+-evoked ATP release is mediated by a mechanism involving gap junction hemichannels.

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Abbreviations $etCO_2$, end-expiratory CO_2; MAP, mean arterial pressure; pFRG, parafacial respiratory group; NA, phrenic nerve activity; RTN, retrotrapezoid nucleus.

Introduction

Carbon dioxide provides the primary stimulus to breathe. Central respiratory chemoreceptors sense changes in tissue CO_2, H^+ and/or HCO_3^- to regulate the rate and depth of breathing (for review see Huckstepp & Dale, 2011). It is now well established that the retrotrapezoid nucleus (RTN) is an important site of chemoreception. Specifically, the RTN contains a subset of neurons that is highly CO_2/H^+ sensitive *in vivo* (Nattie *et al.* 1993; Mulkey *et al.* 2004; Takakura *et al.* 2008) and *in vitro* (Mulkey *et al.* 2004; Ritucci *et al.* 2005), is glutamatergic (Mulkey *et al.* 2004; Weston *et al.* 2004) and projects to respiratory centres to directly influence breathing (Mulkey *et al.* 2004; Abbott *et al.* 2009). The mechanism by which RTN neurons sense pH involves inhibition of an unidentified voltage-independent K^+ conductance (Mulkey *et al.* 2004, 2007). Evidence also indicates that purinergic signalling contributes to the ventilatory response to CO_2 (Thomas *et al.* 1999; Thomas & Spyer, 2000; Gourine *et al.* 2003; Gourine, 2005). At the level of the RTN, hypercapnia has been shown to evoke the discrete release of ATP near the ventral medullary surface (Gourine *et al.* 2005, 2010; Huckstepp *et al.* 2010*b*), and ATP has been shown to contribute to respiratory drive by increasing activity of CO_2/H^+-sensitive RTN neurons (Mulkey *et al.* 2006; Gourine *et al.* 2010; Wenker *et al.* 2010).

There is increasing evidence that astrocytes are the source of purinergic drive to RTN chemoreceptors (Gourine *et al.* 2010; Huckstepp *et al.* 2010*b*; Wenker *et al.* 2010); however, the extent to which ATP contributes to RTN chemoreception is questionable and the mechanisms underlying purinergic modulation of RTN chemoreceptors are not clear (Mulkey & Wenker, 2011). For example, a recent study reported that H^+ sensitivity of RTN neurons could be blocked with a P2 receptor antagonist (Gourine *et al.* 2010), suggesting that RTN chemoreception is entirely dependent on purinergic signalling. The same study also suggested that the purinergic drive to RTN neurons is dependent on extracellular Ca^{2+}; it showed that acidification triggered a Ca^{2+} wave that propagated between ventral surface astrocytes, possibly by activating Ca^{2+}-permeable channels (e.g. P2X1 or P2X3), to potentiate additional ATP release and subsequently activate Phox2b-expressing RTN neurons *in vitro* and increased respiratory activity *in vivo*. Conversely, we have shown *in vitro* that RTN neurons are highly pH sensitive in the presence of P2 receptor blockers (Mulkey *et al.* 2004, 2006; Wenker *et al.* 2010), and that purinergic signalling contributes to only a portion of neuronal CO_2/H^+ sensitivity (Wenker *et al.* 2010). Still further evidence obtained using the brainstem–spinal cord preparation reported that purinergic signalling does not contribute to CO_2 responsiveness of neurons in the parafacial respiratory group (pFRG)/RTN of newborn rats (Onimaru *et al.* 2012). In addition, the mechanism underlying CO_2-evoked ATP release in the RTN appears to be mediated by direct gating of gap junction hemichannels in a Ca^{2+}-independent manner (Huckstepp *et al.* 2010*b*).

The objectives of this study are to establish the extent to which purinergic signalling contributes to RTN chemoreception both *in vivo* and *in vitro*, and to gain insight into the mechanisms underlying CO_2-evoked ATP release and downstream activation of RTN neurons. To make these determinations, we recorded phrenic nerve activity *in vivo* or the firing rate response of CO_2/H^+-sensitive RTN neurons in the brain slice preparation during exposure to hypercapnia alone and in the presence of P2 receptor blockers. In addition, we used a pharmacological approach to manipulate potential mechanisms of ATP release and activity of downstream P2 receptors. We find that purinergic signalling is an important component of RTN chemoreception; application of P2 receptor antagonists into the RTN reduced the ventilatory response to CO_2 by ~30% *in vivo*, and blunted the firing rate response of RTN neurons to both 10% and 15% CO_2 also by 30%. This purinergic drive to breathe was independent of temperature, stimulus strength and changes in the extracellular Ca^{2+} gradient, but could be blocked with gap junction hemichannel antagonists. These results are most consistent with a recent study by Huckstepp and colleagues (Huckstepp *et al.* 2010*b*), and suggest that astrocytes but not neurons mediate CO_2-evoked ATP release by a mechanism involving gap junction hemichannels.

Methods

In vivo preparation

Animal use was in accordance with guidelines approved by the University of São Paulo Animal Care and Use Committee and conforms to the principles of UK regulations, as described in Drummond (2009). All efforts were made to minimize the number of animals used and their suffering. All *in vivo* experiments were performed in male Wistar rats weighing 250–280 g; a total of 38 rats were used for these *in vivo* experiments. The surgical procedures and experimental protocols were similar to those previously described (Takakura & Moreira, 2011; Takakura *et al.* 2011). Briefly, general anaesthesia was induced with 5% halothane in 100% O_2. A tracheostomy was made and the halothane concentration was reduced to 1.4–1.5% until the end of surgery. The femoral artery was cannulated (polyethylene tubing, 0.6 mm o.d., 0.3 mm i.d., Scientific Commodities, Lake Havasu City, AZ, USA) for measurement of arterial pressure (AP). The femoral vein was cannulated for administration of fluids and drugs. The occipital plate was removed, and a micropipette was placed in the medulla oblongata via a dorsal transcerebellar approach for microinjection of drugs. A skin incision was made over the lower jaw for placement of a bipolar stimulating electrode, next to the mandibular branch of the facial nerve, as previously described (Moreira *et al.* 2006; Takakura *et al.* 2011). The phrenic nerve was accessed by a dorsolateral approach after retraction of the right shoulder blade. To prevent any influence of artificial ventilation on phrenic nerve activity (PNA), the vagus nerve was cut bilaterally. Additionally, a complete baro- and peripheral chemoreceptor deafferentation was performed by sectioning the vagosympathetic trunks, the superior laryngeal nerves and the glossopharyngeal nerves (proximal to the junction with the carotid sinus nerves). Upon completion of the surgical procedures, halothane was replaced with urethane (1.2 g kg^{-1}) administered slowly I.V. All rats were ventilated with 100% O_2 throughout the experiment. Rectal temperature was maintained at 37°C. End-tidal CO_2 was monitored throughout each experiment with a capnometer (CWE, Inc., Ardmore, PA, USA) that was calibrated twice per experiment with a calibrated CO_2–N_2 mix. This instrument provided a reading of $<0.1\%$ CO_2 during inspiration in animals breathing 100% O_2, and an asymptotic, nearly horizontal reading during expiration. The adequacy of anaesthesia was monitored during a 20 min stabilization period by testing for the absence of withdrawal responses, pressor responses and changes in PNA to a firm toe pinch. After these criteria were satisfied, the muscle relaxant pancuronium was administered at an initial dose of 1 mg kg^{-1} I.V. and the adequacy of the anaesthesia was thereafter gauged solely by the lack of increase in AP and PNA rate or amplitude to a firm toe pinch. Approximately hourly supplements of one-third of the initial dose of urethane were needed to satisfy these criteria throughout the recording period (2 h).

In vivo recordings of physiological variables

As previously described (Mulkey *et al.* 2004; Moreira *et al.* 2006; Takakura *et al.* 2011), mean arterial pressure (MAP), phrenic nerve activity (PNA) and end-expiratory CO_2 ($etCO_2$) were digitized with a micro1401 (Cambridge Electronic Design), stored on a computer and processed off-line with version 6 of Spike 2 software (Cambridge Electronic Design). Integrated phrenic nerve activity (iPNA) was obtained after rectification and smoothing ($\tau = 0.015$ s) of the original signal, which was acquired with a 30–300 Hz bandpass filter. PNA amplitude (PNA amp) and PNA frequency (PNA freq) were expressed for each rat on a scale from 0 (value during apnoea) to 100 (value while breathing 10% CO_2).

Hypercapnia was produced by addition of pure CO_2 to the 100% O_2 supplied by artificial ventilation to increase the maximum end-expiratory CO_2 to 9.5–10%. End-expiratory CO_2 was maintained for 5 min, and CO_2 was removed. Each rat was submitted to three sessions of hypercapnia: one session 10 min after bilateral saline injections into the RTN, and two additional sessions, 10 and 60 min after bilateral injections of saline, PPDAS or MRS2179 into the RTN.

Histology

At the end of each *in vivo* experiment, rats were deeply anaesthetized with halothane and perfused through the heart with PBS (pH 7.4) followed by paraformaldehyde (4% in 0.1 M phosphate buffer, pH 7.4). The brains were removed and stored in fixative for 24 h at 4°C. The medulla was cut in 40-μm-thick coronal sections with a vibrating microtome (Vibratome 1000S Plus, USA). Sections were stored at −20°C in a cryoprotectant solution. The injection sites were confirmed with an Axioskop 2 microscope (Zeiss, Oberkochen, Germany). Sections from different brains were aligned with respect to a reference section, which was the most caudal section containing an identifiable cluster of facial motor neurons. To this reference section was assigned a value of 11.6 mm caudal to bregma (bregma −11.6 mm, Paxinos & Watson, 1998). Levels rostral or caudal to this reference section were determined by adding or subtracting the number of intervening sections × 40 μm.

Brain slice preparation

All procedures were performed in accordance with National Institutes of Health and University of Connecticut Animal Care and Use Guidelines and conformed to the principles of UK regulations, as described in Drummond (2009). A total of 64 rat pups were used for these *in vitro* experiments. Slices containing the RTN were prepared as previously described (Mulkey *et al.* 2004). Briefly, neonatal rats (7–12 days postnatal) were decapitated under ketamine–xylazine anaesthesia and transverse brainstem slices (300 μm) were cut using a microslicer (DSK 1500E; Dosaka, Kyoto, Japan) in ice-cold substituted Ringer solution containing (in mM): 260 sucrose, 3 KCl, 5 $MgCl_2$, 1 $CaCl_2$, 1.25 NaH_2PO_4, 26 $NaHCO_3$, 10 glucose and 1 kynurenic acid. Slices were incubated for ~30 min at 37°C and subsequently at room temperature in normal Ringer solution (in mM): 130 NaCl, 3 KCl, 2 $MgCl_2$, 2 $CaCl_2$, 1.25 NaH_2PO_4, 26 $NaHCO_3$ and 10 glucose. Both substituted and normal Ringer solutions were bubbled with 95% O_2–5% CO_2 (extracellular pH (pH_o) 7.35).

Slice-patch electrophysiology

Individual slices were transferred to a recording chamber mounted on a fixed-stage microscope (Zeiss Axioskop FS) and perfused continuously (~2 ml min^{-1}) with a bath solution of normal Ringer solution (same as incubation Ringer solution above) bubbled with 95% O_2–5% CO_2 (pH_o 7.35). The pH of the bicarbonate-based bath solution was decreased to 6.90 by bubbling with 15% CO_2 or 7.10 by bubbling with 10% CO_2. All recordings were made with an Axopatch 200B patch-clamp amplifier, digitized with a Digidata 1322A A/D converter, and recorded using pCLAMP 10.0 software (Molecular Devices). Recordings were obtained at room temperature (~22°C) with patch electrodes pulled from borosilicate glass capillaries (Warner Instruments) on a two-stage puller (P89; Sutter Instrument) to a DC resistance of 4–6 MΩ when filled with an internal solution containing the following (in mM): 120 KCH_3SO_3, 4 NaCl, 1 $MgCl_2$, 0.5 $CaCl_2$, 10 Hepes, 10 EGTA, 3 Mg-ATP and 0.3 GTP-Tris (pH 7.2) plus 0.2% biocytin; electrode tips were coated with Sylgard 184 (Dow Corning). All recordings of neuronal firing rate were performed in the loose-patch configuration to ensure minimal alteration of the intracellular milieu. Firing rate histograms were generated by integrating action potential discharge in 10 s bins and plotted using Spike 5.0 software. Excitatory postsynaptic currents (EPSCs) were measured from chemosensitive neurons (identified in loose-patch) in whole-cell voltage clamp ($I_{hold} = -60$ mV) and analysed off-line with AxoGraph X using the following parameters: amplitude = −25 pA, rise constant = 0.7 ms and decay constant = 1.6 ms, with a threshold of detection set to −7 SD.

Drugs

All drugs were purchased from Sigma. For *in vivo* experiments, the non-specific P2 receptor antagonist pyridoxalphosphate-6-azophenyl-2′,4′-disulfonic acid (PPADS), the P2Y1-receptor antagonist MRS2179 and the P2Y1-receptor agonist MRS2365 were diluted to 100 μM in sterile saline (pH 7.4) and injected into the RTN using single-barrel glass pipettes (tip diameter of 20 μm) connected to a pressure injector (Picospritzer III, Parker Hannifin Corp, Cleveland, OH, USA). For each injection we delivered a volume of 50 nl over a period of 5 s. These glass pipettes also allowed recordings of field potential properties that were used to help direct the electrode tip to the desired site. Injections in the RTN region were guided by recordings of the facial field potential (Brown & Guyenet, 1985), and were placed 250 μm below the lower edge of the field, 1.7 mm lateral to the midline and 200 μm rostral to the caudal end of the field. Recordings were made on one side only; the second injection was made 1–2 min later at the same level on the contralateral side. We included a 5% dilution of fluorescent latex microbeads (Lumafluor, New City, NY, USA) with all drug applications to mark the injection sites and verify the spread of the injections (Takakura & Moreira, 2011; Takakura *et al.* 2011). For the *in vitro* experiments, we bath applied PPADS (100 μM) or suramin (100 μM) to block P2 receptors, MRS2179 (3 μM) to block P2Y1 receptors, and carbenoxolone (CBX, 100 μM) or cobalt (500 μM) to block gap junction hemichannels. A low Ca^{2+}, high Mg^{2+} synaptic block solution was used to block potential ATP release from neurons and to limit Ca^{2+} influx into astrocytes from the extracellular space. The composition of the synaptic block medium used in this study is similar to normal Ringer solution except that $MgCl_2$ was increased to 11.4 mM, $CaCl_2$ was decreased to 0.2 mM, and to maintain osmolality, NaCl was decreased to 124 mM. The efficacy of this synaptic blocking medium is well established (Richards & Sercombe, 1970; Mason, 1980; Hatton, 1982) and confirmed here by recording its effects on spontaneous EPSCs. In addition, the specific P2Y1 receptor agonist (MRS2365; 100 μM in Hepes-buffered medium, pH 7.3) was delivered focally using low-resistance pipettes connected to a Picospritzer III (Parker Instrumentation, Cleveland, OH, USA) and manoeuvered into close proximity of the target neurons. Application times were 600 ms, and vehicle control experiments were performed to ensure agonist responses were not attributable to pressure artifacts.

Statistics

Data are reported as mean ± standard error of the mean. Statistical analysis was performed using Sigma Stat version 3.0 software (Jandel Corporation, Point Richmond, CA, USA). *t* test, paired *t* test or one-way ANOVA followed by

the Newman–Keul multiple comparisons test were used as appropriate ($P < 0.05$).

Results

This study consists of three sets of experiments. First, to determine the extent to which purinergic signalling contributes to central chemoreception, we tested the effects of P2 receptor antagonists (PPADS or suramin) on the hypercapnic ventilatory response *in vivo*, and on the firing rate response of RTN neurons to 10% and 15% CO_2 at room or body temperature *in vitro*. Second, to determine if purinergic drive to RTN neurons is dependent on synaptic input or requires extracellular Ca^{2+}, as previously reported, we compared effects of P2 receptor antagonists on neuronal CO_2/H^+ responsiveness under control conditions and in low Ca^{2+}–high Mg^{2+} synaptic block medium. Third, to determine if purinergic drive to CO_2/H^+-sensitive RTN neurons is mediated by hemichannels, we tested neuronal CO_2/H^+ sensitivity in the presence of the gap junction blockers CBX or cobalt. Note that chemosensitive RTN neurons have been shown to express the transcription factor Phox2b (Stornetta *et al.* 2006; Lazarenko *et al.* 2009); however, Phox2b has also been shown to be expressed by other cells in relatively close proximity to the RTN, including catecholaminergic neurons (C1 and A5), facial motor neurons and the superior salivatory nucleus (Kang *et al.* 2007). Therefore, we chose to functionally identify CO_2/H^+-sensitive RTN neurons based on their characteristic firing rate response to CO_2/H^+. Neurons were considered chemosensitive if they were spontaneously active in 5% CO_2 (pH_o 7.35) and responded to 15% CO_2 (pH_o ~6.90) with at least 1.5 Hz increase in firing rate. This level of CO_2/H^+ responsiveness is similar to what we, and others, have reported for RTN chemosensitive neurons (Ritucci *et al.* 2005; Mulkey *et al.* 2006; Wenker *et al.* 2010). RTN neurons that did not exhibit this minimum firing rate response to 15% CO_2 were considered non-chemosensitive and excluded from this study.

Purinergic drive to RTN chemoreceptors *in vivo*

As expected, when urethane-anaesthetized, sino-aortic denervated and vagotomized rats ($n = 6$ per group) are exposure to hypercapnia (10% CO_2) PNA amplitude and PNA frequency increased by 100 ± 2% and 99 ± 2% (Fig. 1*A*, *D* and *E*). Injections of PPADS (3 mM in 50 nl) were placed bilaterally in the RTN in these rats (Fig. 1*B* and *C*). The injection centre was 250 μm below the facial motor nucleus and 200 μm rostral to the caudal end of this nucleus, targeting the region that contains the highest density of CO_2-sensitive RTN neurons (Mulkey *et al.* 2004; Takakura & Moreira, 2011; Takakura *et al.* 2011). Application of PPADS into the RTN did not change resting PNA activity (99 ± 7% of control value; $P > 0.05$), but did reduce the hypercapnia-induced increase in PNA amplitude (62 ± 9% of the control) and PNA frequency (77 ± 3% of the control) (Fig. 1*D* and *E*).

As shown in Fig. 1*A*, exposure to hypercapnia also caused hypotension (−11 ± 7 mmHg) followed by a gradual return of mean arterial pressure (MAP) to control level, 30 to 40 s later. Immediately after hypercapnia ended, the MAP increased by 21 ± 6 mmHg, and returned to control values within 5 min (Fig. 1*A* and *F*). Injection of PPADS into the RTN did not change resting MAP (124 ± 6 mmHg compared with saline 126 ± 5 mmHg), but did reduce the hypercapnia-induced pressor response from 21 ± 6 to 8 ± 4 mmHg (Fig. 1*A* and *F*).

Injections located outside the RTN region often (3 out of 4) reached the facial motor nucleus or dorsal to it and one injection (1 out of 4) was located in the parapyramidal region (data not shown). Bilateral injections of PPADS in the facial motor nucleus or in the parapyramidal region did not change the hypercapnic-induced respiratory or pressor responses in these animals (data not shown).

Purinergic drive to RTN chemoreceptors *in vitro*

Previous evidence indicates that temperature greatly increased pH sensitivity of RTN neurons *in vitro* (Guyenet *et al.* 2005). Therefore, we wanted to determine if the contribution of purinergic drive to CO_2/H^+-sensitive RTN neurons also increased with temperature. We found that increasing temperature from ~22°C to 35°C increased baseline activity by 2.01 ± 0.18 Hz (Fig. 2*A*) and increased the firing rate response to 15% CO_2 from 1.9 ± 0.09 Hz to 3.3 ± 0.23 Hz ($n = 4$) (Fig. 2*B*). The temperature coefficient (Q_{10}) for the CO_2/H^+ sensitivity of RTN neurons was estimated to be ~1.5 ± 0.1 ($n = 9$); this value is very similar to the pH-sensitive Q_{10} of RTN neurons over a similar pH range (~1.55, extrapolated from Fig. 8*D*; Guyenet *et al.* 2005]. At 35°C, exposure to the P2 receptor antagonists PPADS (100 μM) or suramin (100 μM) decreased firing rate by 0.73 ± 0.18 Hz, consistent with the hypothesis that CO_2/H^+-sensitive RTN neurons receive tonic purinergic input (Wenker *et al.* 2010). Prior to testing CO_2/H^+ sensitivity at 35°C, a hyperpolarizing current (~5 pA) was delivered to approximate the control level of neuronal activity. In the continued presence of PPADS or suramin at 35°C (with baseline activity adjusted by DC current injection to near-control levels), exposure to 15% CO_2 increased neuronal activity by 2.38 ± 0.23 Hz ($n = 8$); this response was significantly less than the CO_2/H^+ response at 35°C in the absence of the P2 receptor antagonist (initial response or after wash out) (Fig. 2*A*–*B*). This purinergic-sensitive component of RTN chemoreception has an estimated Q_{10} of ~1.3.

It is worth noting that this value is similar to that described for connexin-mediated ATP release or current flux ($Q_{10} = 1.2$–1.4; Bukauskas *et al.* 1995; Valiunas *et al.* 1999; Leybaert *et al.* 2003). In addition, heating from 22°C to 35°C did not significantly increase effects of P2 receptor antagonists on CO_2/H^+ sensitivity of RTN neurons; PPADS and suramin decreased chemoreceptor activity by $27.3 \pm 4.8\%$ ($n = 12$) and $27.9 \pm 4.5\%$ ($n = 8$) at 22°C and 35°C, respectively (Fig. 2*C*). These results indicate that purinergic drive to CO_2/H^+-sensitive RTN neurons does not vary over this temperature range; therefore, all subsequent *in vitro* experiments were performed at room temperature.

To address the possibility that purinergic drive to RTN neurons is stimulus dependent, we compared effects of suramin on the firing rate response of RTN neurons to 10% and 15% CO_2. As shown in Fig. 3*A* and *B*, exposure to 10% (pH_o 7.10) and 15% CO_2 (pH_o 6.90) increased chemoreceptor activity by 1.83 ± 0.17 Hz and 2.48 ± 0.20 Hz ($n = 3$), respectively. In addition, suramin had similar effects on the firing rate response of RTN neurons to 10% and 15% CO_2. Previous (Wenker *et al.* 2010) and current results (Fig. 3*C* and *D*) show that ~10 min incubation in suramin (100 μM) decreased the response of RTN neurons to 10% and 15% CO_2 by $33.5 \pm 3.3\%$ and $31.9 \pm 7.2\%$, respectively (Fig. 3*C* and *D*), suggesting that purinergic

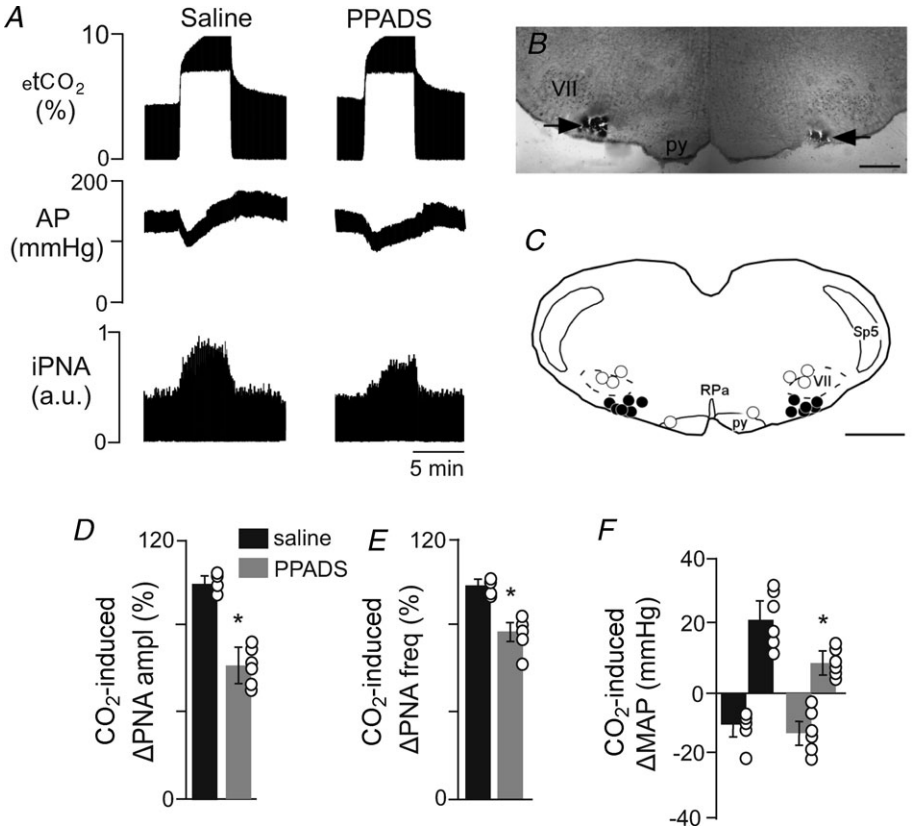

Figure 1. P2 receptor antagonist injections into the RTN attenuate effects of hypercapnia on arterial pressure and PNA in anaesthetized vago-sino-aortic denervated rats
A, recordings from one rat showing the effect of injection of PPADS into the RTN on changes in arterial pressure (AP) and phrenic nerve activity (PNA) elicited by an increase of end-expiratory CO_2 from 5 to 10%. Responses were recorded 10 min after bilateral injection of saline into the RTN, and 10 min after bilateral injection of PPADS (3 mM, 50 nl each side) in the RTN. *B*, photomicrograph of a coronal section showing bilateral injections in the RTN. Scale bar is 500 μm. *C*, computer-assisted plot of the centre of injection sites revealed by presence of fluorescent microbeads included in the injectate (coronal plane at Bregma −11.6; Paxinos & Watson, 1998). Scale bar is 1 mm. *D*–*F*, summary data ($n = 6$) show changes in PNA amplitude (ΔPNA ampl) (*D*), PNA frequency (ΔPNA freq) (*E*) and mean arterial pressure (ΔMAP) (*F*) elicited by stepping the end-expiratory CO_2 from 5 to 10% during saline or PPADS injections into the RTN. Differences expressed as a percentage of the response to the CO_2 challenge elicited during saline injection. *Different from saline ($P < 0.05$). Abbreviations: py, pyramid; RPa, raphe pallidus; Sp5, spinal trigeminal tract; VII, facial motor nucleus.

drive to these cells is relatively constant over this CO_2 range.

Changes in the extracellular Ca^{2+} gradient do not affect purinergic drive to chemoreceptors

Evidence suggests that H^+-evoked ATP release triggers Ca^{2+} influx into ventral surface astrocytes, possibly via P2X receptors, and this leads to bulk ATP release and subsequent activation of CO_2/H^+-sensitive RTN neurons (Gourine *et al.* 2010). To explore this possibility further, we tested the effects of a high $MgCl_2$ and low $CaCl_2$ medium on the CO_2/H^+ sensitivity of RTN neurons. We used low (200 μM) rather than zero Ca^{2+} in order to minimize the effects of low Ca^{2+} on gap junctions. In addition, this medium has been shown to effectively block Ca^{2+}-dependent exocytosis with little disruption of intrinsic membrane characteristics (Richards & Sercombe, 1970). As before, CO_2/H^+-sensitive RTN neurons were identified based on their robust firing rate response to 15% CO_2 in normal Ringer solution. Exposure to low Ca^{2+}–high Mg^{2+} medium alone did not consistently increase baseline activity, suggesting that this low level of extracellular Ca^{2+} did not facilitate gap junction-mediated ATP release. However, as noted below low Ca^{2+}–high Mg^{2+} medium can inhibit excitatory input to RTN chemoreceptors (Fig. 4*C*), and so potentially offset excitatory effects of connexin-mediated ATP release. A second exposure to 15% CO_2, this time after ~10 min incubation in low Ca^{2+}–high Mg^{2+} solution, also elicited a strong firing rate response (Fig. 4*A* and *B*), indicating that a 10-fold decrease in the extracellular Ca^{2+} gradient did not affect CO_2/H^+ sensitivity (Fig. 4*B*). To confirm

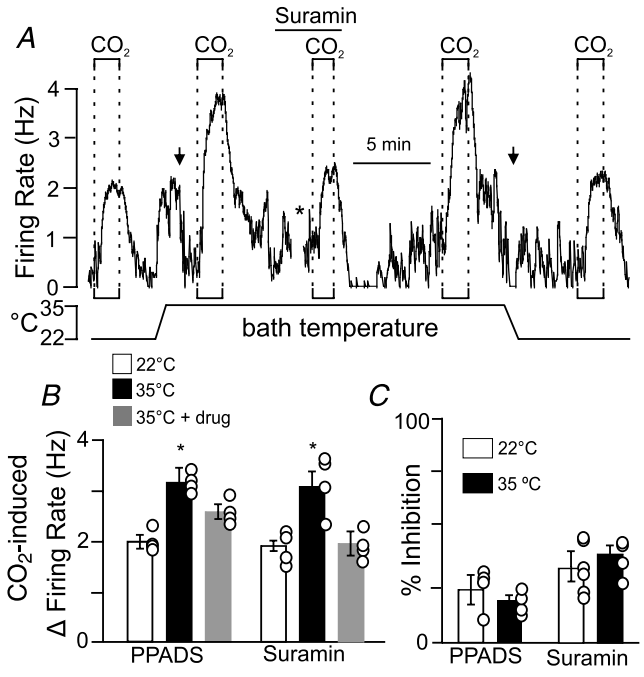

Figure 2 Purinergic contribution to CO_2/H^+ sensitivity of RTN neurons is not temperature dependent
A, traces of firing rate and bath temperature show CO_2/H^+ responsiveness at room temperature (~22°C) and 35°C with and without suramin (Sur, 100 μM), a P2 receptor antagonist. At room temperature, increasing bath CO_2 from 5 to 15% increased firing rate ~2 Hz. Heating to 35°C increased firing rate ~2 Hz. Prior to testing CO_2/H^+ sensitivity at 35°C we delivered a hyperpolarizing current (−5 nA; left arrow) into the cell to match firing rate to control activity. Under these conditions exposure to 15% CO_2 increased firing rate ~3.5 Hz. A 10 min incubation in suramin (*) decreased the firing rate response to 15% CO_2 by 40%. CO_2/H^+ sensitivity returned to control levels in wash. *B*, average data show the CO_2/H^+-induced firing rate response at room temperature (a portion of these results were published previously Wenker *et al.* 2010) and at 35°C with or without the P2 receptor antagonist PPADS (100 μM, $n = 4$) or suramin (100 μM, $n = 4$). Based on these results we calculated the Q_{10} of CO_2/H^+ sensitivity and purinergic drive to RTN neurons to be ~1.5 and ~1.3, respectively. *C*, purinergic drive to chemosensitive RTN neurons shown as % inhibition of CO_2/H^+ sensitivity by PPADS ($n = 11$) or suramin ($n = 11$) at 22°C and 35°C. Note that increasing temperature did not increase the purinergic drive to chemosensitive RTN neurons.

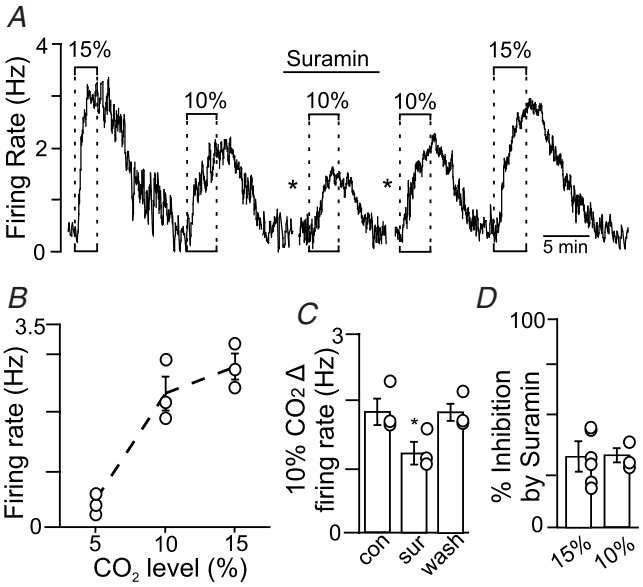

Figure 3. Purinergic drive does not increase with increased CO_2 intensity
A, firing rate trace shows that exposure to 10 and 15% CO_2 increased neuronal activity by 2.9 and 2.1 Hz, respectively. In the presence of suramin (100 μM), exposure to 10% CO_2 increased neuronal activity only 1.6 Hz. Responsiveness to 10 and 15% CO_2 was fully recovered when suramin was washed out. The asterisks denote 10 min time breaks. *B*, average data ($n = 3$) show that exposure to 10 and 15% CO_2 increased neuronal activity in a relatively linear manner. *C*, summary data ($n = 3$) show that suramin decreased the firing rate response to 10% CO_2. *D*, summary data ($n = 3$) show that suramin decreased the responsiveness to 10 and 15% CO_2 by similar amounts, indicating that purinergic drive to chemosensitive RTN neurons does not increase in response to this range of stimulus intensities.

that Ca^{2+}-dependent exocytosis was effectively attenuated by these incubation conditions, we recorded excitatory post-synaptic currents (EPSCs) from RTN chemoreceptor neurons during exposure to the low Ca^{2+}–high Mg^{2+} solution. As expected, ~5 min perfusion with low Ca^{2+}–high Mg^{2+} solution caused a robust and reversible decrease in EPSC frequency with only a modest effect on amplitude (Fig. 4*C*–*E*). These results suggest that the CO_2/H^+ sensitivity of RTN neurons, including the purinergic component, is not dependent on neuronal vesicle release.

Gap junction hemichannels contribute to CO_2/H^+-evoked ATP release in the RTN

There is evidence that connexin 26 hemichannels are directly gated by CO_2 (Huckstepp *et al.* 2010*a*) and contribute to CO_2-evoked ATP release in the RTN (Huckstepp *et al.* 2010*b*). To test whether connexin-mediated transmitter release contributes to the CO_2/H^+ sensitivity of RTN neurons, we used CBX and cobalt to block hemichannels. We found that ~10 min incubation in CBX (100 μM) decreased neuronal CO_2/H^+ sensitivity by 0.74 ± 0.18 Hz ($n = 6$) or $27.7 \pm 5.5\%$ (Fig. 5*A* and *B*). Likewise, incubation in cobalt (500 μM) decreased neuronal CO_2/H^+ sensitivity by 0.64 ± 0.08 Hz ($n = 5$) or $26.36 \pm 2.73\%$ (Fig. 5*C* and *D*). Note that both gap junction blockers decreased neuronal CO_2/H^+ sensitivity by an amount similar to the previously reported effects of P2 receptor antagonists (PPADS and suramin); at room temperature P2 receptor antagonists decreased CO_2/H^+ sensitivity of RTN neurons by 0.7 ± 0.18 Hz or $27.3 \pm 4.8\%$ (Wenker *et al.* 2010). Furthermore, in combination suramin and cobalt decreased CO_2/H^+ sensitivity of RTN neurons by $28 \pm 3.0\%$ (Fig. 6*A* and *B*). The per cent inhibition by suramin and cobalt acting together was not different from that of either blocker alone (Fig. 6*C*), further suggesting that the majority of CO_2-evoked ATP release is mediated by gap junction hemichannels. Together, these results strongly suggest that gap junction hemichannels mediate CO_2-evoked ATP release in the RTN.

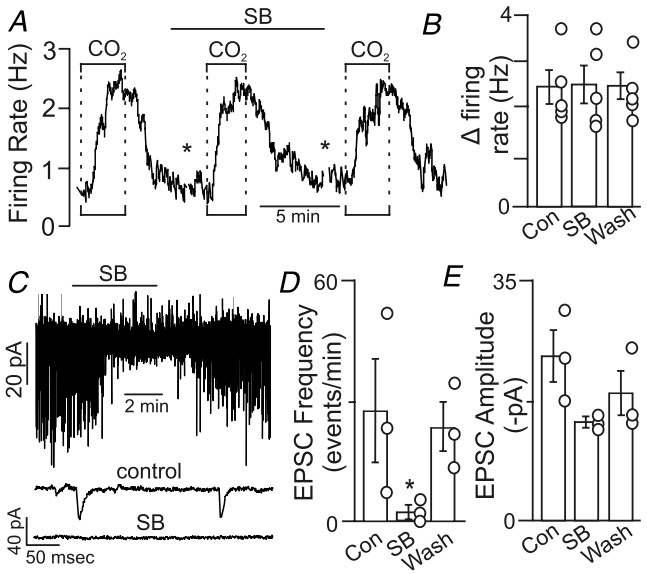

Figure 4. Purinergic drive to RTN neurons is not dependent on extracellular Ca^{2+}
A, firing rate trace shows that the CO_2/H^+ sensitivity of an RTN neuron was unaffected by low Ca^{2+}–high Mg^{2+} synaptic block (SB) medium. The asterisks denote 8 min time breaks. *B*, average data ($n = 5$) show that SB medium did not significantly affect CO_2/H^+ sensitivity ($P = 0.982$). *C*, traces of holding current (at a potential of −60 mV) show that exposure to SB medium blocked spontaneous excitatory post-synaptic currents (EPSCs) in chemosensitive RTN neurons. *D* and *E*, as expected, SB medium inhibited EPSC frequency (*D*, $n = 3$) but not EPSC amplitude (*E*, $n = 3$). Average decay time constant was 4.8 ± 0.2 ms (not shown). These results suggest that extracellular Ca^{2+} is not required for the purinergic drive to breathe.

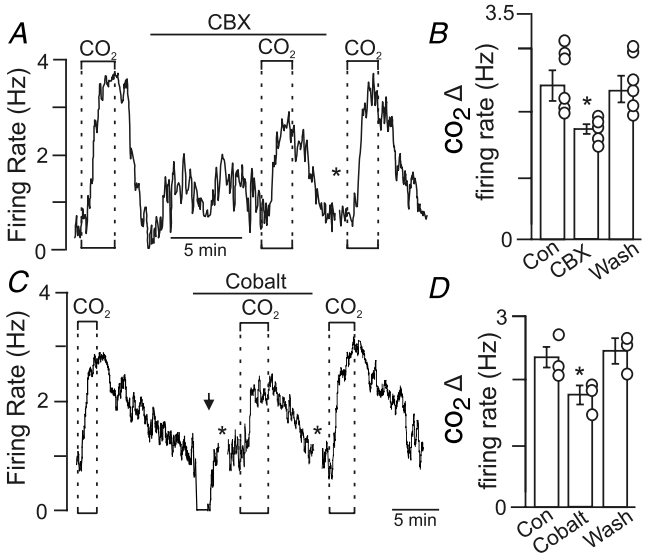

Figure 5. Gap junction blockers decrease purinergic drive to RTN neurons
A, trace of firing rate shows that under control conditions, exposure to 15% CO_2 increased the neuronal activity ~3 Hz. A second exposure to 15% CO_2, this time after 10 min incubation with carbenoxolone (CBX, 100 μM), increased firing rate only ~2 Hz. The asterisk indicates a gap in recording during the CBX wash. *B*, average data ($n = 6$) show that CBX decreased CO_2/H^+ sensitivity by ~30%. *C*, trace of firing rate shows that exposure to 15% CO_2 increased neuronal activity 2.1 Hz. After returning to control conditions, exposure to cobalt (500 μM) inhibited neuronal activity; therefore a depolarizing current (5 nA; arrow) was injected into the cell to approximate baseline activity under control conditions. In the continued presence of cobalt (with a holding current of 5.0 nA), a second exposure to 15% CO_2 this time only increased firing rate ~1.5 Hz. CO_2 responsivness was fully recovered after washing out cobalt for ~10 min. *D*, average data ($n = 5$) show that cobalt decreased CO_2/H^+ sensitivity by ~30%.

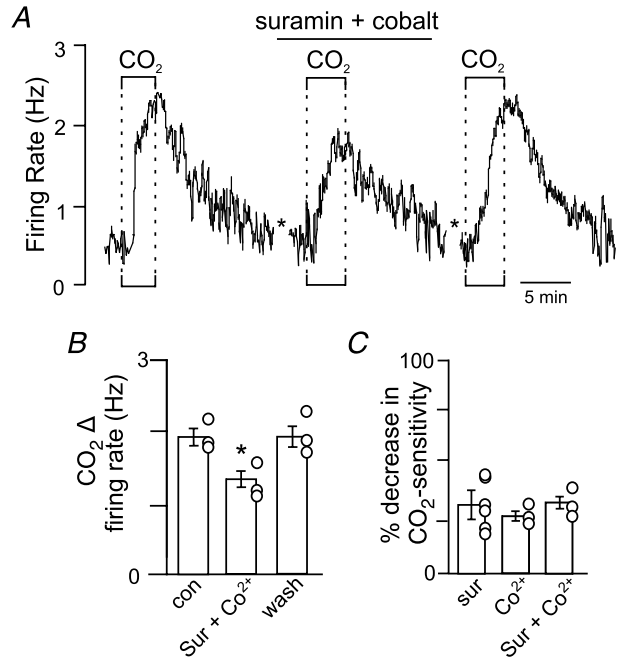

Figure 6. In combination, P2 receptor antagonist and gap junction blockers decrease CO_2/H^+ sensitivity by an amount similar to each blocker alone
A, trace of firing rate shows that under control conditions exposure to 15% CO_2 increased neuronal activity ∼2 Hz. A second exposure to 15% CO_2, this time in the presence of suramin to block P2 receptors and cobalt to block gap junction hemichannels, increased firing rate only ∼1 Hz or 72% of control. The asterisk indicates an 8 min time break. CO_2 sensitivity fully recovered after washing out suramin and cobalt. *B*, summary data ($n = 3$) show that in combination suramin and cobalt decreased CO_2/H^+ sensitivity of RTN neurons by 28 ± 3.0%. *C*, the per cent inhibition by suramin and cobalt acting together is not different from that of either blocker alone.

P2Y1 receptors in the RTN do not contribute to central chemoreception *in vitro* or *in vivo*

Evidence suggests that P2Y receptors mediate the excitatory effects of ATP on CO_2/H^+-sensitive RTN neurons (Mulkey *et al*. 2007). Of these, P2Y1 receptors have been shown to function as the primary substrate for ATP-mediated activation of inspiratory neurons in the preBötzinger complex (Lorier *et al.* 2007, 2008), a region critically involved in inspiratory rhythm generation (Smith *et al.* 1991). Therefore, we considered the possibility that P2Y1 receptors also contribute to up-stream purinergic modulation of CO_2/H^+-sensitive RTN neurons. To make this determination, we tested effects of MRS2179 (a potent and specific P2Y1 receptor blocker) on CO_2 sensitivity of RTN neurons *in vitro* and *in vivo*. In the brain slice preparation, incubation (∼10 min) in MRS2179 (3 μM) had negligible effects on baseline activity and CO_2/H^+ sensitivity of RTN neurons (Fig. 7*A* and *B*). In addition, focal application of a specific P2Y1 receptor agonist (MRS2365, 100 μM) had no effect on activity of CO_2/H^+-sensitive RTN neurons (Fig. 7*A*). These results suggest P2Y1 receptors are not expressed by CO_2/H^+-sensitive RTN neurons and do not contribute to their CO_2 sensitivity.

To determine if P2Y1 receptors contribute to CO_2-induced changes in breathing or blood pressure *in vivo*, we measured CO_2 responsivness after bilateral RTN injections of saline or MRS2179. As described above, under control conditions (i.e. after saline injections), exposure to hypercapnia (10% CO_2) increases PNA and causes hypotension (−16 ± 6 mmHg) followed by an increase in MAP (24 ± 5 mmHg) (Figs 1 and 8). Bilateral RTN injections of MRS2179 (100 μM in 50 nl) did not change resting MAP

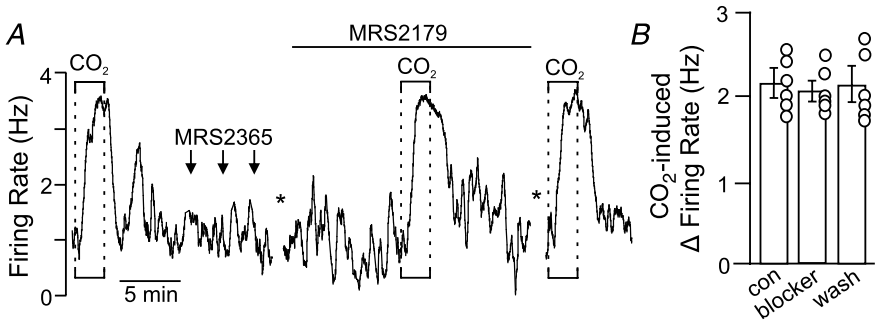

Figure 7. Purinergic drive to RTN neurons does not depend on P2Y1 receptors
A, trace of firing rate shows that under control conditions exposure to 15% CO_2 increased neuronal activity ∼3 Hz. A second exposure to 15% CO_2, this time in the presence of the P2Y1 receptor-specific antagonist MRS2179 (3 μM), also increased firing rate by ∼3 Hz. The asterisk indicates a 10 min time break during which the tissue was incubated in MRS2179. Arrows indicate when the P2Y1-specific agonist MRS2365 (100 μM in 7.3 Hepes buffer) was focally applied. *B*, summary data ($n = 6$) show that MRS2179 had no effect on the CO_2/H^+ sensitivity of RTN neurons.

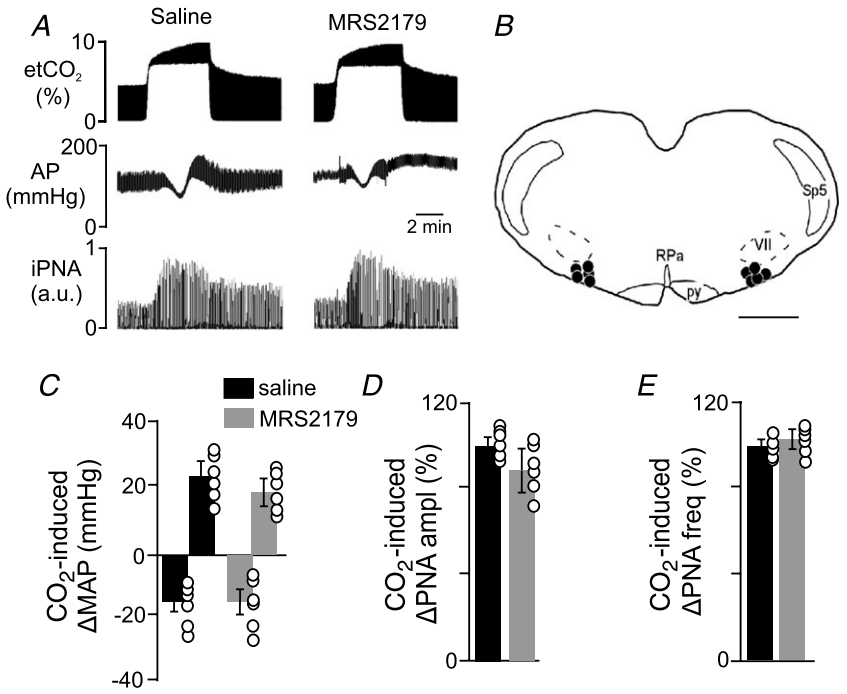

Figure 8. P2Y1 receptors do not contribute to CO_2-induced changes in breathing or blood pressure
A, traces of end-expiratory CO_2 ($etCO_2$), arterial pressure (AP) and integrated PNA (iPNA) show CO_2 responses 10 min after RTN injections of saline or MRS2179 (100 μM, 50 nl each side). *B*, computer-assisted plot of the centre of injection sites (Bregma −11.6; Paxinos & Watson, 1998). Scale bar in *B*, 1 mm. *C–E*, summary data ($n = 5$) showing changes in mean arterial pressure (ΔMAP) (*C*), PNA amplitude (ΔPNA ampl) (*D*) and PNA frequency (ΔPNA freq) (*E*) elicited by stepping the end-expiratory CO_2 from 5 to 10% after RTN injections of saline or MRS2179. Differences are expressed as a percentage of the response to CO_2 after saline injection. Abbreviations: py, pyramid; RPa, raphe pallidus; Sp5, spinal trigeminal tract; VII, facial motor nucleus.

(119 ± 5 mmHg compared with saline 121 ± 3 mmHg) or baseline PNA activity (102 ± 5% of control). In addition, injections of MRS2179 did not affect the CO_2-induced pressor response (24 ± 5 mmHg *vs.* saline 19 ± 4 mmHg) or the CO_2-induced increase in PNA amplitude (86 ± 15% of control) and PNA frequency (108 ± 5% of the control) (Fig. 8*A* and *C–E*). However, unilateral RTN injections of MRS2365 (100 μM in 50 nl) increased MAP by 23 ± 1 mmHg (compared with 4 ± 1 mmHg in control experiments) and increased PNA amplitude and frequency by 59 ± 6% and 53 ± 5%, respectively (Fig. 9*C–E*). Further, bilateral injections of MRS2179 entirely blocked the effects of MRS2365 (Fig. 9*A–E*), thus confirming effectiveness of the antagonist. These results are consistent with our *in vitro* evidence that P2Y1 receptors are not expressed by CO_2/H^+-sensitive RTN neurons and do not contribute to central CO_2 sensitivity.

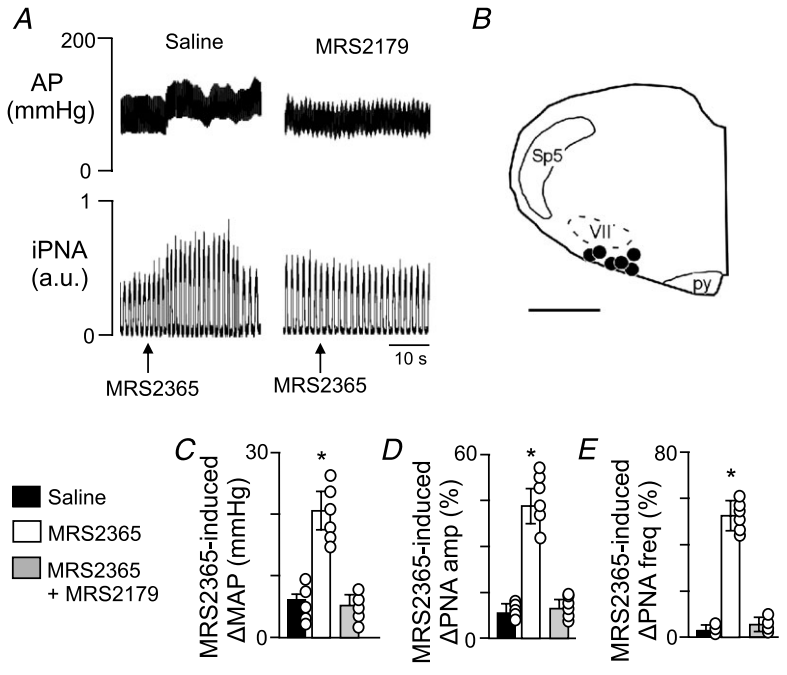

Figure 9. RTN injections of a specific P2Y1 agonist increased arterial pressure and PNA in anaesthetized vago-sino-aortic-denervated rats
A, traces of arterial pressure (AP) and integrated phrenic nerve activity (iPNA) show that unilateral RTN injections of the specific P2Y1 receptor agonist MRS2365 (100 μM in 50 nl) increased AP and PNA. *B*, computer-assisted plot of injection sites (coronal plane at Bregma −11.6 (Paxinos & Watson, 1998). Scale bar in *B*, 1 mm. *C–E*, summary data ($n = 6$) show MRS2365-induced ΔMAP (*B*), ΔPNA amp (*C*) and ΔPNA freq (*D*) after RTN injections of saline or MRS2179. Differences expressed as a percentage of the response to the CO_2 challenge elicited during saline injection. Abbreviations: py, pyramid; Sp5, spinal trigeminal tract; VII, facial motor nucleus.

Discussion

There is compelling evidence that CO_2-evoked ATP release from ventral surface astrocytes contributes to respiratory drive by activating chemosensitive RTN neurons (Gourine, 2005; Gourine *et al.* 2010; Wenker *et al.* 2010). Here, we demonstrate *in vivo* that blocking P2 receptors in the RTN lessened the changes in PNA amplitude, PNA frequency and pressor responses elicited by central chemoreflex activation. Additionally, we show *in vitro* that bath application of P2 receptor antagonists (PPADS and suramin) blunted the firing rate response of RTN neurons to 10% and 15% CO_2 by 30%. We show that the contribution of purinergic signalling to chemosensitive RTN neuronal activity is independent of temperature and stimulus strength. These results clearly indicate that purinergic signalling contributes in part to the integrated output of the RTN during hypercapnia. We also found that the purinergic drive to chemosensitive RTN neurons was wholly retained in low Ca^{2+} synaptic block medium but was significantly attenuated by gap junction blockers (CBX and cobalt), suggesting that astrocytes release ATP via gap junction hemichannels. Our results also show that P2Y1 receptors do not contribute to CO_2/H^+ sensitivity of the RTN; however, P2Y1 receptors are expressed in the region and their activation can influence respiratory and sympathetic outflow of the RTN. The identity and function of these P2Y1-expressing neurons remain unknown. We conclude that RTN chemoreception is determined primarily by CO_2/H^+-sensitive neurons but is augmented by gap junction-mediated, CO_2-evoked ATP release most probably from astrocytes.

Experimental limitations

Our *in vivo* experiments were performed is anaesthetized rats and it is well known that anaesthetics can modify centrally mediated cardio-respiratory reflexes. Therefore, it will be important for future studies to confirm the role of purinergic signalling in central chemoreception using conscious animals. In addition, we did not measure ATP directly but rather used the activity of RTN chemoreceptors as a functional measure of ATP release. This approach is reasonable considering that CO_2-evoked ATP release in the region of the RTN is well established (Gourine, 2005; Gourine *et al.* 2010; Huckstepp *et al.* 2010*b*) and the focus of the current study is on the purinergic drive to RTN neurons, which has received only limited attention. This study was also limited by the lack of pharmacological tools that allow for the specific manipulation of astrocytes without confounding effects on neurons. Nevertheless, the results presented here provide important direction for future experiments that take advantage of astrocyte-specific transgenic animal models, e.g. IP3-receptor type 2 (IP3R2) knockout mice (Petravicz *et al.* 2008) or the dominant negative synaptobrevin 2 model (Pascual *et al.* 2005), to further dissect neuron–astrocyte interactions in the context of chemoreception.

Contribution of purinergic signalling to RTN chemoreception

Bilateral injection of PPADS into the RTN of adult rats decreased the ventilatory response to CO_2 by ∼30%. These results are entirely consistent with previous evidence showing that direct application of PPADS onto the exposed ventral surface also decreased the hypercapnic ventilatory response *in vivo* by up to ∼30% (Gourine *et al.* 2005). In addition, we show by using brain slices from neonatal rat pups that bath application of PPADS or suramin decreased the response of functionally identified CO_2/H^+-sensitive RTN neurons by ∼30% regardless of stimulus strength or bath temperature. Considering that P2 receptor blockers had similar effects on chemosensitivity in neonatal (postnatal days 7–12) and adult rats suggests that the purinergic drive to RTN neurons does not increase over this age range. However, astrocyte are known to undergo dramatic changes during the first postnatal week, and in other brain regions it is not until ∼day 4 that characteristically mature astrocytes first appear (i.e. electrically passive) (Zhou *et al.* 2006). In the RTN, CO_2/H^+-sensitive astrocytes exhibit an electrically passive signature and presumably these cells are the source of ATP released in this region during hypercapnia (Wenker *et al.* 2010). Therefore, if mature astrocytes are absent from the RTN in newborn animals, than we expect CO_2/H^+ sensitivity to be determined exclusively by intrinsic neuronal properties. This is exactly what Onimaru *et al.* (2012) demonstrated using the *in vitro* brainstem–spinal cord preparation from newborn rats; bath application of P2 receptor blockers (MRS2179 or PPADS) had no effect on CO_2 responsivness of pFRG/RTN neurons. Our results clearly show in neonatal and adult rats that CO_2-evoked ATP release contributes to a portion of the hypercapnic ventilatory response by increasing baseline activity of chemosensitive RTN neurons at any given level of CO_2. However, it remains questionable whether purinergic signalling directly contributes to the H^+-sensing mechanism of RTN neurons. In addition, the purinergic-independent component of RTN chemoreception (∼70%) was retained when synaptic activity was blocked which strengthens the possibility that RTN neurons are CO_2/H^+ sensitive (Mulkey *et al.* 2004, 2006).

It is well established that hypercapnia produces biphasic changes in vascular tone, i.e hypotension followed by hypertension. The initial hypotension probably results from a direct effect of CO_2/H^+ on vasculature smooth muscle cells. Compensatory activation of sympathetic activity allows arterial pressure to recover to near control

levels during hypercapnia, and accounts for the increase in arterial pressure observed at the end of the hypercapnia episode (Moreira *et al.* 2006; Takakura & Moreira, 2011). Application of PPADS into the RTN region decreased the sympathetic-mediated pressure response to hypercapnia, suggesting a novel role of purinergic signalling in regulation of vascular tone during hypercapnia. For example, it is possible that CO_2-evoked ATP release provides excitatory drive to the nearby RVLM/C1 presympathetic neurons. In the presence of PPADS this excitatory drive would be reduced and so potentially decrease sympathetic output. However, this possibility remains speculative and in need of further investigation.

The identity of P2 receptors expressed by CO_2/H^+-sensitive RTN neurons remains unknown. It was shown previously that P2Y1 receptors mediate the excitatory effects of ATP on neurons in the preBötzinger complex (Lorier *et al.* 2007, 2008). However, we found that P2Y1 receptors are not expressed by chemosensitive RTN neurons and do not contribute to CO_2/H^+ sensitivity of these cells *in vitro* or to CO_2-induced changes in breathing or blood pressure *in vivo*. Therefore, P2Y1 receptors do not contribute to RTN chemoreception. However, activation of P2Y1 receptors in the RTN increased breathing and MAP, suggesting that P2Y1 receptors are expressed in the region and, when activated, can influence respiratory and sympathetic outflow. Considering that ADP not ATP is the preferential ligand for P2Y1 receptors, we speculate that ATP released during hypercapnia is not readily broken down to ADP and adenosine, thus limiting the contribution of these unknown P2Y1-expressing neurons to regulation of breathing or blood pressure. A focus of future work will be to determine the identity and function of these P2Y1-expressing RTN neurons. Nevertheless, the absence of an identifiable G-protein-coupled purinergic receptor on chemosensitive RTN neurons suggests that one or more P2X receptors may contribute to purinergic modulation of chemosensitive RTN neurons, possibly P2X1 and/or P2X3 (Gourine *et al.* 2010).

Requisite role of gap junction hemichannels in purinergic drive to RTN chemoreceptors

Our evidence that gap junction blockers (CBX and cobalt), but not synaptic block medium, decreased chemosensitive RTN neuronal activity by an amount proportional to inhibition by P2 receptor antagonists, suggests that the purinergic drive to RTN neurons is dependent on hemichannels. We consider it unlikely that communication between astrocytes via gap junctions is a major determinant of purinergic drive to neurons in this region for two reasons. First, RTN astrocytes are unusual in that they exhibit limited dye-coupling compared with astrocytes in other brain regions (Wenker *et al.* 2010), suggesting that these cells are not extensively gap junction coupled. In addition, previous evidence showed that cobalt preferentially blocks hemichannel current with little effect on transjunctional current through gap junctions in embryonic stem cells (Huettner *et al.* 2006). Our results are consistent with a recent *in vivo* study that showed that application of connexin hemichannel antagonists near the RTN inhibited CO_2-evoked ATP release and decreased the whole animal ventilatory response to CO_2 by ∼25% (Huckstepp *et al.* 2010*b*).

There is convincing evidence that CO_2-evoked ATP release in the RTN is dependent on connexin 26 (Cx26) (Huckstepp *et al.* 2010*a*). The mechanism by which CO_2 influences the gating of Cx26 hemichannels is not fully understood. Evidence suggests that molecular CO_2 binds directly to Cx26 to increase the open probability of the hemichannel (Huckstepp *et al.* 2010*a*). However, it has also been shown that CO_2/H^+ sensitivity of RTN astrocytes is determined, in part, by inhibition of Kir4.1–Kir5.1 channels (Wenker *et al.* 2010), suggesting that membrane depolarization could trigger ATP release. This mechanism could be parsimonious with the current data considering that certain hemichannels exhibit voltage-dependent gating (Sáez *et al.* 2005). However, rat Cx26 does not demonstrate voltage dependence (Gonzalez *et al.* 2006); thus, if Cx26 is the main conduit for ATP release from RTN astrocytes then acid-induced changes in membrane potential might not contribute. Consistent with this possibility, a recent *in vivo* study reported that Kir5.1 knockout mice exhibit normal ventilatory response to CO_2 *in vivo* (Trapp *et al.* 2011), suggesting that astrocytic membrane depolarization is not required for CO_2-evoked ATP release. However, it should be recognized that Kir5.1 channels are expressed in other organs, including the kidneys, and as described by Trapp *et al.* these conventional knockout mice have profound metabolic acidosis which could conceivably interfere with this acid-sensing mechanism. Thus, the link between CO_2/H^+-induced changes in astrocytic membrane potential and ATP release remains to be definitively established.

Role of astrocytes in RTN purinergic signalling

There is general agreement that astrocytes (or more precisely glial-like cells that express astrocytic markers) are the most likely source of ATP release (Gourine *et al.* 2010, Huckstepp *et al.* 2010*b*; Wenker *et al.* 2010). For example, Gourine and colleagues demonstrated, using astrocyte-specific expression of channel rhodopsin 2, a light-sensitive cation channel, that generating calcium waves in astrocytes results in ATP release that can activate RTN neurons *in vitro* and increase breathing *in vivo* (Gourine *et al.* 2010). Likewise, we have shown that

fluorocitrate-mediated activation of astrocytes resulted in increased activity of chemosensitive RTN neurons by a purinergic-dependent mechanism (Wenker *et al.* 2010). In addition, Huckstepp and colleagues showed that hypercapnia opened connexin hemichannels on glial fibrillary acidic protein (GFAP)-expressing cells (Huckstepp *et al.* 2010*a*), thus suggesting that direct CO_2 gating of connexin hemichannels results in ATP release from astrocytes. We show here that CO_2 sensitivity of RTN neurons is fully retained in low Ca^{2+}–high Mg^{2+} solution, thus further suggesting that that purinergic drive to RTN neurons is not dependent on excitatory synaptic transmission.

In summary, the RTN contains a population of CO_2/H^+-sensitive neurons that sends excitatory glutamatergic projections to respiratory centres to stimulate breathing (Mulkey *et al.* 2004) and modulate sympathetic drive to the heart and vasculature (Moreira *et al.* 2006; Takakura & Moreira, 2011). In addition to activation by CO_2/H^+, RTN neurons are also modulated by various neurotransmitters, including ATP (Mulkey *et al.* 2006; Gourine *et al.* 2010; Huckstepp *et al.* 2010*b*). Based on previous and present results, astrocytes (but not neurons) most probably mediate CO_2-evoked ATP release by a mechanism involving gap junction hemichannels.

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Author contributions

I.C.W.: experimental design; collection and analysis of *in vitro* data; revising the manuscript. C.R.S.: collection and analysis of *in vivo* data; revising the manuscript. A.C.T.: experimental design; collection and analysis of *in vivo* data; revising the manuscript. T.S.M.: experimental design; collection, analysis and interpretation of *in vivo* data; revising the manuscript. D.K.M.: experimental design; data analysis; revising the manuscript; drafting the manuscript. All authors approved the final version.

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P2Y1 Receptors Expressed by C1 Neurons Determine Peripheral Chemoreceptor Modulation of Breathing, Sympathetic Activity, and Blood Pressure

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P2Y1 Receptors Expressed by C1 Neurons Determine Peripheral Chemoreceptor Modulation of Breathing, Sympathetic Activity, and Blood Pressure

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Abstract—Catecholaminergic C1 cells of the rostral ventrolateral medulla (RVLM) are key determinants of the sympathoexcitatory response to peripheral chemoreceptor activation. Overactivation of this reflex is thought to contribute to increased sympathetic activity and hypertension; however, molecular mechanisms linking peripheral chemoreceptor drive to hypertension remain poorly understood. We have recently determined that activation of P2Y1 receptors in the RVLM mimicked effects of peripheral chemoreceptor activation. Therefore, we hypothesize that P2Y1 receptors regulate peripheral chemoreceptor drive in this region. Here, we determine whether P2Y1 receptors are expressed by C1 neurons in the RVLM and contribute to peripheral chemoreceptor control of breathing, sympathetic activity, and blood pressure. We found that injection of a specific P2Y1 receptor agonist (MRS2365) into the RVLM of anesthetized adult rats increased phrenic nerve activity (≈55%), sympathetic nerve activity (38±6%), and blood pressure (23±1 mm Hg), whereas application of a specific P2Y1 receptor antagonist (MRS2179) decreased peripheral chemoreceptor–mediated activation of phrenic nerve activity, sympathetic nerve activity, and blood pressure. To establish that P2Y1 receptors are expressed by C1 cells, we determine in the brain slice preparation using cell-attached recording techniques that cells responsive to MRS2365 are immunoreactive for tyrosine hydroxylase (a marker of C1 cells), and we determine in vivo that C1-lesioned animals do not respond to RVLM injection of MRS2365. These data identify P2Y1 receptors as key determinants of peripheral chemoreceptor regulation of breathing, sympathetic nerve activity, and blood pressure. (***Hypertension*****. 2013;62:263-273.)** ● **Online Data Supplement**

Key Words: hypertension ■ medulla oblongata ■ purinergic effects ■ sleep apnea, obstructive

Peripheral chemoreceptors located in the carotid sinus sense changes in arterial CO_2 and O_2 and communicate this information to cardiorespiratory centers to regulate breathing and sympathetic outflow to ensure adequate ventilation–perfusion matching in tissues. There is strong evidence that overactivation of the peripheral chemoreflex by repeated bouts of hypoxia contributes to hypertension and cardiovascular mortality associated with obstructive sleep apnea.[1–4] However, despite intense investigation, molecular mechanisms linking peripheral chemoreceptor drive to hypertension remain poorly understood.

It is well known that peripheral chemoreceptor afferents first synapse in the caudal portion of the nucleus tractus solitarii (cNTS)[5,6] before relaying this information primarily to the rostral ventrolateral medulla (RVLM), including at the level of the retrotrapezoid nucleus (RTN).[7–9] This brain stem region contains ≥2 functionally discrete but anatomically overlapping populations of neurons: CO_2/H^+-sensitive RTN neurons that seem to function as respiratory chemoreceptors[10] and C1 and non-C1 cells that control sympathetic vasomotor tone and arterial pressure.[11–14] Evidence suggests that both RTN chemoreceptors[9] and C1 cells[15] receive peripheral chemoreceptor drive. The transmitter basis for this drive is thought to depend largely on glutamate because NTS terminals in this region are immunoreactive for vesicular glutamate transporter 2 (VGLUT2, a marker of glutamatergic cells)[16,17], and bilateral injections of kynurenic acid (glutamate receptor blocker) blunted peripheral chemoreceptor–mediated activation of breathing and blood pressure.[9,18] However, purinergic signaling has also been implicated in the peripheral chemoreflex,[19–22] including at the level of the RVLM.[23] For example, chemosensitive RTN neurons[24,25] and presympathetic RVLM neurons[26] are activated by purinergic agonists, application of purinergic agonists into the RVLM increased breathing[27,28] and blood pressure[29,30] in anesthetized rats, and inhibition of P2 receptors in the nearby Bötzinger and

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pre-Bözinger complex blunted the respiratory response evoked by peripheral chemoreceptor activation in awake rats.[23] These results suggest that purinergic signaling contributes to the peripheral chemoreflex mechanism. However, the identity of P2 receptors regulating the peripheral chemoreflex at this level of the brain stem is unknown.

We have recently determined that P2Y1 receptors are expressed in the RVLM.[31] Although these receptors do not influence cardiorespiratory responses to hypercapnia, application of a P2Y1 receptor agonist into this region mimicked effects of peripheral chemoreceptor activation by increasing breathing and blood pressure.[31] Therefore, we hypothesize that P2Y1 receptors are differentially expressed by C1 neurons and function as key determinants of peripheral chemoreceptor drive through this region. Consistent with this possibility, we find that inhibition of P2Y1 receptors in the RVLM decreased breathing, sympathetic nerve activity (SNA), and blood pressure responses to cyanide-induced activation of peripheral chemoreceptors in anesthetized rats. Furthermore, NTS terminals at this level of the RVLM are immunoreactive for vesicular nucleotide transporter (VNUT). To establish that P2Y1 receptors are expressed by C1 cells, we determine in the brain slice preparation that cells responsive to specific P2Y1 receptor agonist (MRS2365) are immunoreactive for tyrosine hydroxylase (TH, a marker of C1 cells), and we determine in vivo that C1-lesioned animals do not respond to RVLM injection of MRS2365. We find that P2Y1 receptors are key determinants of peripheral chemoreceptor regulation of breathing, SNA, and blood pressure.

Methods

All procedures were performed in accordance with National Institutes of Health and the University of Connecticut and University São Paulo Animal Care and Use Guidelines. An expanded Methods section is available in the online-only Data Supplement.

Results

This study consists of both in vivo and in vitro experiments. First, to determine whether purinergic signaling in the RVLM contributes to peripheral chemoreceptor regulation of breathing, sympathetic activity, or blood pressure, we measured these parameters during cyanide-induced activation of peripheral chemoreceptors after bilateral RVLM injections of saline, a nonspecific P2 receptor blocker (pyridoxalphosphate-6-azophenyl-2′,4′-disulfonic acid [PPADS]) or a specific P2Y1 receptor blocker (MRS2179).[32] To further support the possibility that purinergic signaling contributes to peripheral chemoreceptor drive, we determine the extent to which NTS terminals in the RVLM are immunoreactive for VNUT and VGLUT2. Although our focus is on the peripheral chemoreflex, we also tested the possibility that purinergic signaling via P2Y1 receptors contributes to other reflexes mediated by C1 cells, including the somatosympathetic reflex and the baroreflex. Second, to determine which neurons express P2Y1 receptors, we used slice-patch recording techniques to measure neuronal responses to focal application of a specific P2Y1 receptor agonist (MRS2365).[33] As in previous studies,[10,31] we define RTN chemoreceptors as cells that respond to 15% CO_2 with ≥1.5 Hz increase in firing rate. Neurons that did not exhibit this minimum firing rate response to 15% CO_2 were considered nonchemosensitive. Previous evidence suggests that the majority of CO_2/H^+-insensitive neurons in this region are presympathetic neurons that regulate blood pressure,[10] approximately two-thirds of which are C1 cells known to express TH and one third are non-C1 cells. Therefore, we use TH immunoreactivity to confirm the identity of MRS2365-responsive cells recorded in vitro, and in parallel we test MRS2365 responsiveness in C1-lesioned animals.

Purinergic Signaling in the RVLM Contributes to Peripheral Chemoreceptor Regulation of Breathing, Sympathetic Outflow, and Blood Pressure

To determine whether purinergic signaling contributes to peripheral chemoreceptor transduction in the RVLM, we measured breathing, sympathetic outflow, and blood pressure responses to peripheral chemoreceptor activation after bilateral RVLM injections of saline or a purinergic receptor antagonist (Figure 1A).[34] The injection center was 250 µm below the facial motor nucleus and 200 µm rostral to the caudal end of this nucleus (Figure 1B).[10,35] Bilateral injections of PPADS (100 µmol/L, 50 nL) into the RVLM did not change baseline mean arterial pressure (MAP; 119±5 mm Hg compared with saline 121±6 mm Hg), splanchnic SNA (sSNA; 101±11% of control), or phrenic nerve activity (PNA) activity (97±4% of control value). However, PPADS treatment strongly inhibited cardiorespiratory responses to peripheral chemoreceptor activation. For example, PPADS attenuated the increase in sSNA (78±10% versus saline 211±13%; P=0.015; n=7), MAP (12±3 mm Hg versus saline 25±6 mm Hg; P=0.037), and PNA amplitude (84±4% versus saline 121±14%; P=0.038) and frequency (45±7% versus saline 118±8%; P=0.026) elicited by cyanide (Figure 1A, 1C–1F).

Previous evidence showed that P2Y1 receptors are expressed in the RVLM.[31] However, P2Y1 receptors do not influence CO_2 responsiveness in vitro or in vivo, suggesting that these receptors relay a purinergic signal disparate from CO_2-evoked ATP release, but still essential to autonomic regulation of cardiorespiratory homeostasis. To determine whether P2Y1 receptors are part of the peripheral chemoreceptor circuit, we repeated the experiments described above using the specific P2Y1 receptor antagonist (MRS2179) alone or in combination with a nonspecific ionotropic glutamate receptor antagonist (kynurenic acid; Figure 2B). As previously reported,[31] bilateral RVLM injections of MRS2179 (100 µmol/L to 50 nL) did not change resting MAP (123±6 mm Hg compared with saline 122±8 mm Hg; P=0.21), sSNA (99±9% of control; P=0.77), or PNA (102±5% of control; P=0.86), suggesting that P2Y1 receptor blockade does not alter basal activity of C1 cells. Interestingly, bilateral injections of MRS2179 into this same region decreased the cyanide-induced pressor response from 22±3 to 15±2 mm Hg (P=0.036; n=6/group; Figure 2A and 2C) and decreased the sympathoexcitatory response from 218±17% to 126±8% (P=0.028; Figure 2A and 2D). Similarly, MRS2179 also decreased peripheral chemoreceptor activation of PNA amplitude and frequency by 25±6% (P=0.037; Figure 2A and 2E) and 33±7% (P=0.032; Figure 2A and 2F), respectively. Consistent with previous evidence,[36] bilateral RVLM injections of kynurenic acid decreased baseline PNA amplitude

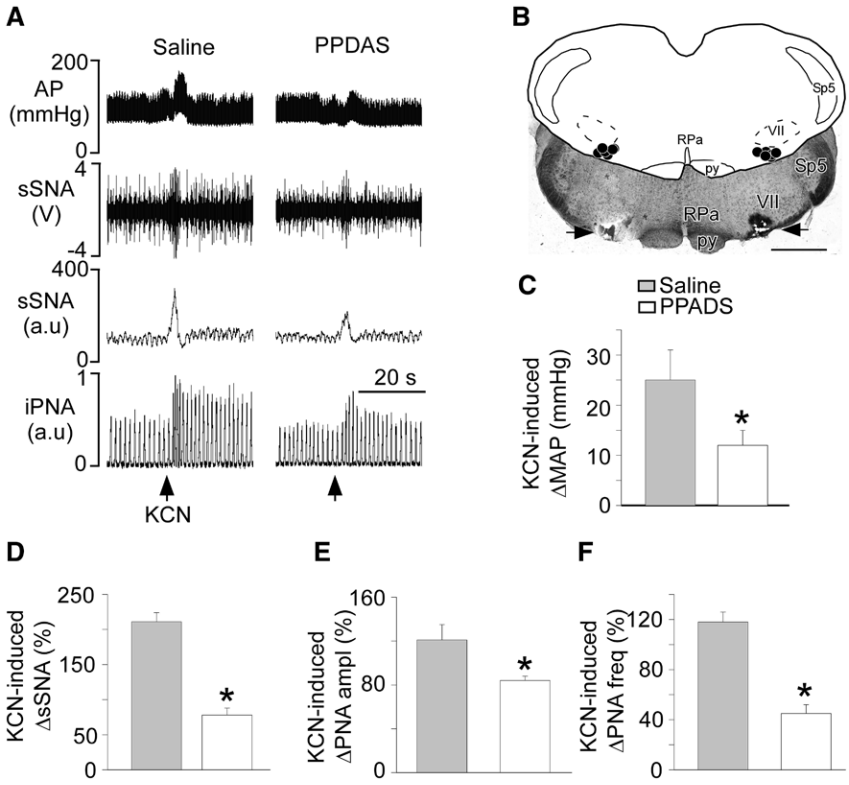

Figure 1. Purinergic signaling in the rostral ventrolateral medulla (RVLM) is necessary for peripheral chemoreceptor regulation of breathing, blood pressure, and sympathetic activity. **A**, Traces of arterial pressure (AP), raw and integrated (∫) splanchnic sympathetic nerve activity (sSNA), and integrated phrenic nerve activity (∫PNA) show the response of a urethane-anaesthetized rat to activation of peripheral chemoreceptors with cyanide (KCN; 40 μg/0.1 mL IV) 5 minutes after bilateral RVLM injections of saline and pyridoxalphosphate-6-azophenyl-2′,4′-disulfonic acid (PPADS; 100 μmol/L, 50 nL). **B**, Computer-assisted plot and representative photomicrograph show PPADS injection sites in the RVLM (Bregma –11.6).[34] Scale bar, 1 mm. **C** through **F**, Summary data (n=6 rats/group) show that RVLM injections of PPADS decreased effects of peripheral chemoreceptor activation on mean AP (MAP; **C**), sSNA (**D**), PNA amplitude (**E**), and PNA frequency (**F**). *P<0.05. VII indicates facial motor nucleus; py, pyramidal tract; RPa, raphe pallidus; and Sp5, spinal tract of trigeminal nerve.

(59±5%) and increased PNA frequency (46±4%), but did not affect sSNA or MAP. Injections of kynurenic acid into the RVLM also blunted the peripheral chemoreceptor–mediated increase in breathing amplitude, SNA, and blood pressure (Figure 2A, 2C–2F). The effect of kynurenic acid on PNA frequency was reduced by MRS2179 (137±6 versus 169±4 breaths/min; Figure 2A and 2F). In addition, the combination of kynurenic acid and MRS2179 further decreased peripheral chemoreceptor–mediated changes in MAP (64% inhibition), sSNA (69% inhibition), and phrenic nerve discharge amplitude (68% inhibition) by more than either blocker alone (Figure 2A, 2C–2E). These results indicate that purinergic

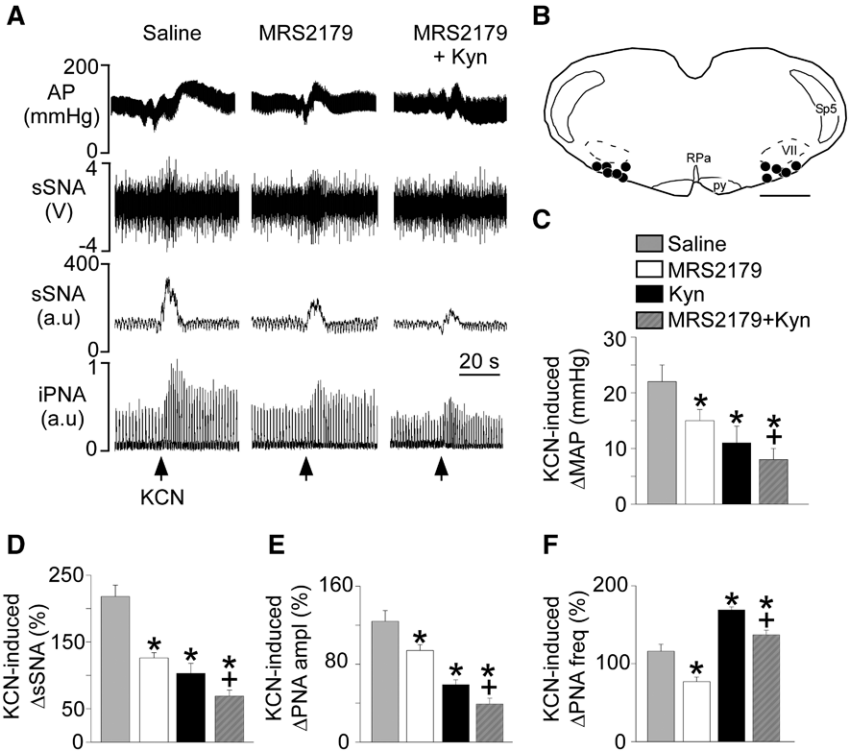

Figure 2. P2Y1 and ionotropic glutamate receptors regulate peripheral chemoreflex in the rostral ventrolateral medulla (RVLM). **A**, Traces of arterial pressure (AP), raw and integrated (∫) splanchnic sympathetic nerve activity (sSNA), and integrated phrenic nerve activity (∫PNA) show the response of a urethane-anaesthetized rat to KCN (40 μg/0.1 mL IV)-induced activation of the peripheral chemoreflex 5 minutes after bilateral RVLM injections of saline (**left**), MRS2179 (100 μmol/L, 50 nL) alone (**middle**), and MRS2179 and kynurenic acid (100 mmol/L, 50 nL; **right**). **B**, Computer-assisted plot of injection sites in the RVLM (Bregma –11.6).[34] Scale bar, 1 mm. Summary data (n=6 rats/group) show that RVLM injections of MRS2179 and kynurenic acid alone and in combination decreased effects of peripheral chemoreceptor activation on mean AP (MAP; **C**), sSNA (**D**), PNA amplitude (**E**), and PNA frequency (**F**). *P<0.05. VII indicates facial motor nucleus; py, pyramidal tract; RPa, raphe pallidus; and Sp5, spinal tract of trigeminal nerve.

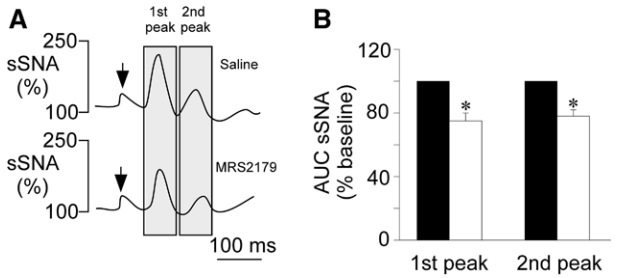

Figure 3. Purinergic signaling via P2Y1 receptors in the rostral ventrolateral medulla (RVLM) contributes to the somatosympathetic reflex. **A**, Grouped effect of sciatic nerve–evoked stimulation of splanchnic sympathetic nerve activity (sSNA) at control period (saline injection) and after bilateral injection of MRS2179 (100 µmol/L to 50 nL; n=5) into the RVLM. Arrows indicate the time of stimulation. **B**, Grouped data illustrating the effects of saline or MRS2179 on the area under the curve (AUC) of the first and second sympathoexcitatory peaks for sSNA (**A**). *P<0.05 compared with control (saline group).

signaling via P2Y1 receptors is an important component of peripheral chemoreceptor drive through the RVLM.

Injections located outside the RVLM region often reached the facial motor nucleus or dorsal to it (3 of 4), and 1 injection (1 of 4) was located in the parapyramidal region (data not shown). Bilateral injections of PPADS, MRS2179, or kynurenic acid into the facial motor nucleus or parapyramidal region did not change the cyanide-induced respiratory or pressor responses in these animals (data not shown).

Purinergic Signaling in the RVLM Contributes to the Excitatory Somatosympathetic Reflex But Not the Inhibitory Baroreflex

To determine whether P2Y1 receptor–dependent modulation of C1 cells is specific to the peripheral chemoreflex, we also tested effects of MRS2179 on 2 other reflexes mediated by C1 cells: the somatosympathetic reflex which is thought to be mediated by glutamate in the RVLM[37] and the baroreflex which is inhibitory and largely mediated by gamma-aminobutyric acid in the RVLM.[38] The somatosympathetic reflex was represented by 2 characteristic excitatory peaks in the sSNA in response to intermittent sciatic nerve stimulation under baseline conditions (control) and after injections of saline or MRS2179 (100 µmol/L to 50 nL). As shown in Figure 3A, latencies of the peaks of sSNA (85±3 and 176±3 ms, respectively; n=4) were not significantly altered by bilateral injection of MRS2179 (sSNA: 89±4 and 179±5 ms, respectively; P=0.145; n=5). However, bilateral RVLM injections of MRS2179 decreased the area under the curve of each sSNA peak by 25±4% (P=0.042) and 22±5% (P=0.044), respectively (Figure 3A and 3B).

The baroreflex was measured by raising arterial pressure with phenylephrine (5 µg/kg, IV) and lowering arterial pressure with sodium nitroprusside (30 µg/kg, IV). A baroreflex curve relating MAP and SNA was constructed for each rat under control conditions and 10 minutes after bilateral injections of saline or MRS2179 (100 µmol/L to 50 nL; n=5). We found that the baroreflex operated around a comparable MAP50 in all groups (MRS2179: 116±9 versus saline 118±6 mm Hg; P=0.46; Table). Injections of MRS2179 into the RVLM did not change the range (135±11% versus saline 138±13%; P=0.37) and the gain (5.3±0.5 versus saline 5.4±1%; P=0.71) of the sympathetic baroreflex (Table).

To confirm in vivo that MRS2179 is specific to P2Y1 receptors and does not disrupt glutamatergic signaling, we test effects of MRS2179 on cardiorespiratory responses to RVLM injections of glutamate in urethane-anesthetized rats. Injection of MRS2179 (100 µmol/L to 50 nL; n=5) in the RVLM did not change the increase in MAP (23±4 mm Hg versus saline 27±2 mm Hg; P=0.064), sSNA (34±8% versus saline 33±9%; P=0.084), PNA amplitude (17±2% versus saline 18±4%; P=0.13), or PNA frequency (14±2% versus saline 16±4%; P=0.077) evoked by unilateral injection of glutamate (10 mmol/L to 50 nL) in the RVLM (Figure S1 in the online-only Data Supplement).

Together, these results suggest that application of MRS2179 into the RVLM does not antagonize glutamate receptors. These results also suggest that purinergic signaling via P2Y1 receptors in the RVLM contributes to excitatory (ie, peripheral chemoreflex and the somatosympathetic reflex) but not inhibitory baroreflex control of sympathetic activity. This study focuses on the peripheral chemoreflex because overactivation of this reflex is thought to contribute to hypertension associated with obstructive sleep apnea.

VNUT Is Expressed by NTS Neuronal Terminals in the RVLM

Our observation that P2Y1 receptors in the region of the RVLM contribute to the peripheral chemoreflex suggests that synapses activated in the RVLM during peripheral chemoreceptor stimulation release purinergic signaling molecules. To build on this possibility, we injected the anterograde tracer biotinylated dextran amine (BDA) into the cNTS (Figure 4A and 4B) and subsequently performed immunohistochemistry to determine whether cNTS terminals in the RVLM express VNUT, the protein responsible for vesicular storage and release of nucleotides.[39] Considering that purinergic

Table. Average Parameters of Sigmoid Baroreflex Curves in Rats Treated With Saline or MRS2179

Group	n	Baseline MAP, mm Hg	MAP50, mm Hg	Upper Plateau, %	Lower Plateau, %	Range, %	Gain max, %/mm Hg
Saline (control)	5	117±3	118±6	157±9	18±4	135±11	5.4±1
MRS2179	5	115±8	116±9	158±7	19±6	138±13	5.3±0.5

Baroreflex analysis after bilateral injection of saline or MRS2179 (100 µmol/L to 50 nL) injection into RVLM. Curves relating splanchnic nerve activity (sSNA) and mean arterial pressure (MAP) were generated by lowering MAP with sodium nitroprusside (SNP, 30 µg/kg, IV) and increasing MAP with phenylephrine (Phe, 5 µg/kg, IV). Baseline sSNA was set to 100%, and minimum sSNA was determined after intravenous injection of hexamethonium (30 mg/kg, IV). RVLM indicates rostral ventrolateral medulla.

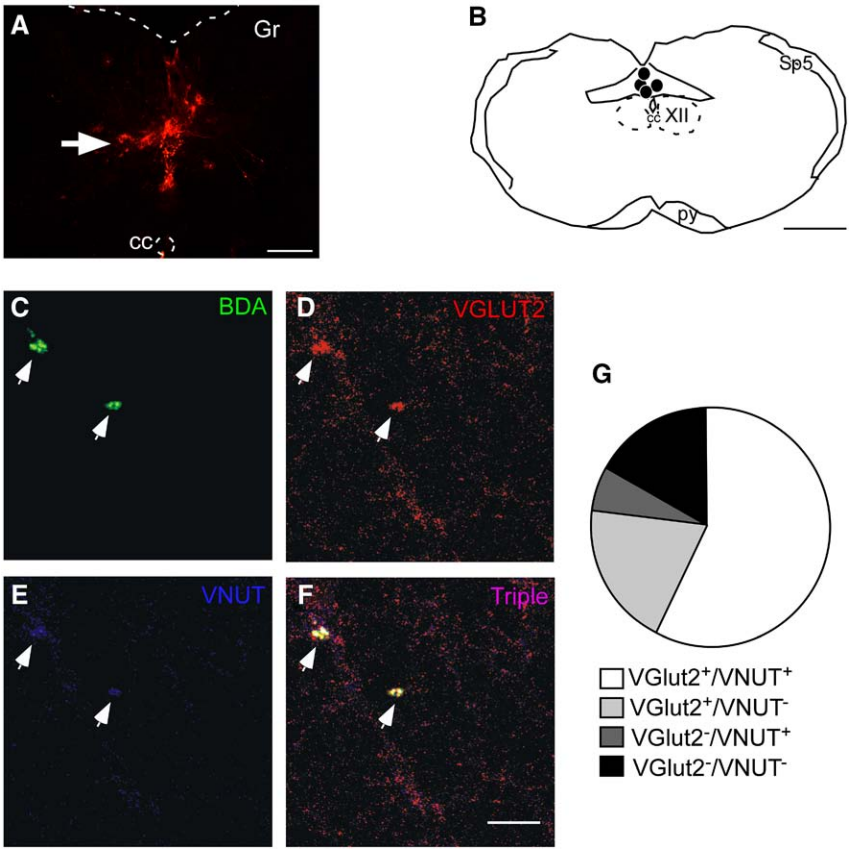

Figure 4. Projections from the caudal nucleus tractus solitarii (NTS) to the rostral ventrolateral medulla (RVLM) at the level of the retrotrapezoid nucleus (RTN) are immunoreactive for vesicular glutamate transporter 2 (VGLUT2) and vesicular nucleotide transporter (VNUT). Fluorescent microbeads were injected with BDA to confirm the location of injection sites. Computer-assisted plot (**A**) and representative photomicrograph (**B**) of the boxed region in **A** shows BDA injection sites in the caudal NTS (cNTS). All injection sites were within 200 μm of the caudal edge of the area postrema (Bregma=−13.8 mm).[34] Scale bar, 200 μm. Digital images show that the majority of BDA-labeled cNTS terminals in the RVLM (green, **C**) are immunoreactive for VGLUT2 (red, **D**) and VNUT (blue, **E**). **F**, Merged image. The double arrow designates a cNTS terminal in the RVLM that was VNUT positive but VGLUT2 negative. Scale bar, 25 μm. **G**, Summary data (n=4 rats) show the proportion of cNTS terminals in the RVLM that were immunopositive for VGLUT2 and VNUT. XII indicates hypoglossal nucleus; cc, central canal; Gr, gracile nucleus; py, pyramidal tract; and Sp5, spinal tract of trigeminal nerve.

nucleotides are known to be coreleased with glutamate at certain central synapses[39] and cNTS projections to the RVLM are known to be glutamatergic,[9] we also assayed for VGLUT2 to determine whether glutamate and nucleotides are colocalized in the same terminals.

We focused our observations on the marginal layer of the ventrolateral medulla between Bregma −11.3 and Bregma −12.2 because this region contains chemosensitive[9,10,25,31] neurons and C1[15,17] cells, and it is known to receive a dense input from the cNTS.[9] BDA-labeled varicosities were assumed to be terminals (putative synapse). This possibility is supported by our evidence that the majority of BDA labeling was immunoreactive for VNUT and VGLUT2. Figure 4C–4F shows examples of this staining where terminals from the cNTS (green) are immunoreactive for VGLUT2 (red) and VNUT (blue). A total of 126 terminals from 31 RVLM regions (n=4 rats) were counted, and 72 (57%) were positive for both VGLUT2 and VNUT, 25 (20%) were VGLUT2-positive only, 8 (6%) were VNUT-positive only, and 21 (17%) lacked any discernible immunoreactivity (Figure 4G). Although it is not surprising to find numerous BDA-labeled terminals in which no other immunoreactivity could be detected, it should be noted that in the absence of a vesicular marker, at least some BDA labeling may reflect cut axons rather than terminals.

C1 Cells But Not RTN Chemoreceptors Express P2Y1 Receptors

To determine the cellular distribution of P2Y1 receptors in the region of the RVLM, we used the brain slice preparation to make cell-attached recordings of action potential frequency in response to 15% CO_2 and focal application of MRS2365 (100 μmol/L). We found that the majority of RVLM neurons (62 of 78) could be differentiated based on responsiveness to either CO_2/H^+ or MRS2365. Note that an increase of ≥0.5 Hz immediately after MRS2365 application was the cutoff for a neuron to be considered MRS2365 sensitive. For example, 22 of 25 chemosensitive neurons (88%; ie, ≥1.5 Hz increase in firing rate during 15% CO_2) demonstrated no appreciable response to focal application of MRS2365 (Figure 5A). Conversely, 37 of 53 CO_2/H^+-insensitive neurons (≈70%) exhibited a robust firing rate response to focal application of MRS2365. Furthermore, responsiveness to MRS2365 was retained in synaptic block solution (Figure 5D and 5E) and blunted by bath application of MRS2179, a specific P2Y1 receptor blocker[32] (3 μmol/L; Figure 5B and 5C). All MRS2365-insensitive but CO_2/H^+-sensitive neurons had baseline firing rates <1 Hz (data not shown), consistent with type 1 RTN chemosensitive neurons.[40] However, 3 CO_2/H^+-sensitive neurons did respond to MRS2365, and each of these exhibited basal activity reminiscent of type II chemoreceptors (ie, ≥1 Hz),[40] suggesting that a small subset of chemosensitive neurons may express P2Y1 receptors. Nevertheless, our results clearly show that the majority of P2Y1 receptors are expressed by CO_2/H^+-insensitive cells (Figure 6A). After recording, we gained whole-cell access to fill a subset of MRS2365-senstive cells with biocytin (included in the pipette internal solution) for later determination of their immunohistochemical phenotype. We found that 9 of 18 MRS2365-sensitive cells were TH-immunoreactive, thus

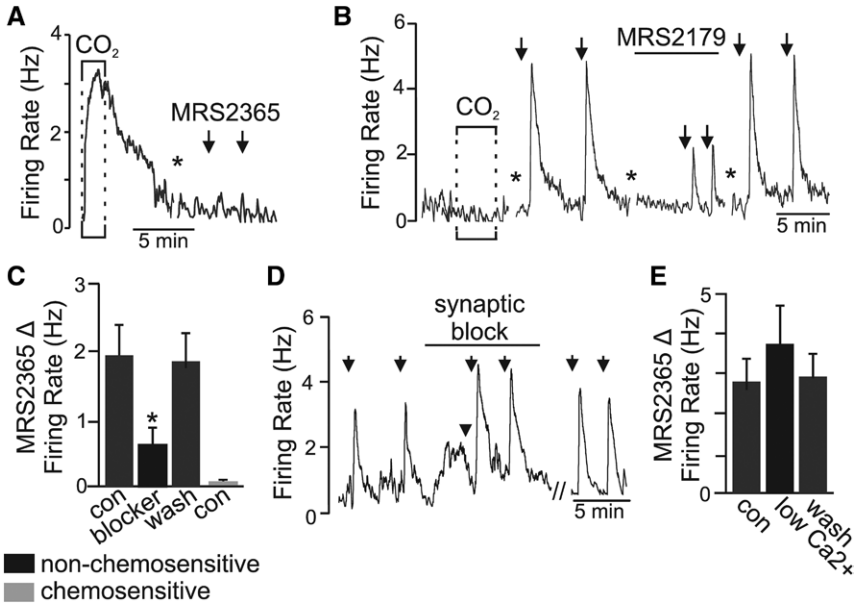

Figure 5. P2Y1 receptors are functionally expressed by CO_2-insensitive rostral ventrolateral medulla (RVLM) neurons but not retrotrapezoid nucleus (RTN) chemoreceptor neurons. **A**, Trace of firing rate from a chemosensitive RTN neuron shows that increasing CO_2 from 5% to 15% increases firing rate by ≈3 Hz. After returning to 5% CO_2, focal application (arrows) of the selective P2Y1 receptor agonist (MRS2365, 100 μmol/L) had no effect on firing rate. **B**, Trace of firing rate from a CO_2-insensitive neuron in the RVLM shows a robust and repeatable firing rate response to MRS2365. MRS2365 responsiveness was blunted by bath application of the selective P2Y1 receptor antagonist (MRS2179, 3 μmol/L) and fully recovered in wash. **C**, Summary data show the MRS2365 firing rate response of CO_2-sensitive neurons (gray bar, n=19) and CO_2-insensitive neurons (black bars, n=5) under control conditions and in the presence of the P2Y1 receptor blocker MRS2179 (blocker). **D**, Firing rate trace from a CO_2-insensitive RVLM neuron shows that MRS2365 responsiveness was retained in the presence of synaptic block solution (high Mg^{2+} and low Ca^{2+} solution, see Methods in the online-only Data Supplement). ▼ designates DC current injection; // designates 10-minute time breaks. **E**, Average data (n=5) show the firing rate response to MRS2365 under control conditions and in the presence of synaptic block solution (low Ca^{2+}). *P<0.05.

confirming that at least half of MRS2365-responsive cells are C1 neurons (Figure 6B–6E). The identity of MRS2365-sensitive cells that were TH^- is less clear. Together, these results suggest that P2Y1 receptors are differentially but not exclusively expressed by C1 neurons.

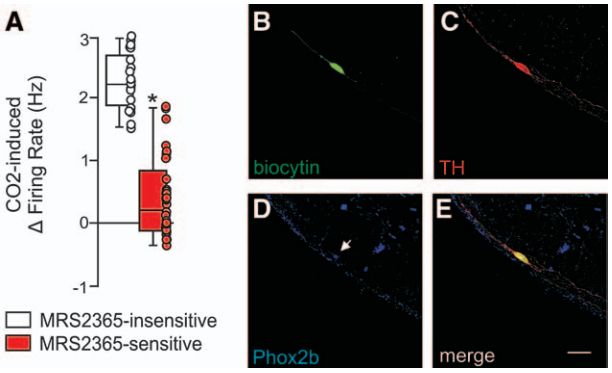

Figure 6. P2Y1 receptors are differentially expressed by C1 cells. **A**, Summary box plot shows that cells in the rostral ventrolateral medulla (RVLM) can be differentiated based on responsiveness to CO_2 (15%) and MRS2365 (100 μmol/L). Chemosensitive retrotrapezoid nucleus neurons were largely MRS2365 insensitive (white bar, n=22), whereas the majority of CO_2-insensitive neurons are activated by MRS2365 (red bar, n=46). After recording, cells were filled with biocytin for later immunohistochemical characterization; C1 cells were identified based on strong tyrosine hydroxylase (TH) immunoreactivity and weak phox2b labeling. Triple immunolabeling shows that a biocytin-filled MRS2365-sensitive cell (**B**, green) is immunoreactive for TH (**C**, red) and weakly immunoreactive for phox2b (**D**, blue); the merged image is shown in **E**. We found that 9 of 18 MRS2365-sensitive cells tested were TH positive. Scale bar, 20 μm.

To further support the possibility that C1 cells preferentially express P2Y1 receptors, we tested MRS2365 responsiveness in C1-lesioned animals. To preferentially destroy C1 cells, we made bilateral RVLM injections of saporin (SAP) (an immunotoxin) that was conjugated to an antibody for dopamine-β-hydroxylase (anti–DβH-SAP; 4.2 ng/100 nL) as previously described.[15,41] Two weeks after anti–DβH-SAP injection, TH labeling was examined within the ventrolateral medulla to confirm specificity of the lesion to C1 cells. To confirm specificity of the C1 lesion, we also examined Phox2b immunoreactivity. Phox2b is a transcription factor strongly expressed by chemosensitive RTN neurons, but only weekly expressed by C1 cells. Therefore, we define C1 cells as TH positive and Phox2b negative (TH^+/$Phox2b^-$). Animals treated with anti–DβH-SAP showed an 86±4% reduction in the number of cells that were TH positive and Phox2b negative compared with saline-injected animals (Figure S2). The number of cells that were immunoreactive for Phox2b was unaffected by DβH-SAP treatment, suggesting that only C1 cells were affected. Furthermore, the toxin did not affect the number of choline acetyltransferase-positive facial motor neurons or TH-positive cells outside the C1 (eg, A2 and A5; Figure S2). These results demonstrate selective lesion of C1 neurons.

Two weeks after DβH-SAP treatment, vagotomized and urethane-anesthetized C1-lesioned animals with intact carotid sinus nerves (n=7) exhibit reduced sSNA (72±6% of control; P=0.032) but normal resting MAP (116±4 mm Hg versus

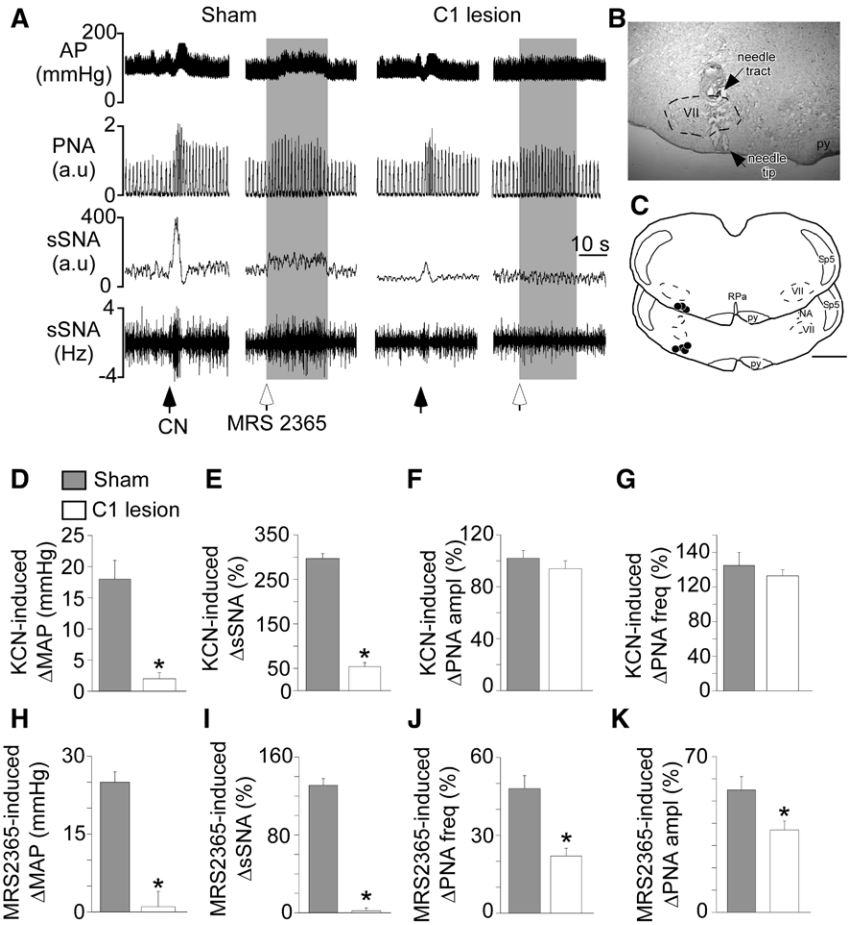

Figure 7. Depletion of C1 neurons decreased pressor and sympathoexcitatory responses to activation of peripheral chemoreceptors and decreased cardiorespiratory responses to activation of P2Y1 receptors in the rostral ventrolateral medulla (RVLM). We made bilateral RVLM injections of anti–dopamine-β-hydroxylase (DβH)-SAP (4.2 ng/100 nL) to selectively lesion C1 cells (see Figure S1). **A**, Traces of arterial pressure (AP), raw and integrated (∫) splanchnic sympathetic nerve activity (sSNA), and integrated phrenic nerve activity (∫PNA) show the response of a urethane-anaesthetized sham rat (**left**) and C1 lesion rat (**right**) to activation of peripheral chemoreceptors with cyanide (KCN: 40 μg/0.1 mL IV) and unilateral RVLM injection of MRS2365 (100 μmol/L, 50 nL). **B**, Representative photomicrograph shows that MRS2365 was injected into the RVLM at the level of the RTN. **C**, Plot of MRS2365 injection sites (Bregma –11.6 and –11.8).[34] Scale bar, 1 mm. Summary data (n=7 rats/group) show mean AP (MAP; **D**), sSNA (**E**), PNA amplitude (**F**), and PNA frequency (**G**) responses of sham and C1-lesioned animals to activation of peripheral chemoreceptors. Note that C1-lesioned animals exhibit reduced pressor and sympathoexcitatory responses but otherwise normal respiratory response to peripheral chemoreceptor activation. Summary data (n=7 rats/group) show MAP (**H**), sSNA (**I**), PNA amplitude (**J**), and PNA frequency (**K**) responses of sham and C1-lesioned animals to unilateral RVLM injection of MRS2365. These results confirm that P2Y1 receptors expressed on C1 cells are required for peripheral chemoreflex in the RVLM. *P<0.05. VII indicates facial motor nucleus; py, pyramidal tract; RPa, raphe pallidus; and Sp5, spinal tract of trigeminal nerve.

saline: 119±8 mm Hg; P=0.11). Consistent with our hypothesis, C1-lesioned animals showed reduced sSNA (2±3% versus saline 131±7%; P=0.0043) and MAP (1±3 mm Hg versus saline: 25±2 mm Hg; P=0.0051) responses to unilateral RVLM injection of MRS2365 (100 μmol/L to 50 nL; Figure 7A–7C, 7H–7K). The respiratory responses of C1-lesioned animals to MRS2365 were also decreased compared with control animals (Figure 7A, 7H–7K), suggesting that C1 cells project to RTN chemoreceptors possibly as a means of integrating sympathetic activity with respiratory drive. We found that C1-lesioned animals showed attenuation in the SNA and MAP, but not in PNA amplitude or frequency, responses elicited by cyanide (Figure 7A, 7D–7G), suggesting that C1 cells are key determinants of the sympathoexcitatory response to peripheral chemoreceptor activation. Although C1 cells also contribute to the peripheral chemoreceptor ventilatory reflex,[36] the cyanide-induced ventilatory responses were retained in C1-lesioned animals suggesting compensation by other components of the respiratory circuit (eg, chemosensitive RTN neurons).

Discussion

Purinergic signaling has been shown to contribute to central and peripheral chemoreflex control of cardiorespiratory function. However, molecular determinants of purinergic modulation of autonomic function remain poorly defined, and potential contribution of purinergic signaling to reflex regulation of blood pressure and sympathetic tone remains unclear. Here, we show that purinergic signaling at the level of the RVLM contributes to peripheral chemoreceptor regulation of breathing, sympathetic outflow, and blood pressure by a P2Y1 receptor–dependent mechanism. We show that P2Y1

receptors are preferentially expressed on C1 cells, but not chemosensitive neurons, and C1-lesioned animals do not respond to RVLM injections of a P2Y1 agonist. In addition, in vivo inhibition of P2Y1 receptor signaling in the RVLM decreased peripheral chemoreceptor–mediated activation of breathing, SNA, and blood pressure, but did not change baroreflex control of sympathetic outflow or cardiorespiratory responses to RVLM injections of glutamate. We also found that pharmacological blockade of P2Y1 receptors in the RVLM caused a modest decrease in the somatosympathetic reflex. These results suggest that pharmacological blockade of P2Y1 does not alter excitability of C1 cells in a nonspecific manner and that P2Y1 receptors contribute to excitatory (glutamatergic) reflex control of C1 cells and sympathetic outflow. Consistent with this possibility, we show that inhibition of both P2Y1 receptors and glutamate receptors virtually abolished cardiorespiratory responses to peripheral chemoreceptor activation, suggesting that purinergic nucleotides are coreleased with glutamate in the RVLM by peripheral chemoreceptor inputs. In addition, our anatomic evidence shows that ≈60% of NTS terminals in the RVLM are immunoreactive for both VGLUT2 and VNUT. This finding is consistent with evidence that ATP is coreleased with glutamate at certain central synapses,[32] and together these results suggest that peripheral chemoreceptor drive is relayed through the RVLM, in part, by a P2Y1-dependent mechanism. Considering that overactivation of peripheral chemoreceptor drive, as occurs during obstructive sleep apnea (OSA), is associated with increased SNA and hypertension,[2,3,42] we propose that P2Y1 receptors could represent a therapeutic target for the treatment of OSA-induced hypertension.

P2Y1 Receptors Regulate the Peripheral Chemoreflex at the Level of the RVLM

It is well established that bulbospinal presympathetic neurons located in the RVLM (ie, C1 and non-C1 cells) are critical determinants of reflex control of the cardiovascular system.[14,43] Present and previous[15] evidence shows that targeted destruction of C1 cells virtually eliminated the sympathoexcitatory response to peripheral chemoreceptor activation in anesthetized rats. Furthermore, selective activation of C1 cells by channelrhodopsin-2 has been shown to increase breathing, sympathetic activity, and blood pressure,[44–46] whereas inhibition of C1 cells by activation of the allostatin receptor did the opposite.[47] However, despite the critical role of C1 cells in regulation of cardiorespiratory function, the identity of neurotransmitters and downstream effectors responsible for peripheral chemoreflex control of autonomic function at the level of the ventrolateral medulla remains incomplete.

Our results indicate that peripheral chemoreflex control of breathing, sympathetic activity, and blood pressure depends on both glutamate and purinergic signaling at the level of the RVLM. Specifically, we show in anesthetized rats that bilateral injections of kynurenic acid significantly decreased the ventilatory, sympathetic, and pressor responses to peripheral chemoreceptor activation. These results are consistent with previous studies that used kynurenic acid[36] or APV ([2*R*]-amino-5-phosphonovaleric acid; specific *N*-methyl-D-aspartate receptor blocker)[48] to attenuate the response of RVLM presympathetic cells to peripheral chemoreceptor drive. In addition, we found that the majority of NTS terminals in the RVLM were immunoreactive for VGLUT2 (77%). Although we do not know whether VGLUT2-positive NTS neurons that innervate the RVLM actually mediate the chemoreflex or contact C1 cells, these results are consistent with previous evidence[9] and suggest that glutamate contributes to peripheral chemoreflex in the RVLM.

We also discovered that purinergic signaling via P2Y1 receptors expressed on C1 cells contributes to peripheral chemoreceptor regulation of breathing and blood pressure. This finding builds on the possibility that ATP is a key transmitter throughout the peripheral chemoreceptor circuit. For example, as part of the first step in peripheral chemotransduction glomus cells—the chemosensory unit of the carotid bodies—release ATP to activate P2X2 and P2X3 receptors on sensory nerve endings, which then relays this excitatory drive to neurons in the cNTS.[22] In the cNTS, application of ATP in awake rats mimicked cardiorespiratory responses (ie, bradycardia, hypertension, and tachypnea) to peripheral chemoreceptor activation.[49,50] Also consistent with a role of purinergic signaling in peripheral chemoreflex, cNTS injections of glutamate receptor antagonists did not block sympathetic or bradycardic components of the chemoreceptor reflex in awake rats[51] or in the working heart-brain stem preparation,[52] whereas simultaneous antagonism of glutamate and P2 receptors in this region reduced pressor and sympathetic responses to chemoreflex activation.[19] In addition, presympathetic neurons in the RVLM are activated by exogenous application of ATP analogs,[26,29,30] and application of purinergic agonists into this region increased sympathetic tone[29] and blood pressure[29,30] in anesthetized rats. Furthermore, inhibition of P2X receptors within the more caudal RVLM (at the level of the Bötzinger complex) blunted the ventilatory, but not pressure response elicited by peripheral chemoreceptor activation in conscious rats.[23] In light of our evidence that P2Y1 receptors expressed on C1 cell mediate the peripheral chemoreceptor pressure response, we propose that differential expression of P2 receptors throughout the ventrolateral medulla could allow for parallel processing of respiratory and cardiovascular components of the peripheral chemoreflex.

It is also possible that P2Y1 receptors regulate other excitatory reflexes at the level of the RVLM. For example, we show that pharmacological blockade of P2Y1 receptors in the RVLM caused a modest reduction in the somatosympathetic reflex by reducing the SNA peak elicited by electric stimulation of the sciatic nerve in anesthetized rats. Previous evidence reported this reflex to be mediated largely by glutamate at this level of the RVLM.[37] Our evidence suggests that purinergic signaling via P2Y1 receptors in the RVLM may also contribute to the increase in sympathetic outflow evoked by stimulation of somatic afferents. Conversely, the inhibitory baroreflex was not affected by RVLM application of a P2Y1 receptor antagonist. These results are consistent with previous evidence that P2Y1 receptor blockade in the RVLM did not affect basal cardiorespiratory parameters[31] and suggests that P2Y1 receptors preferentially regulate excitatory reflex control of C1 cells.

P2Y1 receptors are best known for their role in paracrine signaling between astrocytes and neurons,[53] but there is some evidence that these receptors are present postsynaptically and contribute to synaptic transmission.[54–56] For example, P2Y1 receptors appear to be expressed postsynaptically in layer V pyramidal neurons, and activation of these receptors has been shown to decrease synaptic strength and plasticity,[54–56] in part, by inhibiting voltage-sensitive Ca^{2+} channels.[55] Our results are consistent with the possibility that P2Y1 receptors are situated postsynaptically on C1 cells; however, contrary to the studies noted above, we find that activation of P2Y1 on C1 cells increased excitability by activating a yet to be identified inward conductance (voltage-clamp data are not shown). It should be noted that P2Y1 receptors may also be expressed by other cells types in the RVLM, including astrocytes, and our evidence that MRS2365 responsiveness was retained in synaptic block media does not rule out a potential indirect contribution of astrocytes to the peripheral chemoreflex.

Physiological Significance

OSA is defined as the occurrence of repetitive episodes of upper airway obstruction during sleep. OSA is a major health problem affecting ≤20% of adults in the United States and is considered a major risk factor for cardiovascular disease[3,4,57,58] (eg, heart failure, stroke, hypertension, and coronary heart disease). The link between OSA and cardiovascular disease is thought to result from repeated apneic/hypoxic-mediated activation of the sympathetic nervous system via peripheral chemoreceptors, which leads to hypertension and other cardiovascular problems.[57] Previous evidence showed that C1 cells are key determinants of the sympathoexcitatory response to peripheral chemoreceptor activation.[15] In addition, an animal model of OSA showed that chronic intermittent hypoxia increased sympathoexcitatory responsiveness to RVLM injections of ATP,[28] suggesting that purinergic signaling in this region contributes to OSA-induced activation of sympathetic activity and hypertension. Here, we show that purinergic signaling contributes to the peripheral chemoreflex at the level of the RVLM by a P2Y1 receptor–dependent mechanism. Therefore, P2Y1 receptors in the RVLM may represent new avenues for the treatment of hypertension resulting from overactivation of peripheral chemoreceptors.

Perspectives

Catecholaminergic C1 cells in the RVLM are key determinants of the sympathoexcitatory response to peripheral chemoreceptor activation. Overactivation of this reflex is thought to contribute to increased sympathetic activity and hypertension; however, molecular mechanisms linking peripheral chemoreceptor drive to hypertension remain poorly understood. Here, we show that P2Y1 receptors are preferentially expressed on C1 cells but not on chemoreceptor neurons, and C1-lesioned animals do not respond to injections of a P2Y1 agonist in the ventrolateral medulla. In addition, inhibition of P2Y1 receptor signaling in the ventrolateral medulla decreased peripheral chemoreceptor-mediated activation of breathing, sympathetic outflow, and blood pressure, as well as the somatosympathetic reflex, but did not change the baroreflex activation. Furthermore, inhibition of both P2Y1 receptors and glutamate receptors virtually abolished cardiorespiratory responses to peripheral chemoreceptor activation, suggesting that purinergic nucleotides are coreleased with glutamate in the RVLM by peripheral chemoreceptor inputs. Our results incorporate the already-established notion that RVLM/C1 neurons are excited by peripheral chemoreceptors via a direct glutamatergic input from cNTS.[9] To this notion, we have added the concept that purinergic signaling also contributes to peripheral chemoreflex control of autonomic function at the level of the RVLM/C1 neurons via a P2Y1-dependent mechanism.

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Disclosures

None.

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Novelty and Significance

What Is New?

- We show for the first time that (1) purinergic signaling via P2Y1 receptors regulates peripheral chemoreceptor control of breathing, sympathetic nerve activity, and blood pressure; (2) activation of P2Y1 receptors in the rostral ventrolateral medulla mimics effects of peripheral chemoreceptor activation in control animals, but not in C1-lesioned animals; and (3) P2Y1 receptors are preferentially expressed on blood pressure–regulating C1 cells but not in respiratory chemoreceptor neurons.

What Is Relevant?

- Overactivation of the peripheral chemoreflex by repeated bouts of hypoxia is thought to contribute to hypertension and cardiovascular mortality associated with obstructive sleep apnea. Our evidence that P2Y1 receptors are differentially expressed by blood pressure–regulating cells and function as key determinants of peripheral chemoreceptor regulation of blood pressure identifies P2Y1 receptors as potential therapeutic targets for the treatment of hypertension associated with conditions, such as obstructive sleep apnea. We expect that our findings will be of great interest to a broad audience in the basic, clinical, and pharmaceutical community.

Summary

Catecholaminergic C1 cells in the rostral ventrolateral medulla are key determinants of the sympathoexcitatory response to peripheral chemoreceptor activation. Overactivation of this reflex is thought to contribute to increased sympathetic activity and hypertension; however, molecular mechanisms linking peripheral chemoreceptor drive to hypertension remain poorly understood. Here, we use a combination of immunohistochemistry and in vivo and in vitro electrophysiological approaches to show that P2Y1 receptors are differentially expressed by C1 cells and function as important determinants of peripheral chemoreceptor regulation of breathing, sympathetic outflow, and blood pressure. These results suggest that P2Y1 receptors expressed on C1 cells represent a therapeutic target for the treatment of hypertension resulting from overactivation of peripheral chemoreceptors.

ONLINE SUPPLEMENT

P2Y1-receptors expressed by C1 neurons determine peripheral chemoreceptor modulation of breathing, sympathetic activity and blood pressure

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Supplemental Methods

In vivo preparation.

Animal use was in accordance with guidelines approved by the University of São Paulo Animal Care and Use Committee. All *in vivo* experiments were performed in male Wistar rats weighing 250-280 g. The surgical procedures and experimental protocols were similar to those previously described[1,2]. Briefly, general anesthesia was induced with 5% halothane in 100% O_2. A tracheostomy was made and the halothane concentration was reduced to 1.4-1.5% until the end of surgery. The femoral artery was cannulated (polyethylene tubing, 0.6 mm o.d., 0.3 mm i.d., Scientific Commodities, Lake Havasu City, Arizona, USA) for measurement of arterial pressure (AP). The femoral vein was cannulated for administration of fluids and drugs. The occipital plate was removed, and a micropipette was placed in the medulla oblongata via a dorsal transcerebellar approach for microinjection of drugs. A skin incision was made over the lower jaw for placement of a bipolar stimulating electrode, next to the mandibular branch of the facial nerve, as previously described[1,2]. The phrenic nerve was accessed by a dorsolateral approach after retraction of the right shoulder blade. To prevent any influence of artificial ventilation on phrenic nerve activity (PNA), the vagus nerve was cut bilaterally as follows.

Splanchnic sympathetic nerve activity (sSNA) was recorded as previously described[1-3]. The right splanchnic nerve was isolated via a retroperitoneal approach, and the segment distal to the suprarenal ganglion was placed on a pair of teflon-coated silver wires that had been bared at the tip (250 μm bare diameter; A-M Systems, Carlsborg, WA, USA). The nerves and wires were embedded in adhesive material (Kwik-Cast Sealant, WPI, USP, Sarasota, FL USA), and the wound was closed around the exiting recording wires.

Upon completion of the surgical procedures, halothane was replaced by urethane (1.2 g/kg) administered slowly i.v. All rats were ventilated with 100% O_2 throughout the experiment. Rectal temperature was maintained at 37°C. End-tidal CO_2 was monitored throughout each experiment with a capnometer (CWE, Inc, Ardmore, PA, USA) that was calibrated twice per experiment with a calibrated CO_2/N_2 mix. This instrument provided a reading of <0.1% CO_2 during inspiration in animals breathing 100% O_2 and provided an asymptotic, nearly horizontal reading during expiration. The adequacy of anesthesia was monitored during a 20 min stabilization period by testing for the absence of withdrawal responses, pressor responses, and changes in PNA to a firm toe pinch. After these criteria were satisfied, the muscle relaxant pancuronium was administered at an initial dose of 1 mg/kg i.v. and the adequacy of the anesthesia was thereafter gauged solely by the lack of increase in AP and PNA rate or amplitude to a firm toe pinch. Approximately hourly supplements of one-third of the initial dose of urethane were needed to satisfy these criteria throughout the recording period (2 hours).

C1 lesion model

The injections of the toxin anti-dopamine beta-hydroxylase-saporin (anti DβH-SAP; Advanced Targeting Systems, San Diego, CA, USA) or saline were made while the rats were anesthetized with a mixture of ketamine (80 mg/kg) and xylazine (7 mg/kg) administered intraperitoneally. Surgery was performed using standard aseptic methods. After surgery, rats were treated with the antibiotic ampicillin (100 mg/kg) and the analgesic ketorolac (0.6 mg/kg, s.c.). The anti DβH-SAP conjugate was administered into the RVLM region by pressure injection using glass pipettes with an external tip diameter of 25 μm. These glass pipettes also allowed recordings of field potentials, which were used to direct the electrode tip to the desired

sites. The rats received bilateral injections (100 nl) placed symmetrically in the RVLM at the level of the C1, i.e., 100-200 μm below the lower edge of the field and 1.8 mm lateral to the midline and 100-200 μm caudal to the caudal end of the facial field. Animals were maintained for 2 weeks before they were used in physiological experiments. Consistent with previous evidence4, the toxin produced no obvious phenotype under resting conditions. The dose of anti DβH-SAP used in the present study (4.2 ng/100 nl) was selected based on previous experiments investigating the effects of injecting anti DβH-SAP into the C1 and A5 region[3, 4]. In addition, we did not observe any obvious gliosis or any other readily observable cytological difference between control and anti DβH-SAP rats (data not shown).

***In vivo* recordings of physiological variables**

As previously described[1-3,5], mean arterial pressure (MAP), phrenic nerve activity (PNA), sSNA and end-expiratory CO_2 ($etCO_2$) were digitized with a micro1401 (Cambridge Electronic Design), stored on a computer, and processed off-line with version 6 of Spike 2 software (Cambridge Electronic Design, Cambridge, UK). Integrated phrenic nerve activity (∫PNA) and integrated splanchnic nerve activity (∫SNA) were obtained after rectification and smoothing (τ = 0.015 and 2s, respectively) of the original signal, which was acquired with a 30-300 Hz bandpass filter. ∫SNA was normalized within animals by assigning a value of 100 to resting SNA and a value of 0 to the minimum value recorded either during administration of a dose of phenylephrine that saturated the baroreflex (5 μg/kg, i.v.) or after ganglionic blockade (hexamethonium; 30 mg/kg, i.v.). Nerve activity was rectified and averaged over 1-s intervals and stored on hard disk for subsequent analysis. Noise was subtracted from the recordings prior to performing any calculations of evoked changes in SNA. A direct physiological comparison of the absolute level of nerve activity across nerves is not possible because of nonphysiological factors (e.g., nerve electrode contact, size of nerve bundle) and the ambiguity in interpreting how a given increase in voltage in one nerve relates to an increase in voltage in another nerve. Thus, all nerve activities were defined to be at their baseline physiological state just prior to their activation. These activities were normalized to 100%, and percent change was used to compare the magnitude of increase or decrease across nerves from these physiological baselines. PNA amplitude (PNA amp) and PNA frequency (PNA freq) were normalized in each experiment by assigning to each of the two variables a value of 100 at saturation of the chemoreflex (high CO_2) and a value of 0 to periods of apnea.

Potassium cyanide was used (KCN, 40 μg/0.1 ml, i.v.) to activate peripheral chemoreceptors because *in vivo* responses to KCN are robust, reversible, and blocked by carotid denervation[6].

Tracer injections

Tracer injections were made while the rats were anaesthetized with a mixture of ketamine (80 mg kg^{-1}) and xylazine (5mg kg^{-1}) administered i.p. Surgery used standard aseptic methods, and after surgery, the rats were treated with the antibiotic ampicillin (100 mg kg^{-1}) and the analgesic ketorolac (0.6 mg kg^{-1}, s.c.). A group of four rats received pressure injections of the anterograde tracer biotinylated dextran amine (BDA-lysine fixable, MW 10000; 10% w/v in 10 mm phosphate buffer, pH 7.4; Molecular Probes) into the commissural part of the nucleus of the solitary tract (cNTS) (25 μm tip diameter glass pipettes). These injections were made 0.4 mm caudal to the calamus scriptorius, in the midline and 0.3-0.5mm below the dorsal surface of the brainstem. These rats were allowed to survive 7-10 days following which they were

anaesthetized with pentobarbital (60 mg kg-1, i.p.) and perfused transcardially with fixative as described below.

Somatosympatheic reflex analysis

The somatosympathetic reflex was activated by electrical stimulation (5–15 V; 50 sweeps; 0.2-ms pulses at 1 Hz) of the sciatic nerve and the average response of sSNA peak was analyzed off-line. The area under the curve (AUC) of the sympathoexcitatory peak, less baseline of sSNA, was determined. sSNA were rectified and smoothed at 5-ms time constant to analyze the somatosympathetic reflex.

Baroreflex analysis

Animals underwent a series of reflex tests that were performed in the same order separated by 5-10 minutes with drug doses established in previous studies[7]. The drugs were prepared in sterile isotonic saline for intravenous (i.v) injections. The baroreflex stimulation was examined by raising arterial pressure with the phenilephrine (5 μg/kg, i.v) and lowering arterial pressure with sodium nitroprusside (30 μg/kg, i.v). A baroreflex curve relating MAP and SNA as constructed for each rat. The SNA at resting MAP was set at 100%, and SNA after hexamethonium (10 mg/kg i.v) was set as the minimum (i.e., 0) value. Boltzman sigmoidal curves, with use of the equation $SNA = (A1 - A2)/\{1 + \exp [A3 \bullet (MAP - A4)]\} + A2$, were fitted to the experimental data points by use of the software program Sigma Plot (Jandel Corporation, Point Richmond, CA), where A1 defines the upper plateau of the curve, A2 defines the lower plateau of the curve, A3 describes the distribution of the gain along the curve, and A4 (MAP50) is the midpoint of the curve. Maximum gain (Gmax) was calculated using the formula $Gmax = A3 \bullet (A1 - A2)/4$[8].

Brain slice preparation

All procedures were performed in accordance with National Institutes of Health and University of Connecticut Animal Care and Use Guidelines. Slices containing the RVLM were prepared as previously described[5]. Briefly, neonatal rats (7-12 days postnatal) were decapitated under ketamine/xylazine anesthesia and transverse brain stem slices (300 μm) were cut using a microslicer (DSK 1500E; Dosaka, Kyoto, Japan) in ice-cold substituted Ringer solution containing (in mM): 260 sucrose, 3 KCl, 5 $MgCl_2$, 1 $CaCl_2$, 1.25 NaH_2PO_4, 26 $NaHCO_3$, 10 glucose, and 1 kynurenic acid. Slices were incubated for ~30 min at 37°C and subsequently at room temperature in normal Ringer solution (in mM): 130 NaCl, 3 KCl, 2 $MgCl_2$, 2 $CaCl_2$, 1.25 NaH_2PO_4, 26 $NaHCO_3$, and 10 glucose. Both substituted and normal Ringer solutions were bubbled with 95% O_2-5% CO_2, extracellular pH (pHo = 7.35).

Slice-patch electrophysiology

Individual slices were transferred to a recording chamber mounted on a fixed-stage microscope (Zeiss Axioskop FS) and perfused continuously (~2 ml min^{-1}) with a bath solution of normal Ringer solution (same as incubation Ringers above) bubbled with 95% O_2-5% CO_2 (pHo = 7.35). The pH of the bicarbonate-based bath solution was decreased to 6.90 by bubbling with 15% CO_2. All recordings were made with an Axopatch 200B patch-clamp amplifier, digitized with a Digidata 1322A A/D converter, and recorded using pCLAMP 10.0 software (Molecular Devices). Recordings were obtained at room temperature (~22 ºC) with patch electrodes pulled from borosilicate glass capillaries (Warner Instruments) on a two-stage puller (P89; Sutter

Instrument) to a DC resistance of 4–6 MΩ when filled with an internal solution containing the following (in mM): 120 KCH_3SO_3, 4 NaCl, 1 $MgCl_2$, 0.5 $CaCl_2$, 10 HEPES, 10 EGTA, 3 Mg-ATP, 0.2% biocytin, and 0.3 GTP-Tris (pH 7.2); electrode tips were coated with Sylgard 184 (Dow Corning). All recordings of neuronal firing rate were performed in the cell-attached configuration to ensure minimal alteration of the intracellular milieu. Firing rate histograms were generated by integrating action potential discharge in 10-s bins and plotted using Spike 5.0 software.

Drugs

All drugs were purchased from Sigma unless otherwise indicated. For *in vivo* experiments, the non-specific P2-receptor antagonist pyridoxalphosphate-6-azophenyl-2',4'-disulfonic acid (PPADS), the P2Y1-receptor antagonist MRS2179 (Tocris Bioscience) and the P2Y1-receptor agonist MRS2365 (Tocris Bioscience) were diluted to 100 µM in sterile saline (pH 7.4) and injected into the RVLM using single-barrel glass pipettes (tip diameter of 25 µm) connected to a pressure injector (Picospritzer III, Parker Hannifin Corp, Cleveland, OH).

For each injection we delivered a volume of 50 or 100 nl over a period of 5s. These glass pipettes also allowed recordings of field potential properties that were used to help direct the electrode tip to the desired site. Injections in the RVLM region were guided by recordings of the facial field potential[9], and were placed 250 µm below the lower edge of the field, 1.7 mm lateral to the midline, and 200 µm rostral to the caudal end of the field. Recordings were made on one side only; the second injection was made 1-2 min later at the same level on the contralateral side. We included a 5% dilution of fluorescent latex microbeads (Lumafluor, New City, NY) with all drug applications to mark the injection sites and verify spread of the injections[1,2]. For *in vitro* experiments, we bath applied MRS2179 (3 µM) to block P2Y1-receptors. In addition, the specific P2Y1-receptor agonist (MRS2365; 100 µM in HEPES buffered medium, pH 7.3) was delivered focally using low-resistance pipettes connected to a Picospritzer III (Parker Instrumentation, Cleveland, OH) and maneuvered into close proximity of the target neurons. Application times were 600 ms, and vehicle control experiments were performed to ensure agonist responses were not attributable to pressure artifacts. A low Ca^{2+} and high Mg^{2+} synaptic block solution was used to block vesicular neurotransmitter release. The composition of the synaptic block medium used in this study is similar to normal Ringer's solution except that $MgCl_2$ was increased to 11.4 mM, $CaCl_2$ was decreased to 0.2 mM, and to maintain osmolality, NaCl was decreased to 124 mM. The efficacy of this synaptic blocking medium is well established[10, 11] and confirmed to work in our preparation previously[12].

Histology: *in vivo* experiments

At the end of each *in vivo* experiment rats were deeply anesthetized with halothane, injected with heparin (500 units, intracardially) and finally perfused through the ascending aorta with 150 ml of phosphate-buffered saline (pH 7.4) followed by formaldehyde (4% in 0.1 M phosphate buffer; pH 7.4) (Electron Microscopy Sciences, Fort Washington, PA, USA). Brains were removed and stored in the perfusion fixative for 24-48 h at 4◦C. Series of coronal brain sections (40 µm) were cut using a vibrating microtome (Vibratome 1000S Plus, USA) and stored in a cryoprotectant solution at -20◦C for up to 2 weeks (20% glycerol plus 30% ethylene glycol in 50 mM phosphate buffer, pH 7.4) pending histological processing[13]. All histochemical procedures were performed using free-floating sections according to previously described protocols[3]. Tyrosine hydroxylase (TH) was detected with a mouse antibody (1:10,000,

Chemicon, Temecula), and Phox2b was detected with a rabbit antibody (1:800, gift from J.-F. Brunet, Ecole Normale Supèrieure, Paris, France). These primary antibodies were detected by incubation with appropriate secondary antibodies tagged with biotinylated donkey anti-mouse (1:1000, Jackson) or (donkey anti-rabbit, Jackson, West Grove, PA, USA), followed by the ABC kit (Vector, Burlingame, CA, USA) and subsequent colourisation with 3-3-di-aminobenzidine (DAB). Choline acetyltransferase (ChAT) was detected with a goat anti-ChAT antibody (1:50, Chemicon, raised against human placental ChAT) and revealed with the DAB colourimetric method using biotinylated donkey anti-goat (1:500, Jackson) and subsequent colourisation with DAB. The specificity of the antibodies has been validated previously[14]. TH-positive neurons with or without Phox2b were plotted and counted in 7 coronal sections per rat. Each section was 240 μm apart, and the middle section was selected to coincide with the caudal end of the facial motor nucleus.

For cell mapping, counting and imaging, a conventional Zeiss Axioskop 2 multifunction microscope (brightfield, darkfield and epifluorescence) was used for all observations. Injection sites (fluorescent microbeads), and TH-labelled neurons were plotted using a previously described computer-assisted mapping technique based on the use of a motor-driven microscope stage controlled by Neurolucida software. The Neurolucida files were exported to NeuroExplorer software (MicroBrightfield, Colchester, VT) to count the various types of neuronal profiles within a defined area of the pons and brainstem. When appropriate, selected Neurolucida files were also exported to the Canvas 9 software drawing program (ACD Systems of America, Miami, FL, USA) for final modifications. Section alignment between brains was done relative to a reference section. To align sections around the C1 region, the most caudal section containing an identifiable cluster of facial motor neurons was identified in each brain and assigned the level of 11.6 mm caudal to Bregma (Bregma -11.6 mm)[15]. Levels rostral or caudal to this reference section were determined by adding a distance corresponding to the interval between sections multiplied by the number of intervening sections. Images were captured with a SensiCam QE 12-bit CCD camera (resolution 1376×1040 pixels, Cooke Corp., Auburn Hills, MI, USA). The neuroanatomical nomenclature is adopted from Paxinos and Watson[15].

Histology: *in vitro* experiments

Recorded slices were fixed in 4% paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.4) for 16 to 24 hours at 4°C and rinsed in phosphate-buffered saline (PBS). Slices were blocked and permeabilized in tris-buffered saline (TBS) containing 10% normal horse serum and 0.5% Triton X-100 for 1 h at room temperature. After three TBS washes, the slices were incubated in primary antibodies; rabbit anti-Phox2b (gift of Dr. Brunet, 1:1000) and sheep anti-tyrosine hydroxylase (Millipore, 1:1000), or VGLUT2 (Millipore, 1:1000) and VNUT (MBL, 1:400), for 48 hours at 4°C. After three TBS washes, the slices were incubated in secondary antibodies (Jackson Labs); alexa488–conjugated strepavidin (1:1,000), Cy3-conjugated goat anti-sheep (1:500) and Cy5-conjugated goat anti-rabbit (1:200), or alexa488-conjugated streptavidin, Cy3-conjugated goat anti-guinea pig (1:500) and Cy5-conjugated goat anti-rabbit for 1 h at room temperature. Slices were washed a final three times in TBS and mounted in Vectashield. Images were collected on a Leica TCS SP2 confocal microscope equipped with 488-, 543-, and 633-nm laser lines and tunable emission wavelength detection. For each slice, biocytin-positive cell, or terminal, was identified and confocal ***z***-stacks were collected sequentially for the other two channels to detect the neuronal antigens. Neurons were scored as TH-, or Phox2b-, immunopositive and NTS terminals were scored as VGLUT2-, or VNUT-, immunopositive if the

immunoreactivity for the protein marker was detected in the biocytin-positive cell body, or terminal. Biocytin neurons and terminals were scored as immunonegative if immunoreactivity for the protein marker was not detected in the biocytin filled cell or terminal, but immunopositive cells and terminals were observed nearby. To determine background fluorescence of the tissue and nonspecific binding of secondary antibodies, we tested secondary antibodies without preincubation in primary antibody.

Statistics

Data are reported as mean ± standard error of the mean. Statistical analysis was performed using Sigma Stat version 3.0 software (Jandel Corporation, Point Richmond, CA). T-test, paired T-test or one way ANOVA followed by the Newman-Keuls multiple comparisons test were used as appropriate ($p<0.05$ unless otherwise stated).

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Supplemental Figures

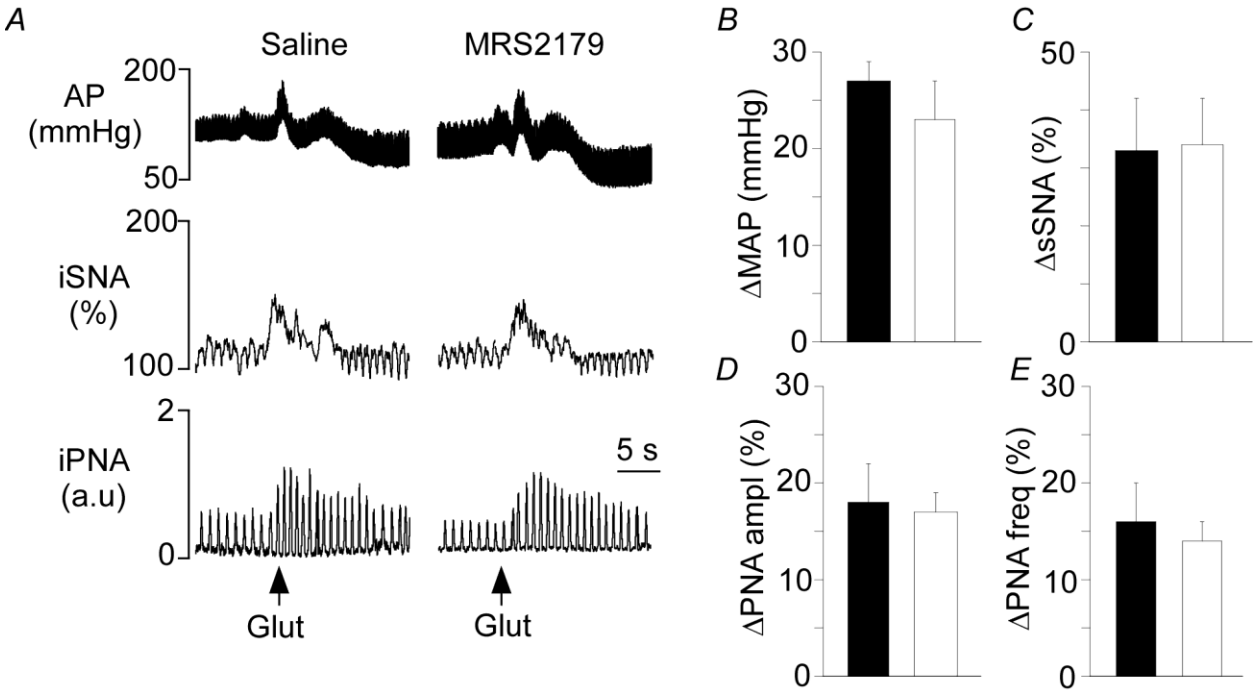

Figure S1: Purinergic blockade do not change the cardiorespiratory effects of activation of RVLM/C1 neurons. A, Recordings from one rat showing the effect of MRS2179 (100 μM - 50 nl) into the RVLM/C1 region on changes in arterial pressure (AP), splanchnic sympathetic nerve activity (sSNA) and phrenic nerve activity (PNA) induced by glutamate (10 mM - 50 nl) injection. Responses were recorded 10 min after bilateral injection of saline or MRS2179 in the RVLM/C1 region. **B,** Changes in mean arterial pressure (ΔMAP), **C,** sSNA (ΔsSNA), **D,** PNA amplitude (ΔPNA ampl) and **E,** PNA frequency (ΔPNA freq) elicited by glutamate injection in the RVLM/C1 during saline or MRS2179 injections into RVLM/C1 region. N = 5 rats/group.

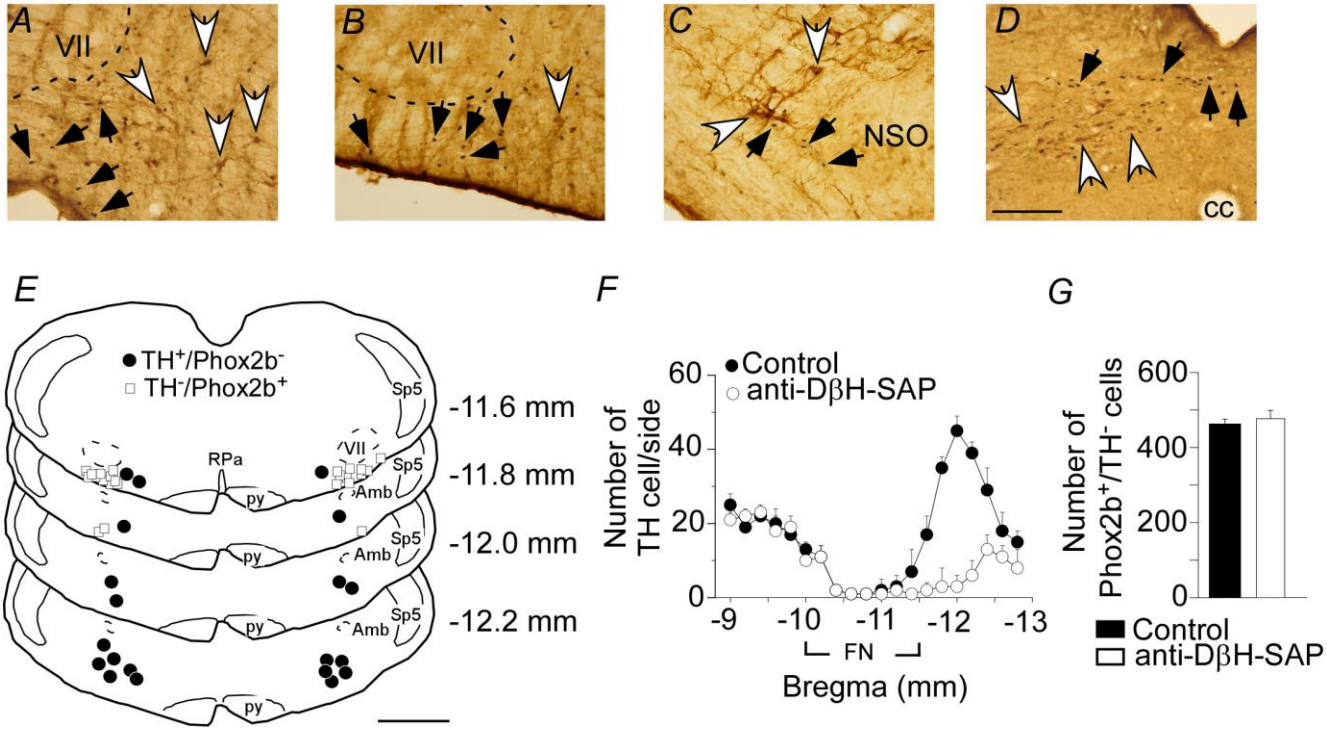

Figure S2: Injection of anti DβH-SAP into the RVLM selectively destroys C1 neurons. C1 cells were identified immunohistochemically as TH-positive and Phox2b-negative cells. **A-B,** photomicrographs at the level of the C1 region (-11.80 mm from Bregma) from control (A) and C1 lesion (B) animals. **C-D**, photomicrographs show normal TH-immunolabeling in the nearby A5 (C) and A2 (D) regions. **E**, computer assisted plots showing the distribution of C1 cells (•) and Phox2b-positive chemosensitive neurons (□) over several coronal planes. Note that the numbers on the right side of each drawing designate caudal distance from bregma. Scale bar is 1 mm. **F**, average number of TH neurons per section from 7 rats. Counts were made from a 1 in 6 series of 40 μm coronal sections. **G,** average number of $Phox2b^{+}/TH^{-}$ neurons (i.e., control). Abbreviations: VII, facial motor nucleus; Amb, ambiguus nucleus; IO, inferior olivary nucleus; py, pyramidal tract, Sp5, spinal trigeminal tract. Black arrows represent the Phox2b-positive neurons and white arrows represent the TH-positive neurons.

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Purinergic signalling contributes to chemoreception in the retrotrapezoid nucleus but not the nucleus of the solitary tract or medullary raphe

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Key points

- Several brain regions are thought to sense changes in tissue CO_2/H^+ to regulate breathing (i.e. central chemoreceptors) including the nucleus of the solitary tract (NTS), medullary raphe and retrotrapezoid nucleus (RTN).
- Mechanism(s) underlying RTN chemoreception involve direct activation of RTN neurons by H^+-mediated inhibition of a resting K^+ conductance and indirect activation of RTN neurons by purinergic signalling, most likely from CO_2/H^+-sensitive astrocytes.
- Here, we confirm that activation of P2 receptors in the RTN stimulates cardiorespiratory activity, and we show at the cellular and systems level that purinergic signalling is not essential for CO_2/H^+ sensing in the NTS or medullary raphe.
- These results support the possibility that purinergic signalling is a unique feature of RTN chemoreception.

Abstract Several brain regions are thought to function as important sites of chemoreception including the nucleus of the solitary tract (NTS), medullary raphe and retrotrapezoid nucleus (RTN). In the RTN, mechanisms of chemoreception involve direct H^+-mediated activation of chemosensitive neurons and indirect modulation of chemosensitive neurons by purinergic signalling. Evidence suggests that RTN astrocytes are the source of CO_2-evoked ATP release. However, it is not clear whether purinergic signalling also influences CO_2/H^+ responsiveness of other putative chemoreceptors. The goals of this study are to determine if CO_2/H^+-sensitive neurons in the NTS and medullary raphe respond to ATP, and whether purinergic signalling in these regions influences CO_2 responsiveness *in vitro* and in *vivo*. In brain slices, cell-attached recordings of membrane potential show that CO_2/H^+-sensitive NTS neurons are activated by focal ATP application; however, purinergic P2-receptor blockade did not affect their CO_2/H^+ responsiveness. CO_2/H^+-sensitive raphe neurons were unaffected by ATP or P2-receptor blockade. *In vivo*, ATP injection into the NTS increased cardiorespiratory activity; however, injection of a P2-receptor blocker into this region had no effect on baseline breathing or CO_2/H^+ responsiveness. Injections of ATP or a P2-receptor blocker into the medullary raphe had no effect on cardiorespiratory activity or the chemoreflex. As a positive control we confirmed that ATP injection into the RTN increased breathing and blood pressure by a P2-receptor-dependent mechanism. These results suggest that purinergic signalling is a unique feature of RTN chemoreception.

C. R. Sobrinho and I. C. Wenker contributed equally to this study.
T.S.M and D.K.M. are co-senior authors.

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Abbreviations AP, arterial pressure; ChAT, choline acetyltransferase; cNTS, caudal portion of the nucleus of the solitary tract; cNTS, commissural NTS; $etCO_2$, end-expiratory CO_2; iPNA, integrated phrenic nerve activity; ISI, interspike interval; MAP, mean arterial pressure; NTS, nucleus of the solitary tract; PNA, phrenic nerve activity; Ppy, parapyramidal region; RPa, raphe pallidus; RTN, retrotrapezoid nucleus.

Introduction

Hypercapnic acidosis (i.e. high CO_2/H^+) provides the primary stimulus to breathe. Central respiratory chemoreceptors sense changes in tissue CO_2/H^+ and send excitatory drive to respiratory centres to directly regulate depth and frequency of breathing (Feldman *et al.* 2003; Huckstepp & Dale, 2011). The process of chemoreception is especially important during sleep and its disruption has been associated with central sleep apnoea (Dempsey *et al.* 2010) and hypoventilation syndrome (Guyenet & Mulkey, 2010). Nevertheless, despite the importance of central chemoreceptors the cellular and molecular mechanism(s) underlying this process have yet to be fully elucidated.

Several brain regions are thought to function as important sites of chemoreception including the caudal portion of the nucleus of the solitary tract (cNTS; Dean *et al.* 1990; Nattie & Li, 2002), medullary raphe (e.g. raphe pallidus, magnus and obscurus; Wang & Richerson, 1999; Nattie & Li, 2001; Hodges *et al.* 2004; Richerson, 2004) and retrotrapezoid nucleus (RTN; Li *et al.* 1999; Mulkey *et al.* 2004; Wang *et al.* 2013). Of these, the mechanism(s) of chemotransduction are best characterized at the level of the RTN where neurons have been shown to sense changes in CO_2/H^+ by two independent but coordinated mechanisms: direct H^+-mediated activation of pH-sensitive neurons by inhibition of a resting K^+ conductance (Mulkey *et al.* 2004, 2007) and indirect activation by purinergic signalling, most likely from CO_2/H^+-sensitive astrocytes (Gourine *et al.* 2010; Huckstepp *et al.* 2010; Wenker *et al.* 2010). Evidence also suggests that purinergic signalling is a unique feature of RTN chemotransduction. For example, it was shown *in vivo* that CO_2/H^+ facilitates ATP release only at discrete locations near the RTN (Gourine *et al.* 2005) and that RTN astrocytes, but not cortical astrocytes, respond to H^+ with increased Ca^{2+}-dependent exocytosis of ATP-containing vesicles (Kasymov *et al.* 2013). However, the role of purinergic signalling in putative chemoreceptor regions outside the RTN has not been thoroughly investigated.

Indeed, it remains possible that purinergic signalling also regulates neuronal activity in other putative chemosensitive regions. For example, P2 receptors are widely distributed throughout the central nervous system, including in the NTS and medullary raphe (Yao *et al.* 2000). In addition, injections of ATP into the cNTS increased breathing (Antunes *et al.* 2005*b*), whereas application of a P2-receptor blocker to the NTS blunted peripheral chemoreflex control of cardiorespiratory function (Paton *et al.* 2002; Braga *et al.* 2007). Furthermore, injection of ATP into the medullary raphe increased breathing (Cao & Song, 2007), whereas injection of a P2-receptor blocker into the rostral medullary raphe blunted the ventilatory response to CO_2 of conscious rats (da Silva *et al.* 2012). These results suggest that purinergic signalling may contribute to the CO_2/H^+-dependent drive to breathe at other putative chemoreceptor regions. Therefore, the main goal of this study was to determine at the cellular and system levels whether purinergic signalling contributes to CO_2/H^+ sensitivity in the cNTS and medullary raphe.

To test this possibility, we recorded the activity of individual CO_2/H^+-sensitive neurons in putative chemosensitive regions *in vitro*, and phrenic nerve activity (PNA) *in vivo* during exposure to hypercapnia alone and in the presence of P2-receptor blockers. To determine if cells in the regions of interest functionally express P2 receptors, we also tested responsiveness to exogenous application of ATP *in vivo* and *in vitro*. We found *in vitro* that CO_2/H^+-sensitive neurons in the cNTS responded to focal ATP application with a ∼3-fold increase in firing rate, and *in vivo* unilateral ATP injection into this region elicited an increase in PNA frequency by 100%. Application of ATP into the medullary raphe had no effect on chemoreceptor activity or cardiorespiratory output. In addition, P2-receptor blockade in the cNTS and medullary raphe had no effect on the ventilatory response to CO_2 *in vivo*, or the firing rate response to 15% CO_2 *in vitro*. In conjunction with previous data that purinergic signalling is a critical component of RTN chemoreception, these results suggest that purinergic signalling is a unique feature of RTN chemoreception.

Methods

In vivo preparation

All *in vivo* experiments conformed to the guidelines approved by the University of São Paulo Animal Care and Use Committee and were performed in male Wistar rats weighing 250–280 g. Surgical procedures and experimental protocols were similar to those previously described (Wenker *et al.* 2012). Briefly, general anaesthesia was induced with 5% halothane in 100% O_2. A tracheostomy was made and the halothane concentration was reduced to 1.4–1.5% until the end of surgery. The femoral artery was cannulated (polyethylene tubing, 0.6 mm o.d., 0.3 mm i.d., Scientific Commodities, Lake Havasu City, AZ, USA) for measurement of arterial pressure (AP). The femoral vein was cannulated for administration of fluids and drugs. The occipital plate was removed, and a micropipette was placed in the medulla oblongata via a dorsal transcerebellar approach for microinjection of drugs. A skin incision was made over the lower jaw for placement of a bipolar stimulating electrode, next to the mandibular branch of the facial nerve, as previously described (Wenker *et al.* 2012). The phrenic nerve was accessed by a dorsolateral approach after retraction of the right shoulder blade. To prevent any influence of artificial ventilation on phrenic nerve activity (PNA), the vagus nerve was cut bilaterally as previously described (Takakura & Moreira, 2011).

Upon completion of the surgical procedures, halothane was replaced by urethane (1.2 g kg^{-1}) administered slowly I.V. All rats were ventilated with 100% O_2 throughout the experiment. Rectal temperature was maintained at 37°C. End-tidal CO_2 was monitored throughout each experiment with a capnometer (CWE, Inc., Ardmore, PA, USA) that was calibrated twice per experiment with a calibrated CO_2/N_2 mix. This instrument provided a reading of <0.1% CO_2 during inspiration in animals breathing 100% O_2 and provided an asymptotic, nearly horizontal reading during expiration. The adequacy of anaesthesia was monitored during a 20 min stabilization period by testing for the absence of withdrawal responses, pressor responses, and changes in PNA to a firm toe pinch. After these criteria were satisfied, the muscle relaxant pancuronium was administered at an initial dose of 1 mg kg^{-1} I.V. and the adequacy of the anaesthesia was thereafter gauged solely by the lack of increase in AP and PNA rate or amplitude to a firm toe pinch. Approximately hourly supplements of one-third of the initial dose of urethane were needed to satisfy these criteria throughout the recording period (2–3 h).

In vivo recordings of physiological variables

As previously described, mean arterial pressure (MAP), phrenic nerve activity (PNA), and end-expiratory CO_2 ($etCO_2$) were digitized with a micro1401 (Cambridge Electronic Design, Cambridge, UK), stored on a computer, and processed off-line with version 6 of Spike 2 software (Cambridge Electronic Design) (Takakura & Moreira, 2011). Integrated phrenic nerve activity (iPNA) was obtained after rectification and smoothing ($\tau = 0.015$ s) of the original signal, which was acquired with a 30–300 Hz bandpass filter. PNA amplitude (PNA ampl) and PNA frequency (PNA freq) were normalized in each experiment by assigning to each of the two variables a value of 100 at saturation of the chemoreflex (high CO_2) and a value of 0 to periods of apnoea.

Brain slice preparation

All procedures were performed in accordance with the National Institutes of Health and University of Connecticut Animal Care and Use Guidelines. Brainstem slices were prepared as previously described (Wenker *et al.* 2012). Briefly, neonatal rats (7–12 days postnatal) were decapitated under ketamine/xylazine anaesthesia and transverse brainstem slices (300 μm) containing the raphe pallidus (RPa) were cut using a microslicer (DSK 1500E; Dosaka, Kyoto, Japan) in ice-cold substituted Ringer solution containing (in mM): 260 sucrose, 3 KCl, 5 $MgCl_2$, 1 $CaCl_2$, 1.25 NaH_2PO_4, 26 $NaHCO_3$, 10 glucose, and 1 kynurenic acid. Slices were incubated for ~30 min at 37°C and subsequently at room temperature in normal Ringer solution (in mM): 130 NaCl, 3 KCl, 2 $MgCl_2$, 2 $CaCl_2$, 1.25 NaH_2PO_4, 26 $NaHCO_3$, and 10 glucose. Both substituted and normal Ringer solutions were bubbled with 95% O_2–5% CO_2, extracellular pH ($pH_o = 7.35$). It should be noted that slices containing the cNTS were prepared as described above but with two exceptions: (1) slices were cut in normal Ringer solution (not substituted Ringer solution); and (2) slices were incubated at room temperature (not 37°C) for 60 min or more. At the end of each *in vivo* experiment, rats were deeply anesthetized with halothane and perfused through the heart with PBS, pH 7.4, followed by paraformaldehyde (4% in 0.1 m phosphate buffer, pH 7.4). The brain of each animal was isolated and sectioned for later histological analysis of injection sites.

Slice-patch electrophysiology

Individual slices were transferred to a recording chamber mounted on a fixed-stage microscope (Zeiss Axioskop FS, Oberkochen, Germany) and perfused continuously (~2 ml min^{-1}) with a bath solution of normal Ringer solution (same as incubation Ringer solution above) bubbled with 95% O_2–5% CO_2 ($pH_o = 7.35$). The pH of the bicarbonate-based bath solution was decreased to 6.90 by bubbling with 15% CO_2. All recordings were made with an Axopatch 200B patch-clamp amplifier, digitized with a Digidata 1322A A/D converter, and recorded using

pCLAMP 10.0 software (Molecular Devices, Union City, CA). Recordings were obtained at room temperature (~22°C) with patch electrodes pulled from borosilicate glass capillaries (Warner Instruments, Hamden, CT) on a two-stage puller (P89; Sutter Instrument Co., Novato, CA) to a DC resistance of 4–6 MΩ when filled with an internal solution containing the following (in mM): 120 KCH_3SO_3, 4 NaCl, 1 $MgCl_2$, 0.5 $CaCl_2$, 10 Hepes, 10 EGTA, 3 Mg-ATP, 0.2% biocytin, and 0.3 GTP-Tris (pH 7.2); electrode tips were coated with Sylgard 184 (Dow Corning, Midland, MI). All recordings of neuronal firing rate were performed in the cell-attached configuration to ensure minimal alteration of the intracellular milieu. Firing rate histograms were generated by integrating action potential discharge in 10 s bins and plotted using Spike 5.0 software. Whole cell voltage clamp recordings (holding potential $V_h = -60$ mV, in tetrodotoxin to block action potentials) were made to verify the serotonergic phenotype of RPa neurons, i.e. medullary raphe neurons are known to exhibit a serotonin-activated inward potassium conductance (Bayliss *et al.* 1997).

Drugs

All drugs were purchased from Sigma-Aldrich (USA). For *in vivo* experiments, the non-specific P2 receptor antagonist pyridoxalphosphate-6-azophenyl-2′,4′-disulfonic acid (PPADS: 100 μM in sterile saline (pH 7.4)) or ATP (10 mM) were injected into the RTN using single-barrel glass pipettes (tip diameter of 25 μm) connected to a pressure injector (Picospritzer III, Parker Hannifin Corp., Cleveland, OH, USA). For each injection we delivered a volume of 50 nl over a period of 5 s. These glass pipettes also allowed recordings of field potential properties that were used to help direct the electrode tip to the desired site. Injections in the RTN region were guided by recordings of the facial field potential (Brown & Guyenet, 1985), and were placed 250 μm below the lower edge of the field, 1.7 mm lateral to the midline, and 200 μm rostral to the caudal end of the field. Recordings were made on one side only; the second injection was made 1–2 min later at the same level on the contralateral side. Injections into the medullary raphe were placed in the raphe pallidus ($n = 6$) and the parapyramidal region ($n = 5$) 150–200 μm below the lower edge of the field, 1.0 mm lateral to the midline, and 200 μm rostral to the caudal end of the field. Injections in the cNTS region were centred ~400 μm caudal to the calamus scriptorius, in the midline and 300–500 μm below the dorsal surface of the brainstem. We included a 5% dilution of fluorescent latex microbeads (Lumafluor, New City, NY, USA) with all drug applications to mark the injection sites and to verify spread of the injections.

For *in vitro* experiments, we bath applied 100 μM of either suramin or PPADS to block P2 receptors. In addition, ATP (1 mM in Hepes-buffered medium, pH 7.3) was delivered focally using low-resistance pipettes connected to a Picospritzer III (Parker Instrumentation, Cleveland, OH, USA). Application times were 600 ms, and vehicle control experiments were performed to ensure agonist responses were not attributable to pressure artifacts.

Statistics

Data are reported as mean ± standard error of the mean. Statistical analysis was performed using Sigma Stat version 3.0 software (Jandel Corporation, Point Richmond, CA, USA). A *t* test, paired *t* test or one-way ANOVA followed by the Newman–Keuls multiple comparisons test was performed as appropriate ($P < 0.05$).

Results

This study was composed of both *in vitro* and *in vivo* experiments. In the brain slice preparation, we investigated whether purinergic signalling could influence the basal activity or CO_2/H^+ sensitivity of putative chemoreceptors by testing the effects of focal ATP application on firing rate and bath application of a P2 receptor blocker (suramin or PPADS) on the CO_2/H^+ responsiveness of neurons in the cNTS and medullary raphe (see Supporting information Fig. S1 for approximate locations of CO_2-sensitive neurons in these regions). To provide a functional basis for these results, we tested *in vivo* the effects of ATP and PPADS on baseline cardiorespiratory activity and CO_2 responsiveness. As a positive control for ATP injections in the cNTS and raphe, we also tested the effects of focal ATP application into the RTN on breathing and blood pressure.

Purinergic signalling in the cNTS increases activity of chemosensitive neurons and respiratory activity but does not contribute to CO_2/H^+ responsiveness

We recorded from CO_2/H^+-sensitive cNTS neurons in slices to characterize the effects of purinergic signalling on baseline activity and CO_2/H^+ responsiveness. As previously described (Nichols *et al.* 2009), a neuron was designated CO_2/H^+ sensitive if it was spontaneously active under control conditions (5% CO_2) and responded to 15% CO_2 with a ≥0.5 Hz increase in firing rate (this corresponds with a chemosensitivity index of ~150%). We did not find any CO_2-inhibited cells but this is not surprising considering that only a small minority of cells in this region have been shown to be inhibited by hypercapnia (Dean *et al.* 1990). The average firing rate of CO_2/H^+-sensitive cells was 0.50 ± 0.12 Hz ($n = 10$) under control conditions and 2.00 ± 0.24 Hz in 15% CO_2 (i.e. CO_2 increased activity by 1.48 ± 0.17 Hz; $n = 10$ cells from 9 animals; Fig. 1*A* and *B*). This level of CO_2/H^+ sensitivity

is nearly identical to what has been described previously for cells in this region (Dean *et al.* 1990; Nichols *et al.* 2009) so we are confident that we were recording from the group of cells thought to function as chemoreceptors. To gain some insight into the neurochemical phenotype of CO_2-sensitive cNTS neurons, we filled recorded cells with biocytin for subsequent immunohistochemical analysis using antibodies for the transcription factor Phox2b and

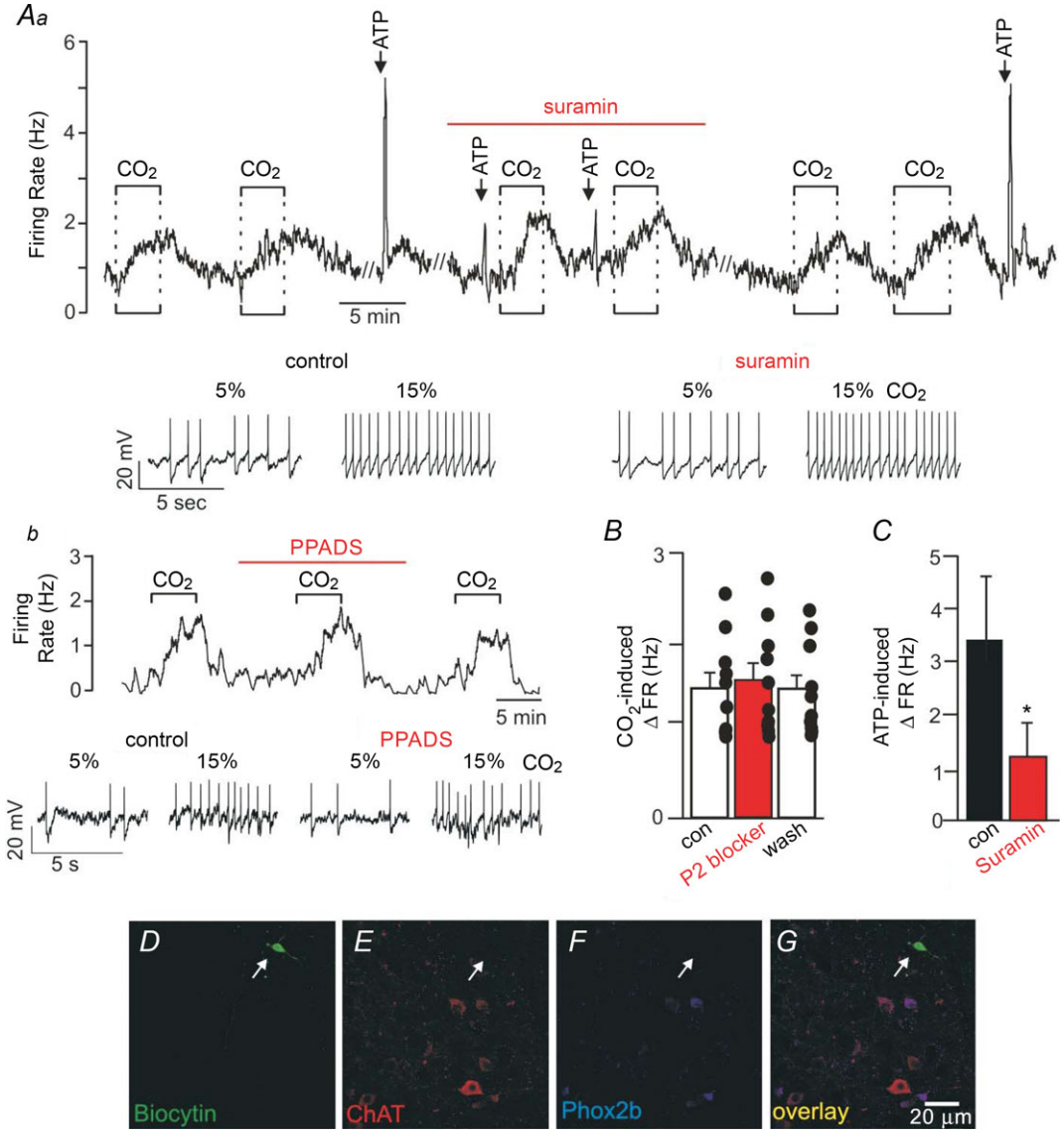

Figure 1. Purinergic signalling modulates activity but not CO_2/H^+ sensitivity of caudal NTS neurons *in vitro*

Aa, firing rate trace and segments of membrane potential from a cNTS neuron show that under control conditions exposure to 15% CO_2 increased neuronal activity by ~1 Hz in a reversible and repeatable manner. Under control conditions, focal application of ATP (1 mM) evoked a rapid and robust increase in firing rate of ~4 Hz. Bath application of the P2-receptor blocker suramin (100 μM) had no effect on basal activity but significantly attenuated the firing rate response to ATP. In the continued presence of suramin additional bouts of 15% CO_2 increased firing rate by an amount similar to under control conditions in the absence of suramin. *Ab*, firing rate trace and segments of membrane potential from a cNTS neuron show that the P2-receptor blocker PPADS (100 μM) also had no effect on baseline activity or CO_2 responsiveness. *B*, summary data ($n = 10$ cells from 9 animals) show that P2-receptor blockers (suramin or PPADS) had no measurable effect on the CO_2/H^+ sensitivity of cNTS neurons. *C*, summary data ($n = 5$) confirm that suramin effectively blunted the ATP responsiveness of these neurons. *indicates different from control ($P < 0.05$). *D–G*, biocytin-labelled CO_2/H^+-sensitive cNTS neuron (*D*, arrow) is immuno-negative for choline acetyltransferase (ChAT) (*E*) and Phox2b (*F*). *G* shows merged images of the same cell as in *D–F*. Each image is the average projection of a confocal stack; around 30 images were taken every 0.26 μm in the *Z* plane. Scale bar is 20 μm.

choline acetyltransferase (ChAT; marker of cholinergic neurons). We found that 6 of 6 CO_2-sensitive cNTS neurons were negative for Phox2b and ChAT (Fig. 1*D*–*G*).

To determine if purinergic signalling contributes to the activity of cNTS neurons, we first measured the firing rate response of CO_2/H^+-sensitive neurons in this region to focal application of ATP (1 mM in Hepes-buffered saline, pH 7.3). Under control conditions all cells tested exhibited a large reversible and repeatable increase in activity (increase of 3.39 ± 1.4 Hz; $n = 6$) in response to ATP (Fig. 1*A* and *C*). Bath application of suramin (100 μM) or PPADS (100 μM) had no discernible effect on baseline activity (firing rate under control conditions and during P2-receptor blockade was 0.50 ± 0.12 and 0.58 ± 0.11 Hz, respectively; $n = 10$ cells from 9 animals), suggesting that these cell do not receive purinergic drive under control conditions. As expected, suramin decreased the firing rate response to ATP by 67.0 ± 7.2%, thus confirming that P2 receptors are effectively blocked under these conditions. However, neither suramin (Fig. 1*Aa*) nor PPADS (Fig. 1*Ab*) blunted the firing rate response to CO_2 (the CO_2-induced change in firing rate under control conditions and during P2-receptor blockade was 1.48 ± 0.17 and 1.57 ± 0.20 Hz, respectively; $n = 10$ cells from 9 animals; Fig. 1*B*). These results indicate that CO_2-sensitive cNTS neurons are activated directly or indirectly by purinergic signalling; however, this signalling mechanism does not contribute to the CO_2 responsiveness of these cells *in vitro*.

It is possible that endogenous purinergic drive to CO_2/H^+-sensitive cNTS neurons was disrupted in our *in vitro* preparation. Therefore, we also tested the effects of cNTS injections of ATP and a P2-receptor blocker (PPADS) on baseline breathing and the ventilatory response to CO_2 in anaesthetized rats. Consistent with our slice data, we found that injection of ATP (10 mM, 50 nl) into the cNTS elicited an increase in phrenic nerve amplitude (PNA ampl) (46 ± 6% *vs.* saline: 6 ± 4%, $P < 0.01$) and frequency (PNA freq) (103 ± 11% *vs.* saline: 5 ± 5%, $P < 0.0001$). ATP injections into this region also increased MAP (21 ± 4 mmHg *vs.* saline: 3 ± 1 mmHg, $P < 0.05$; Fig. 2*A* and *C*–*E*). Also consistent with our slice data, bilateral injections of a P2-receptor blocker (PPADS; 3 mM, 50 nl; $n = 6$ animals per condition (saline or ATP)) into the cNTS did not change resting PNA (99 ± 7% of control, $P > 0.05$) or MAP (124 ± 5 mmHg *vs.* saline: 123 ± 7 mmHg, $P > 0.05$; Figs 2*A* and 3*A*). Furthermore, PPADS injections into the cNTS did not affect the cardiorespiratory response to CO_2; exposure to 10% CO_2 increased PNA amplitude (103 ± 9% *vs.* saline: 99 ± 13%, $P > 0.05$), PNA frequency (107 ± 6% *vs.* saline: 105 ± 4%, $P > 0.05$) and MAP (18 ± 7 mmHg *vs.* saline: 21 ± 4 mmHg, $P > 0.05$) (Fig. 3*A*–*E*).

For these experiments, injections of ATP or PPADS were centred about 400 μm caudal to the calamus scriptorius (Figs 2*B* and 3*B*). A single injection of ATP or PPADS was administered in or near the midline. Based on the distribution of the fluorescent microbeads (Fig. 2*B*), the injectate spread bilaterally ∼500 μm from the injection centre and ∼300 μm from the injection centre in the rostrocaudal direction.

Purinergic signalling in the medullary raphe does not modulate activity of CO_2-sensitive neurons *in vitro* or contribute to the chemoreflex *in vivo*

We recorded from CO_2/H^+-sensitive medullary raphe neurons in slices to characterize the effects of purinergic signalling on baseline activity and CO_2 responsiveness. For these experiments we targeted the raphe pallidus at approximately the same rostrocaudal level as the RTN (−11.6 mm from bregma). Cells in this region were considered CO_2/H^+ sensitive (activated or inhibited) if they responded to 15% CO_2 with ≥20% change in firing rate (this corresponds with a chemosensitivity index of ≥120% or ≤80%). This criteria is similar to previous definitions of raphe chemosensitivity (Richerson, 1995; Wang & Richerson, 1999). It should be noted that cells were presumed to be serotonergic based on (i) location, (ii) CO_2/H^+ sensitivity, (iii) stereotypic firing behaviour as evidenced by a low interspike interval (ISI) coefficient of variation (Mason, 1997), and in several cases (iv) expression of a characteristic serotonin-activated inward potassium conductance (Bayliss *et al.* 1997) measured in the whole cell voltage clamp configuration (data not shown). The majority of cells in this region did not respond to 15% CO_2, as expected for raphe neurons in slices isolated from animals ≤12 days of age (Wang & Richerson, 1999). Raphe neurons that were stimulated by hypercapnia ($n = 9$ cells from 8 animals) had an average spontaneous discharge rate of 0. 84 ± 0.19 Hz in 5% CO_2 that increased to 1.35 ± 0.24 Hz in 15% CO_2 (Fig. 4*A* and *B*). Raphe neurons that were inhibited by hypercapnia ($n = 3$) had a spontaneous firing rate of 0.70 ± 0.13 in 5% CO_2 that decreased to 0.28 ± 0.06 in 15% CO_2 (Supporting information Fig. S2).

To determine if purinergic signalling contributes to the activity of these cells, we next measured the CO_2/H^+ responsiveness of raphe pallidus neurons when P2 receptors are blocked with suramin or PPADS. Bath application of either suramin (100 μM) or PPADS (100 μM) had no measurable effect on the baseline activity or CO_2/H^+ sensitivity of these cells. For example, during P2 receptor blockade CO_2/H^+-activated cells responded to hypercapnia with an increase in firing rate from 0.96 ± 0.25 Hz to 1.49 ± 0.33 Hz ($n = 9$ cells from 8 animals; Fig. 4*A*, *B* and *D*). This response corresponds to an activity increase of 0.53 ± 0.10 Hz which is not significantly different from the CO_2/H^+-induced

increase under control conditions (0.51 ± 0.08). We were not able to successfully recover recorded cells for immunohistochemical characterization, therefore to identify CO_2-activated cells as serotonergic-like we used a linear discriminant function to analyse interspike interval (ISI) as previously described (Mason, 1997); serotonergic cells fire in a highly stereotypic manner and so have a low ISI coefficient of variation (ISI_{CV}) and negative discriminant score compared to non-serotonergic cells which have a higher ISI_{CV} and positive discriminant score (Mason, 1997). We found that CO_2-activated raphe neurons had an average ISI_{CV} of 0.22 ± 0.2 and all cells had negative discriminant scores that ranged from −312 to −1817, i.e., these data points appear below the discriminant line shown in Fig. 4*C*. These results confirm that all nine raphe cells are serotonergic-like. We also found that in the presence of suramin CO_2/H^+-inhibited raphe neurons respond to 15% CO_2 with a decrease in firing rate from 0.76 ± 0.12 Hz to 0.39 ± 0.12 Hz (Supporting information Fig. S2). This corresponds to a 0.39 ± 0.12 Hz decrease in activity which is similar to CO_2/H^+-mediated inhibition under control conditions (Supporting information Fig. S2). Furthermore, in a separate series of experiments, we found that 11 (7 CO_2/H^+-activated and 6 CO_2/H^+-inhibited) of 13 CO_2/H^+-sensitive raphe pallidus neurons showed no measurable response to focal application of ATP (1 mM in Hepes-buffered saline, pH 7.3; Fig. 4*A* and *C*). Two raphe neurons (one CO_2-activated and one CO_2-inhibited) responded to ATP with an increase in firing rate (data not shown). Together, these results indicate that purinergic signalling does not contribute to CO_2/H^+ sensing by raphe pallidus neurons *in vitro*.

To determine if purinergic signalling in the medullary raphe contributes to the chemoreflex, we tested the effects of medullary raphe injections of ATP and PPADS on baseline breathing and the ventilatory response to CO_2 in urethane-anaesthetized rats. For these experiments, we targeted the raphe pallidus (RPa; $n = 6$) and the parapyramidal region (Ppy) ($n = 5$) because serotonergic

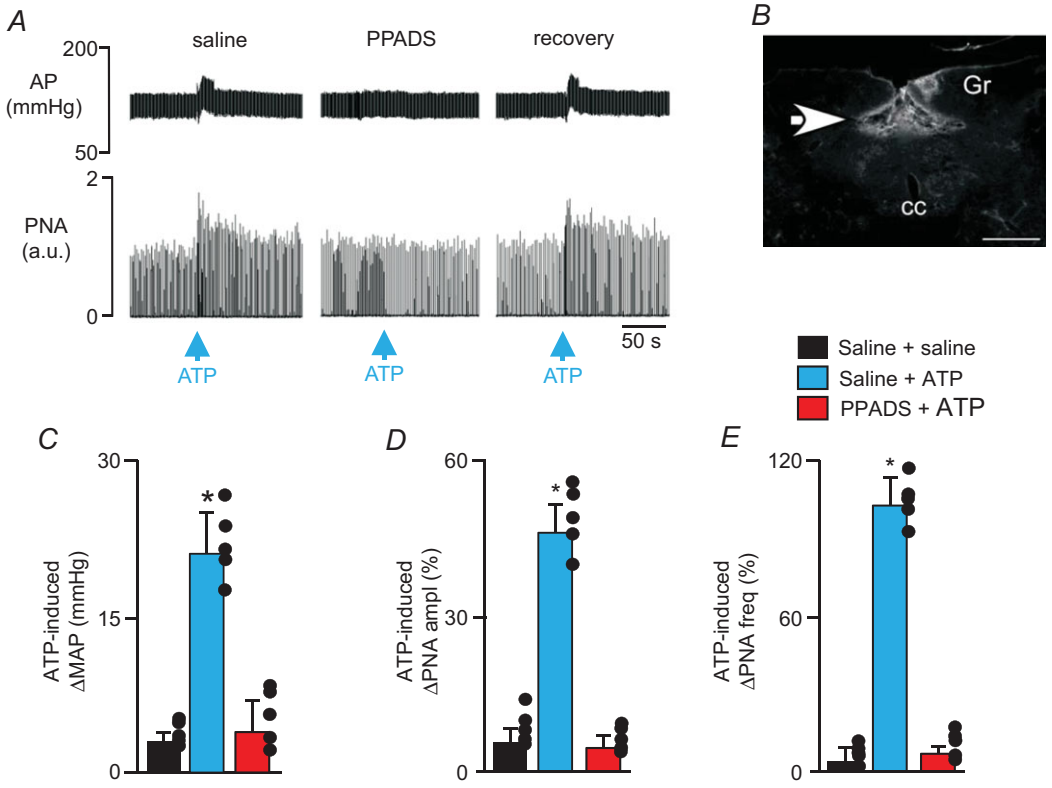

Figure 2. Injection of ATP into the cNTS increased breathing and blood pressure in vago-sino-aortic denervated rats by a P2-receptor-dependent mechanism
A, recordings of AP and PNA show that application of ATP (10 mM, 50 nl) into the cNTS increased breathing and blood pressure when preceded by injection of saline into this same region (i.e. control condition) but not after injection of PPADS into this region. Cardiorespiratory responses to ATP partly recovered after washing PPADS for ∼1 h. *B*, histology section showing the distribution of fluorescent microbeads within the cNTS. *C–E*, summary data ($n = 5$) show changes in mean arterial pressure (ΔMAP) (*C*), PNA amplitude (ΔPNA ampl) (*D*) and PNA frequency (ΔPNA freq) (*E*) elicited by injection of saline or PPADS + ATP into the cNTS. *indicates different from control ($P < 0.05$). Abbreviations: Gr, gracile nucleus; cc, central canal. Scale bar in *A* is 50 s and in *B* is 500 μm. a.u., arbitrary units.

cells in these regions reportedly function as chemoreceptors (Richerson, 2004) and are thought to be located within diffusion distance (estimated to be ~400 μm from the ventral surface; Spyer & Gourine, 2009) from sites of CO_2/H^+-evoked ATP release on the ventral surface. Injections of PPADS were placed bilaterally in the medullary raphe in these rats (Fig. 5*B*). The injection centre was 200–230 μm below the facial motor nucleus, 200 μm rostral to the caudal end of this nucleus and 1 mm lateral to the midline as previously demonstrated (Mulkey *et al.* 2004; Takakura & Moreira, 2013; Fig. 5*B*). Consistent with our *in vitro* data, we found that bilateral injections of PPADS (3 mM, 50 nl) into either the RPa or Ppy had no effect on baseline PNA or the ventilatory response to CO_2. For example, bilateral injections of PPADS (3 mM, 50 nl; $n = 8$ per group) into the medullary raphe did not change resting PNA or resting MAP (Fig. 5*A*). In addition, bilateral injections of PPADS into the medullary raphe did not affect the hypercapnia-induced increase in PNA amplitude (106 ± 11% *vs.* saline: 105 ± 14%, $P > 0.05$), PNA frequency (102 ± 8% *vs.* saline: 101 ± 5%, $P > 0.05$) and MAP (22 ± 4 mmHg *vs.* saline: 22 ± 6 mmHg, $P > 0.05$; Fig. 5*A* and *C–E*). Also consistent with our slice data, we found that injections of ATP (10 mM, 50 nl) into the medullary raphe had no effect on PNA ampl, PNA freq or blood pressure (Fig. 6*A–E*).

Application of ATP into the RTN increases cardiorespiratory activity

There is strong evidence that purinergic signalling contributes to RTN chemoreception (Gourine *et al.*

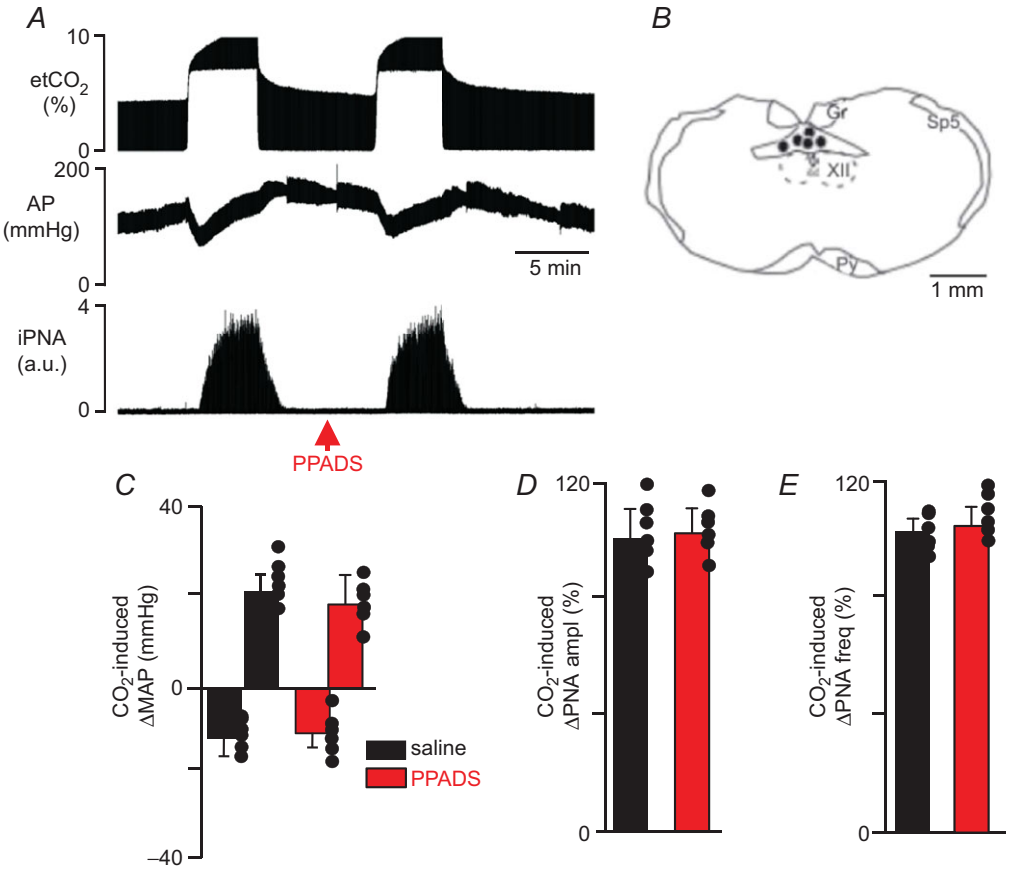

Figure 3. P2-receptor blockade at the level of the cNTS had no effect on hypercapnia-induced changes in cardiorespiratory activity of anaesthetized vago-sino-aortic denervated rats
A, recordings of end-expiratory CO_2 ($etCO_2$), arterial pressure (AP) and integrated phrenic nerve activity (iPNA) show the pressure and ventilatory responses of a rat to hypercapnia under control conditions (i.e. after saline injection) and 5 min after PPADS (3 mM, 50 nl) was injected into the cNTS. *B*, computer-assisted plots of the centre of the injection sites revealed by the presence of fluorescent microbeads included in the injectate (coronal projection on plane bregma –14.3 mm; Paxinos & Watson, 1998). *C–E*, summary data ($n = 6$) show that cNTS injections of PPADS had no effect on CO_2-induced changes in mean arterial pressure (ΔMAP) (*C*), PNA amplitude (ΔPNA ampl) (*D*) and PNA frequency (ΔPNA freq) (*E*). Abbreviations: Gr, gracile nucleus; Py, pyramids; Sp5, spinal trigeminal tract; XII, hypoglossal motor nucleus; cc, central canal. Black dots represent the injections sites in the cNTS. Scale bar in *A* is 5 min and in *B* is 1 mm.

2005, 2010; Mulkey *et al.* 2006; Huckstepp *et al.* 2010; Wenker *et al.* 2010, 2012). We confirm this possibility by testing the effects of unilateral injections of ATP into the RTN on cardiorespiratory activity. These experiments also serve as a positive control for similar experiments in the commissural NTS (cNTS) and medullary raphe (described above). Consistent with previous evidence (Gourine *et al.* 2005; Mulkey *et al.* 2006), we found that unilateral injection of ATP into the RTN increased MAP (14 ± 3 mmHg *vs.* saline: 2 ± 3 mmHg, $P < 0.05$), PNA amplitude by 26 ± 5% and PNA frequency by 21 ± 4% (Fig. 7*A–E*). The cardiorespiratory responses elicited by ATP were eliminated by previous injections of PPADS within the RTN (Fig. 7*A–E*). These results confirm that

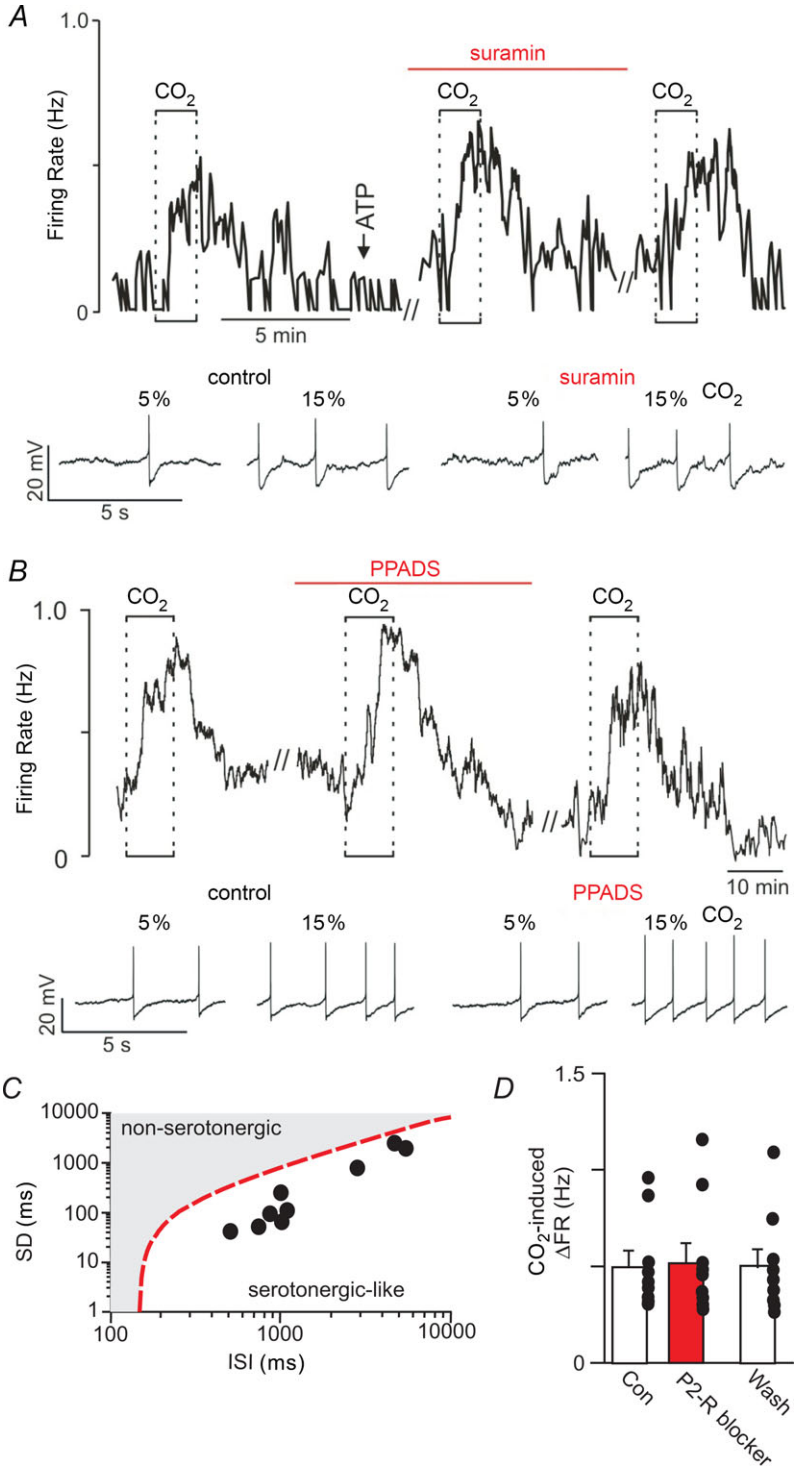

Figure 4. Purinergic signalling does not modulate activity or CO_2/H^+ sensitivity of raphe pallidus neurons *in vitro*
A, trace of firing rate and segments of membrane potential from a CO_2/H^+-activated RPa neuron show that exposure to 15% CO_2 increased firing rate ∼0.5 Hz under control conditions and when P2 receptors were blocked with suramin (100 μM). In addition, focal application of ATP (1 mM) had no effect on neuronal activity (arrow). *B*, firing rate trace and segments of membrane potential show that PPADS (100 μM) also did not affect CO_2 responsivness of this CO_2/H^+-activated RPa neuron. *C*, to confirm that all CO_2/H^+-activated raphe neurons were serotonergic-like we analysed the interspike interval (ISI) using a linear discriminant function (y(ISI, standard deviation) = 146 – ISI + 0.98SD) as previously described (Mason, 1997). *C* shows a log–log plot of the mean interspike interval *vs.* the SD of the interspike interval for all CO_2-activated RPa neurons used in this study. The discriminant line (dotted line) occurs when the equation is set equal to zero and reportedly defines the optimal linear boundary between serotonergic and non-serotonergic cell types (Mason, 1997). All raphe neurons used in this study had negative discriminant scores, thus predicting that they are serotonergic. *D*, summary data ($n = 9$) shows that P2-receptor blockade with suramin or PPADS had no effect on the CO_2/H^+-activated RPa neurons. //designates 10 min time breaks where data is not shown.

purinergic signalling at the level of the RTN regulates cardiorespiratory activity.

Discussion

There is compelling evidence that CO_2/H^+-evoked ATP release from ventral surface astrocytes contributes to respiratory drive by activating chemosensitive RTN neurons (Gourine *et al.* 2005; Huckstepp *et al.* 2010; Wenker *et al.* 2012). Here, we confirm that activation of P2 receptors in the RTN increases cardiorespiratory activity, and we test at the cellular and systems level whether purinergic signalling also modulates the activity or CO_2/H^+ sensitivity of neurons in two other brainstem regions thought to contribute to central chemoreception (i.e. the cNTS and medullary raphe). As expected, application of ATP into the RTN elicited a strong increase in breathing and a mild increase in blood pressure by a P2-receptor-dependent mechanism, thus further supporting the possibility that purinergic signalling in the ventrolateral medulla can modulate the activity of respiratory chemoreceptors (Thomas & Spyer, 2000; Gourine *et al.* 2005; Huckstepp *et al.* 2010; Wenker *et al.* 2012) and blood pressure-regulating C1 cells (Wenker *et al.* 2013). Similar to previous evidence in the RTN, CO_2/H^+-sensitive cNTS neurons also respond to focal ATP application with a robust increase in firing rate, and *in vivo* ATP injection into the cNTS increased breathing. Conversely, CO_2/H^+-sensitive medullary raphe neurons did not respond to ATP nor did injection of ATP into this region elicit a cardiorespiratory response. Contrary to evidence in the RTN, inhibition of P2 receptors in the cNTS and medullary raphe had no effect on basal activity and CO_2/H^+ sensitivity *in vitro*, or baseline cardiorespiratory activity and CO_2 responsiveness *in vivo*. Therefore, previous and present results suggest that purinergic signalling is a unique feature of RTN chemoreception.

Experimental limitations

Our *in vivo* experiments were performed in anaesthetized rats and it is possible that anaesthetics modify centrally mediated cardiorespiratory reflexes. Therefore, it will be important for future studies to confirm the role of purinergic signalling in central chemoreception using

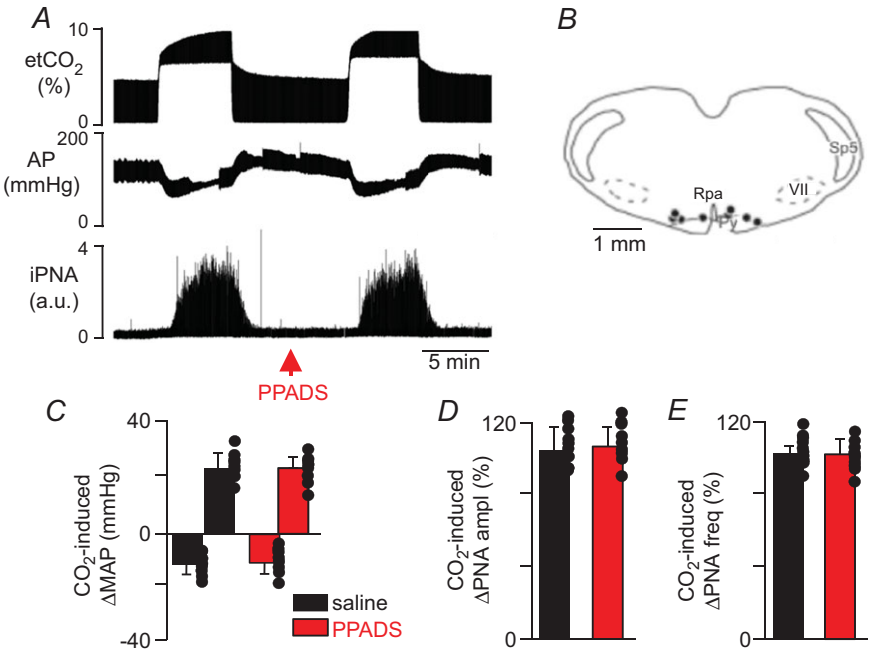

Figure 5. P2 receptor antagonist injections into the medullary raphe had no effect on hypercapnia-induced changes in arterial pressure and PNA in vago-sino-aortic denervated rats
A, recordings from one rat show effects of injection of PPADS into the RPa/Ppy on changes in phrenic nerve activity (PNA) elicited by an increase of end-expiratory CO_2 from 5 to 10%. Responses were recorded 5 min after injection of saline (50 nl) or PPADS (3 mM, 50 nl) into the RPa/Ppy. *B*, computer-assisted plot shows the centre of the injection sites (coronal projection on plane bregma −11.6 mm; Paxinos & Watson, 1998). *C–E*, summary data ($n = 11$) show that PPADS injection into the RPa/Ppy had no effect on CO_2-induced changes in mean arterial pressure (ΔMAP) (*C*), PNA amplitude (ΔPNA ampl) (*D*) or PNA frequency (ΔPNA freq) (*E*). Abbreviations: py, pyramid; RPa, raphe pallidus; Sp5, spinal trigeminal tract; VII, facial motor nucleus. Black dots represent the injections sites in the RPa/Ppy. Scale bar in *A* is 5 min and in *B* is 1 mm.

conscious animals. In addition, different age ranges were used for *in vitro* (7- to 12-day-old pups) and *in vivo* (adult rats) experiments; however, we consider this a minor issue since the extent to which purinergic signalling contributes to chemoreception at the level of the RTN was very similar between preparations (Wenker *et al.* 2012). Another limitation of this study is that we did not assess astrocyte function directly using Ca^{2+} imaging so we are unable to provide insight into mechanisms underlying the differential role of purinergic signalling in RTN chemoreception. However, previous studies have shown that RTN astrocytes, but not cortical or NTS astrocytes, respond to H^+ with an increase in intracellular Ca^{2+} (Kasymov *et al.* 2013), therefore it is possible that RTN astrocytes are functionally specialized to sense and respond to changes in CO_2/H^+.

The RTN is an important site of chemoreception and intense effort has been made to identify a unique CO_2/H^+-sensing mechanism in this region. Evidence indicates that chemosensitive RTN neurons sense and respond to changes in tissue CO_2/H^+ directly (Lazarenko *et al.* 2009; Onimaru *et al.* 2012; Wang *et al.* 2013), in part by H^+-mediated inhibition of TASK–2 channels (Wang *et al.* 2013), and indirectly by CO_2/H^+-evoked ATP release, most likely from astrocytes (Gourine *et al.* 2005, 2010; Huckstepp *et al.* 2010; Wenker *et al.* 2010, 2012). Evidence also suggests that a subset of RTN astrocytes are uniquely specialized to release ATP in a CO_2/H^+-dependent manner. For example, CO_2/H^+-evoked ATP release has been shown to occur at discrete regions on the ventral surface near the RTN but not in deeper brainstem regions or near the dorsal surface (Gourine *et al.* 2005; Huckstepp *et al.* 2010). In addition, evidence suggests that a subset of ventral surface astrocytes but not cortical or NTS astrocytes respond to H^+ with an increase in intracellular Ca^{2+} and vesicular release of ATP (Kasymov *et al.* 2013). The results presented here build on the possibility that purinergic signalling is a unique component of RTN chemoreception by showing that P2-receptor blockade in two putative chemoreceptor regions did not affect CO_2 responsiveness *in vitro* or *in vivo*.

The caudal portion of the NTS is thought to contribute to central chemoreception because a subset of neurons in this region respond to CO_2 with increased firing rate (Dean *et al.* 1990; Nichols *et al.* 2009) and focal acidification of this region has been shown to stimulate breathing in

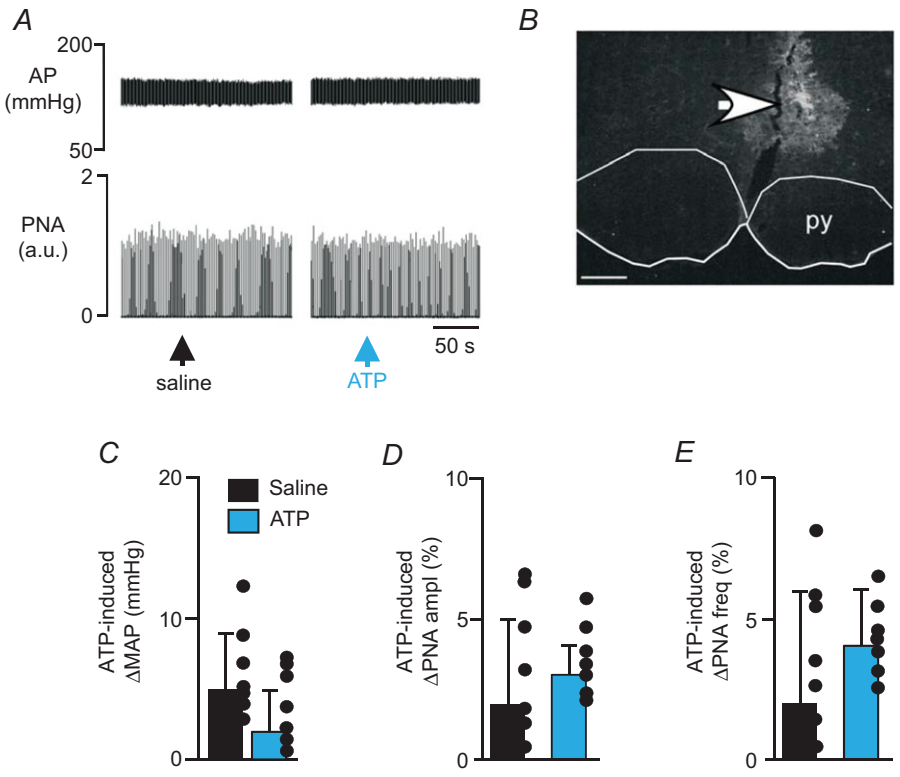

Figure 6. Injection of ATP into the medullary raphe had no effect on breathing or blood pressure
A, recordings of AP and PNA show that application of ATP (10 mM, 50 nl) into the medullary raphe (RPa/Ppy) did not affect blood pressure or respiratory motor output. *B*, histology section showing the distribution of fluorescent microbeads within the RPa. *C–E*, summary data ($n = 7$) show that RPa/Ppy injections of ATP had no effect on mean arterial pressure (ΔMAP) (*C*), PNA amplitude (ΔPNA ampl) (*D*) or PNA frequency (ΔPNA freq) (*E*). Scale bar in *A* is 50 s and in *B* is 50 μm.

anaesthetized (Coates *et al.* 1993) and unanaesthetized animals (Nattie & Li, 2002). Although the cellular and molecular identity of CO_2/H^+ sensors in the cNTS are unknown, it is well established that cells in this region, including Phox2b immunoreactive neurons (Stornetta *et al.* 2006), relay peripheral chemosensory inputs to other components of the respiratory circuit; thus cells in this area are well positioned to regulate cardiorespiratory activity in response to hypercapnia.

Previous evidence suggests that purinergic signalling in the cNTS influences cardiorespiratory activity. For example, several types of P2 receptors are expressed in the cNTS (Yao *et al.* 2000) and injection of ATP or related analogues into the cNTS of awake rats mimicked cardiorespiratory responses (i.e. bradycardia, hypertension and tachypnoea) to peripheral chemoreceptor activation (Paton *et al.* 2002; de Paula *et al.* 2004; Antunes *et al.* 2005*a*). Purinergic signalling also contributes to pulmonary stretch receptor-mediated activation of second-order relay neurons in the NTS to control inspiration (Gourine *et al.* 2008). Consistent with the possibility that purinergic signalling in the cNTS contributes to cardiorespiratory control, we show that ATP injections into this region increased cardiorespiratory activity. We also show that CO_2/H^+-sensitive cNTS neurons are strongly activated by focal ATP application. It should be noted that the neurochemical phenotype of CO_2/H^+-sensitive cNTS neurons remains unknown, since these cells were immuno-negative for Phox2b and ChAT. Nevertheless, blocking P2 receptors in this region did not affect the firing rate response to CO_2/H^+ *in vitro*, or the ventilatory response to CO_2 *in vivo*. These results indicate that purinergic signalling can increase the activity of CO_2/H^+-sensitive cNTS neurons and stimulate cardiorespiratory activity, but this mechanism does not contribute to CO_2 responsiveness. It remains possible that astrocytes in this region contribute to the CO_2/H^+ responsiveness of cNTS neurons, albeit by an ATP-independent mechanism. For example, recent evidence showed that acidification decreased glutamate uptake by NTS astrocytes and facilitated glutamatergic slow excitatory potentials that could potentially increase integrated output of the NTS to the rest of the respiratory network (Huda *et al.* 2013).

Serotonergic medullary raphe neurons can strongly influence breathing (Hodges & Richerson, 2010) and are thought to function as respiratory chemoreceptors in part because neurons in this area have been shown to be acid sensitive *in vitro* (Richerson, 1995, 2004; Wang & Richerson, 1999; Wang *et al.* 2001), focal acidification of this region increased breathing in unanaesthetized awake (Hodges *et al.* 2004) or sleeping (Nattie & Li,

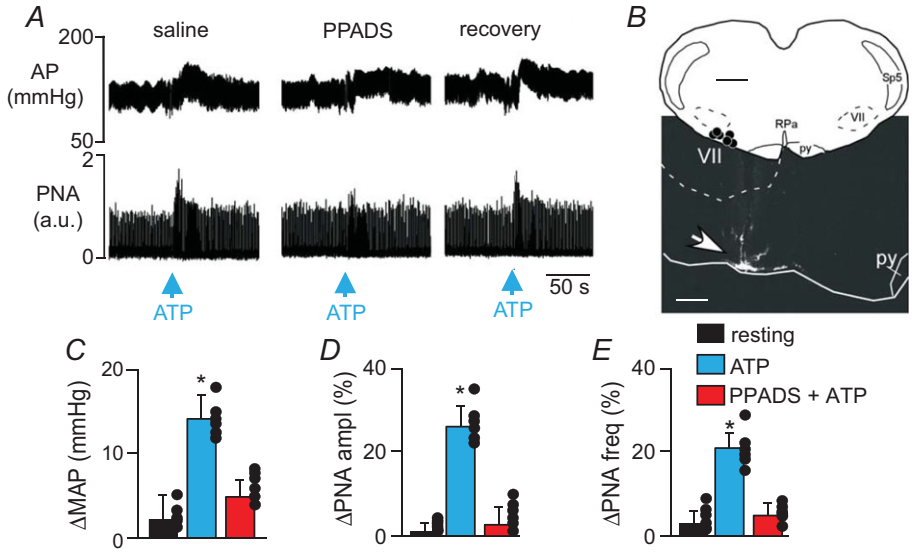

Figure 7. ATP injections into the RTN increase cardiorespiratory activity
A, recordings of arterial pressure (AP) and phrenic nerve activity (PNA) show that under control conditions unilateral injection of ATP (10 mM, 50 nl) into the RTN increased breathing and blood pressure. Injection of PPADS (3 mM, 50 nl) into the RTN blocked cardiorespiratory responses to subsequent applications of ATP. *B*, computer-assisted plot and representative histological section show centre of injection sites (coronal projection on plane bregma –11.6 mm; Paxinos & Watson, 1998). *C–E*, summary data ($n = 6$) show changes in mean arterial pressure (ΔMAP) (*C*), PNA amplitude (ΔPNA ampl) (*D*) and PNA frequency (ΔPNA freq) (*E*) elicited by injection of saline or ATP into the RTN. *indicates different from control ($P < 0.05$). Abbreviations: py, pyramid; RPa, raphe pallidus; Sp5, spinal trigeminal tract; VII, facial motor nucleus. Black dots represent the injections sites in the RPa/Ppy. Scale bar in *A* is 50 s and top and bottom scale bars in *B* are 1 mm and 300 μm, respectively.

2001) animals, and genetic deletion (Hodges *et al.* 2008) or selective inhibition (Ray *et al.* 2011) of serotonergic neurons decreased the ventilatory response to CO_2. As previously described (Richerson, 1995; Wang & Richerson, 1999), we found a subset of medullary raphe neurons in slices from neonatal rat pups that responded to 15% CO_2 with a modest increase or decrease of firing rate.

There is some evidence that CO_2/H^+ sensitivity of raphe neurons is partially dependent on purinergic signalling. For example, a study in anaesthetized rats showed that microinjection of ATP into the raphe magnus and pallidus decreased or increased respiratory activity, respectively (Cao & Song, 2007). In addition, injection of PPADS into the medullary raphe blunted the ventilatory response to CO_2 of conscious rats (da Silva *et al.* 2012). Considering that P2 receptors are ubiquitously expressed in the central nervous system, including at the level of the medullary raphe (Yao *et al.* 2000), we were surprised to find that CO_2/H^+-sensitive neurons (i.e. activated or inhibited) in the raphe pallidus did not respond to focal application of ATP *in vitro*, and injection of ATP into the raphe pallidus or the parapyramidal region had no effect on baseline breathing *in vivo*. In addition, blocking P2 receptors in these medullary raphe regions did not perturb CO_2 sensitivity *in vitro* or *in vivo*. These results suggest that purinergic signalling does not influence activity or CO_2 sensitivity at these raphe nuclei under our experimental conditions.

In summary, purinergic signalling contributes to the mechanism by which RTN neurons sense and respond to changes in CO_2/H^+; however, this signalling mechanism is not essential for CO_2/H^+ sensing in the cNTS or medullary raphe. These results are the first evidence for a unique CO_2/H^+-sensing mechanism in the RTN.

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Additional information

Competing interests

None declared.

Author contributions

All *in vitro* experiments were performed at the University of Connecticut and all *in vivo* experiments were perforemd at the University of Sao Paulo C.R.S: collection and analysis of *in vivo* data; revising the manuscript; final approval of the manuscript. I.C.W.: experimental design; collection and analysis of *in vitro* data; revising the manuscript; final approval of the manuscript. E.M.P.: collection and analysis of *in vitro* data; revising the manuscript; final approval of the manuscript. A.C.T.: experimental design; collection and analysis of *in vivo* data; revising the manuscript; final approval of the manuscript. T.S.M.: experimental design; collection, analysis and interpretation of *in vivo* data; revising the manuscript; final approval of the manuscript. D.K.M.: experimental design; data analysis; drafting the manuscript; revising the manuscript; final approval of the manuscript.

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SYMPOSIUM REVIEW

Independent purinergic mechanisms of central and peripheral chemoreception in the rostral ventrolateral medulla

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Abstract The rostral ventrolateral medulla oblongata (RVLM) contains two functionally distinct types of neurons that control and orchestrate cardiovascular and respiratory responses to hypoxia and hypercapnia. One group is composed of the central chemoreceptor neurons of the retrotrapezoid nucleus, which provides a CO_2/H^+-dependent drive to breathe and serves as an integration centre and a point of convergence of chemosensory information from other central and peripheral sites, including the carotid bodies. The second cluster of RVLM cells forms a population of neurons belonging to the C1 catecholaminergic group that controls sympathetic vasomotor tone in resting conditions and in conditions of hypoxia and hypercapnia. Recent evidence suggests that ATP-mediated purinergic signalling at the level of the RVLM co-ordinates cardiovascular and respiratory responses triggered by hypoxia and hypercapnia by activating retrotrapezoid nucleus and C1 neurons, respectively. The role of ATP-mediated signalling in the RVLM mechanisms of cardiovascular and respiratory activities is the main subject of this short review.

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Abbreviations Cx26, connexin 26; NTS, nucleus of the solitary tract; RTN, retrotrapezoid nucleus; RVLM, rostral ventrolateral medulla oblongata.

Thiago S. Moreira received a PhD in Physiology from the Federal University of Sao Paulo (UNIFESP), Brazil. In 2009, he joined the Institute of Biomedical Science at the University of Sao Paulo, Brazil as an Assistant Professor and in 2014 became Associate Professor. During recent years, his research has focused on the brainstem network that co-ordinates respiration and circulation. **Ian C. Wenker** holds an MS from Wright State University and a PhD from the University of Connecticut. For his PhD thesis, he studied intrinsic properties of brainstem cardiorespiratory neurons and astrocytes with brain slice patch-clamp recording techniques and earned a predoctoral fellowship from the American Heart Institute. He is now a postdoctoral fellow at the University of Virginia, exploring the brainstem circuitry involved in autonomic reflex control of the cardiorespiratory system.

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Introduction

Respiratory chemoreception is the ability of an organism to sense changes in blood gases (i.e. $O_2/CO_2/H^+$) to provide for the homeostatic control of respiratory and cardiovascular systems and can be divided into central and peripheral components. Central chemoreception relies on specialized cells within the brainstem that sense CO_2/H^+ and output to increase breathing and sympathetic nerve activity (Guyenet *et al.* 2010; Moreira *et al.* 2011). Peripheral chemoreceptors of the carotid body sense changes in $O_2/CO_2/H^+$ and also regulate breathing and sympathetic outflow, via synapses through the central nervous system (Kumar & Prabhakar, 2012). It is well established that the rostral ventrolateral medulla (RVLM) contains two important subsets of neurons involved in cardiorespiratory control during the chemoreflexes, namely the CO_2/H^+-sensitive neurons of the retrotrapezoid nucleus (RTN) that function as central respiratory chemoreceptors (Mulkey *et al.* 2004; Guyenet *et al.* 2010) and presympathetic catecholaminergic C1 neurons that control sympathetic vasomotor tone in response to a number of reflexes, including the peripheral chemoreflex (Guyenet, 2006; Guyenet *et al.* 2013).

The role of ATP as a neurotransmitter was first described in the enteric nervous system several decades ago (Burnstock *et al.* 1970). Since then, purinergic signalling has been found to contribute at all levels of the nervous system, including enteric, autonomic and central (Burnstock, 2007). The mechanisms of ATP signalling are equally diverse. They include many ionotropic (P2X) and metabotropic (P2Y) receptor subtypes (Fredholm *et al.* 1994), as well as varying methods of transmission, including vesicular, volume-regulated anion channel and gap junction hemichannel release of ATP from neuronal and non-neuronal cells (Burnstock, 2007).

This short review addresses the role of purinergic signalling in the RTN chemoreceptor and C1 presympathetic neuronal control of the central and peripheral chemoreflexes. To learn more about purinergic signalling in respiratory control, the reader is referred to reviews by Erlichman and colleagues (2010) and Funk (2013). In addition, this review focuses on central purinergic mechanisms; however, purinergic signalling is also critical peripherally in the carotid bodies (Piskuric & Nurse, 2013).

Purinergic signalling in the RVLM: the RTN and central chemoreception

The defining properties of central respiratory chemoreceptors include the following: (i) intrinsic CO_2/H^+ sensitivity *in vivo* and *in vitro*; (ii) an excitatory neurochemical phenotype; and (iii) projection to the respiratory pattern generator. While there are a number of chemosensitive respiratory neurons that are likely to contribute to central chemoreception (Nattie & Li, 2012), the chemosensitive neurons of the RTN fulfil all three of these criteria and are the focus of this review. They are highly activated by increasing arterial P_{CO_2} *in vivo*, independently of respiratory activity (Mulkey *et al.* 2004; Takakura *et al.* 2006). Retrotrapezoid nucleus neurons are directly activated by CO_2/H^+, as demonstrated in neuronal recordings from brain slices (Mulkey *et al.* 2004; Onimaru *et al.* 2012) and acutely dissociated preparations (Wang *et al.* 2013). Retrotrapezoid nucleus neurons are glutamatergic, and their selective stimulation *in vivo* results in rapid and robust breathing activity (Abbott *et al.* 2009; Kanbar *et al.* 2010), while selective inhibition blunts whole-animal breathing responses to hypercapnia (Marina *et al.* 2010; Takakura *et al.* 2014), thus indicating that RTN chemoreceptors provide an excitatory drive to breathe.

As denoted above, at least some of the CO_2/H^+ sensitivity of RTN neurons is intrinsic, and this appears to be mediated partly by TWIK-related acid-sensitive K-2 channels (TASK-2; Wang *et al.* 2013). However, adult RTN neurons receive numerous excitatory and inhibitory inputs, including polysynaptic inputs from the carotid body, pulmonary receptors, hypothalamus, nucleus of the solitary tract (NTS), periaqueductal grey matter, spinal cord, dorsolateral pons and raphe nuclei (Rosin *et al.* 2006; Takakura *et al.* 2006; Moreira *et al.* 2007; Barna *et al.* 2014). If some of these inputs are chemosensitive themselves (e.g. carotid body inputs surely are and some NTS inputs could be), then part of the *in vivo* chemosensitivity of RTN neurons could be synaptically driven. However, pharmacological blockade of excitatory inputs has little to no effect on their CO_2/H^+ sensitivity *in vivo*, at least in an anaesthetized, hyperoxic state (Mulkey *et al.* 2004), underscoring their intrinsic chemosensitivity.

In the past decade, a role for paracrine release of ATP (i.e. purinergic signalling) in the RVLM has been found to be crucial for proper central chemoreception (Thomas & Spyer, 1999, 2000; Spyer *et al.* 2004; Gourine *et al.* 2005). Work from our group confirmed and extended some of these earlier studies (Fig. 1). We found that blocking P2 receptors in the RTN produces a reduction in the amplitude and frequency of phrenic nerve activity and in the pressor responses elicited by hypercapnia in anesthetized and conscious rats (Wenker *et al.* 2012; Sobrinho *et al.* 2014; B. F. Barna, A. C. Takakura, D. K. Mulkey and T. S. Moreira, unpublished results). At the cellular level, bath application of P2-receptor antagonists blunted the CO_2/H^+-evoked firing rate response of RTN neurons in brain slice recordings (Gourine *et al.* 2010; Wenker *et al.* 2010, 2012). The contribution of purinergic signalling to chemosensitive RTN neuronal activity was found to be independent of temperature and stimulus strength and was wholly retained when synaptic activity was blocked using high-Mg^{2+}, low-Ca^{2+} solution (Wenker *et al.* 2012).

Our group also found that connexin hemichannel blockers were effective at blunting the purinergic component of RTN neuronal CO_2/H^+ sensitivity (Wenker *et al.* 2012). This observation is congruent with a series of experiments from the laboratory of Nicholas Dale, where they demonstrated that CO_2-evoked ATP release at the ventral surface is likely to be mediated by CO_2-sensitive connexin 26 (Cx26) hemichannels (Huckstepp *et al.* 2010*a*, 2010*b*; Meigh *et al.* 2013). Using ATP-sensing microelectrodes, they found that CO_2, and not H^+, was the stimulus for ATP release in brain slices containing the ventral surface, and this process is dependent on functional connexin hemichannels (Huckstepp *et al.* 2010*b*). In cultured HeLa cells, transfection with Cx26 and preloading with ATP was enough to recapitulate the CO_2-dependent ATP release observed in brain slices (Huckstepp *et al.* 2010*a*). Furthermore, in a series of elegant molecular studies they were able to demonstrate that CO_2 binds directly to Cx26 channels, resulting in their opening (Meigh *et al.* 2013).

Although the above experiments provide evidence for a purinergic role in central chemoreception and the ability of Cx26 to sense CO_2 and release ATP, major questions remain. For instance, which cells are releasing ATP? Based on experiments using synaptic blockade (Mulkey *et al.* 2004; Wenker *et al.* 2012), it is clear that fast chemical synapses do not provide for the purinergic drive in the RTN region. The common interpretation has been that

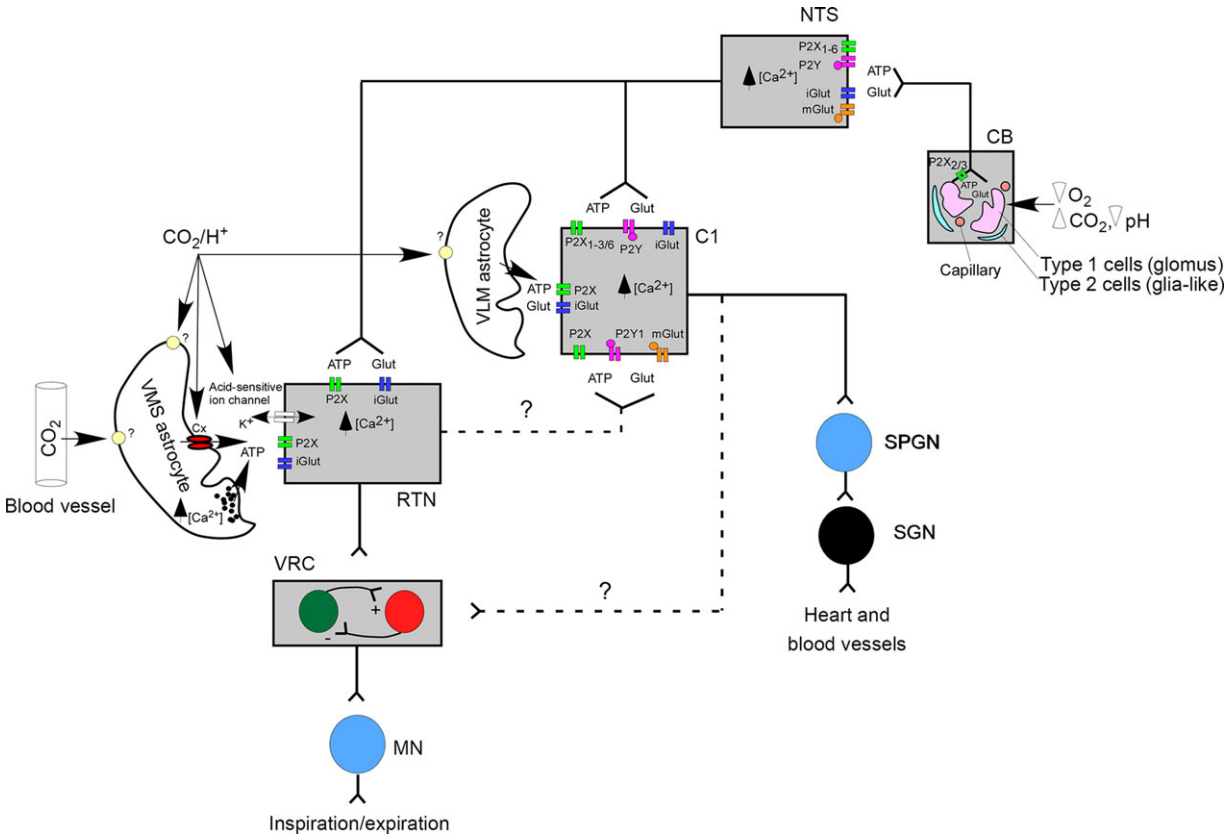

Figure 1. Schematic model of the possible medullary mechanisms involved in the control of cardio-respiratory responses caused by raising cerebral arterial P_{CO_2} and lowering arterial P_{O_2}
Signals from central or peripheral chemoreceptors may directly or indirectly affect the activity of several medullary areas, including the NTS, C1 region, RTN and VRC, which affect sympathetic discharge to the heart and blood vessels and motorneurons to the respiratory muscles (Pankratov *et al.* 2006; Braga *et al.* 2007; Moraes *et al.* 2011; Wenker *et al.* 2013). An essential step for hypercapnia-induced increase in breathing is activation of RTN neurons by CO_2/H^+, directly or indirectly from VMS astrocytes, which in turn send excitatory signals to activate the VRC, either directly or through activation of metabotropic and ionotropic glutamate/purinergic receptors in the C1 region (Takakura & Moreira, 2011; Wenker *et al.* 2013). Release of ATP by astrocytes may be a calcium-dependent exocytotic process triggered by intracellular acidification and/or a leak through connexin channels (primarily connexin 26) opened by molecular CO_2 via carbamylation (Huckstepp *et al.* 2010*a*, 2010*b*). Signals from the RTN that activate metabotropic receptors in the C1 region may also increase sympathetic activity to the cardiovascular system. Abbreviations: ATP, adenosine triphosphate; C1, C1 adrenergic region; CB, carotid body; Glut, glutamate; iGlut, ionotropic glutamatergic receptors; mGlut, metabotropic glutamatergic receptors; MN, motor neuron; NTS, nucleus of the solitary tract; P2X, ionotropic purinergic receptors; P2Y, metabotropic purinergic receptors; RTN, retrotrapezoid nucleus; SGN, sympathetic ganglionic neurons; SPGN, sympathetic preganglionic neurons; VLM, ventrolateral medulla; VMS, ventral medullary surface; and VRC, ventral respiratory columm.

ATP is released by astrocytes, because astrocytes have been found to release ATP in response to a number of different physiological stressors (Butt, 2011; Ota *et al.* 2013). Work by Gourine and colleagues (2010) demonstrated that optogenetic stimulation of astrocytes produced ATP release and respiratory effects when the light was directed at the ventral surface. The investigators also found that ATP was released in the RVLM in response to CO_2/H^+ stimulation, via Ca^{2+}-dependent vesicular release. The pharmacology used to block vesicular release could affect any cell type, and astrocyte-specific loss-of-function experiments was not done. By itself, this leaves open the possibility that other cell types could be responsible for the CO_2/H^+-evoked ATP release. However, the ATP release was unaffected by tetrodotoxin, a blocker of neuronal action potentials, and genetically identified astrocytes were found to elevate intracellular Ca^{2+} in response to CO_2/H^+. In addition, cultured brainstem astrocytes have demonstrated H^+-mediated ATP release (Kasymov *et al.* 2013). Thus, although it remains possible that ATP could be released by other cell types, astrocytes appear to be the likely candidates. In separate experiments, Nicholas Dale's laboratory also produced data supporting astrocytes as the ATP-releasing cells (Huckstepp *et al.* 2010*a*, 2010*b*). Looking at fluorescent dye uptake into cells (dyes that can traverse Cx26 channels) during elevated CO_2, they found that it mostly co-localized with glial fibrillary acid protein, a marker for astrocytes (Huckstepp *et al.* 2010*b*). However compelling the apparent CO_2-dependence of these data, it is of course only correlative, and future studies will require cell-specific loss of function, as has been done in the cortex (Lalo *et al.* 2014), to confirm that astrocytes are indeed responsible for the purinergic drive to breath.

Another open question is, by what mechanism(s) does purinergic signalling alter the function of RTN chemosensitive neurons? For example, purinergic receptor subtypes and downstream cellular mechanisms of membrane depolarization (e.g. ion channels) are incompletely understood. Based on the purinergic agonist profile described by Mulkey and colleagues (2006), RTN neurons are excited by direct activation of P2Y receptors and inhibited by indirect activation (i.e. via interneurons) of P2X receptors. However, based on the purinergic antagonist profile of the CO_2/H^+ responses of neurons in the RVLM, Gourine and colleagues (2010) suggested that the receptors might be of the P2X variety. The former case may be open to some contention because newer, more subtype-selective pharmacological agents have since been developed for purinergic receptors (Fredholm *et al.* 1994). Our group's only results using these agents show that P2Y1 receptors (for a review of purinergic receptor subtypes see Fredholm *et al.* 1994) do not contribute to CO_2/H^+ sensitivity of the RTN (Wenker *et al.* 2012), although, serendipitously, they do regulate the activity of local catecholamine neurons in the RVLM (see next section; Fig. 1). The use of the ever-improving purinergic pharmacology and cell specific loss-of-function genetics will no doubt improve our understanding of purinergic mechanisms in central chemoreception.

Purinergic signalling in the RVLM: the C1 neurons and the peripheral chemoreflex

The increased sympathetic outflow elicited by peripheral chemoreceptors is mediated primarily by activation of the presympathetic neurons of the RVLM, the majority of which are C1 neurons (Fig. 1; Guyenet *et al.* 2013). In support of this idea, selective lesion of the C1 neurons with dopamine-β-hydoxylase-conjugated saporin toxin virtually abolishes the sympathoexcitatory response to peripheral chemoreflex activation (Schreihofer & Guyenet, 2000). The cardiorespiratory effects of peripheral chemoreceptors are mediated in part by direct glutamatergic inputs from the NTS to C1 neurons. Indirect pathways also exist, and the best documentation is a di-synaptic input that relays via the chemosensitive neurons of the RTN (Koshiya *et al.* 1993; Sun & Reis, 1995; Paton *et al.* 2001; Moreira *et al.* 2006; Takakura *et al.* 2006; Takakura & Moreira, 2011).

In addition to glutamatergic neurotransmission, the C1 neuronal activity can be modulated by purinergic signalling, both by exogenous agonists and endogenously, during autonomic reflex control. In studies from the last two decades, spinally projecting RVLM neurons were found to express P2Y and P2X receptors functionally (Sun *et al.* 1992; Ralevic *et al.* 1999), and activation of these receptors in the RVLM also increases cardiorespiratory parameters (i.e. fictive breathing and blood pressure) in anaesthetized rats (Ralevic *et al.* 1999). Later, it was purported that these P2X receptors were important for RVLM reflex control of cardiorespiratory function. For example, inhibition of P2X receptors within the ventrolateral medulla blunted the ventilatory but not the pressure response elicited by peripheral chemoreceptor activation in conscious rats (Moraes *et al.* 2011). The interpretation that P2X receptors are responsible is based on the relatively low pyridoxal-phosphate-6-azophenyl-2′,4′-disulfonate concentration used, which *in vivo* is dubiously selective over P2Y receptors. Nevertheless, if it is true, this could be explained by P2X receptor expression on nearby RTN chemoreceptor neurons (Gourine *et al.* 2010) or by differential expression of P2X and P2Y receptors amongst RVLM neurons (as in Ralevic *et al.* 1999).

More recently, a role for P2Y signalling in C1 neuronal control of the peripheral chemoreflex has been put forward by our group. Specifically, we found that P2Y1 receptors are robustly expressed by C1 neurons but not by

nearby RTN chemoreceptors *in vitro* (Wenker *et al.* 2013). This was determined by the fact that action potential firing of CO_2/H^+-sensitive (i.e. RTN chemosensitive) neurons in this region were unaffected by application of a P2Y1-specific agonist, whereas CO_2/H^+-insensitive neuronal firing was greatly increased. In addition, many of these CO_2/H^+-insensitive, P2Y1-expressing neurons were immunoreactive for tyrosine hydroxylase, a marker for C1 neurons in this region. The expression of P2Y1 receptors by C1 neurons was confirmed *in vivo* by showing that the cardiorespiratory responses induced by P2Y1 agonist injection in the RVLM were blunted in C1-lesioned animals (Wenker *et al.* 2013). Additionally, selective inhibition of P2Y1 receptors in the RVLM decreased peripheral chemoreceptor-mediated activation of breathing and sympathetic outflow. Importantly, this did not change cardiorespiratory outflow during baroreflex or RVLM stimulation, indicating that pharmacological blockade of P2Y1 receptors does not directly alter excitability of C1 cells and that ATP is released during the chemoreflex to stimulate P2Y1 receptors (Wenker *et al.* 2013). Corroborating this idea, we found that approximately 60% of caudal NTS neuron varicosities in the RVLM are immunoreactive for both vesicular glutamate and nucleotide transporters (VGLUT2 and VNUT; Wenker *et al.* 2013), which at other central synapses are sufficient machinery to allow for ATP and glutamate co-release (Gordon *et al.* 2009).

Together, these results suggest that peripheral chemoreceptor drive is relayed, in part, by ATP and glutamate co-release from NTS neuron terminals acting on P2Y1 and ionotropic glutamatergic receptors expressed on C1 neurons (Fig. 1). Interestingly, this purinergic mechanism appears to be distinct from those involved in RTN chemoreception. By this, we mean that although ATP is released in the RVLM during the central CO_2 chemoreflex, P2Y1 receptors do not appear to influence the cardiorespiratory effects of this reflex (Wenker *et al.* 2013), Of course, this has been tested only in anaesthetized hyperoxic conditions. It is known that while central and peripheral chemoreflexes operate via separate sensors, they do influence the activity of one another (Blain *et al.* 2010). Thus, in different conditions (i.e. CO_2/O_2 levels or conscious state) it is possible that the P2 receptors in the RVLM could contribute to central–peripheral chemoreflex interactions.

Finally, it is important to point out that astrocytes in the RVLM are capable of releasing ATP to affect C1 neurons. Optogenetic stimulation of astrocytes within the ventrolateral medulla excites presympathetic C1 neurons via an ATP-dependent mechanism (Marina *et al.* 2013). This is particularly interesting because evidence exists that glial cells release ATP in response to various stimuli, including hypoxia (Aley *et al.* 2006), and hypoxia produces ATP release in the RVLM (Gourine *et al.* 2005). Thus, depending on the conditions, purinergic signalling of a number of varieties could co-ordinate the output of RVLM neurons.

Conclusions and clinical perspectives

In this review, we have discussed a number of independent purinergic mechanisms of RTN and C1 neurons that influence breathing and autonomic control of the chemoreflexes. It is not surprising that purinergic signalling is so important in this region, because it contributes to autonomic control via varying mechanisms at several levels of the nervous system, both peripherally and centrally (Gourine *et al.* 2009).

The central and peripheral chemical drive to breathe is associated with several widespread autonomic disorders. Deficits in central chemical drive are associated with central sleep apnoea, a debilitating disease with few therapies besides constant positive airway pressure (Dempsey *et al.* 2014). In addition, disruption of the drive to breath is thought to contribute to mortality of certain pathologies, including sudden infant death syndrome, stroke and epilepsy (Kinney *et al.* 2009; Davis *et al.* 2013; Massey *et al.* 2014). Finally, in obstructive sleep apnoea, certain forms of hypertension and heart failure, the sensitization of peripheral chemoreceptor drive, particularly the sympathetic component, is observed, and this overactivity is thought contribute to the pathology (Narkiewicz *et al.* 1999; Schultz *et al.* 2007).

In recent years, purinergic signalling has been proposed to be an excellent system to target for therapies of numerous pathologies (Jacobson & Boeynaems, 2010; Burnstock, 2014), mainly due to novel pharmacological agents being developed. As more detailed understanding of the purinergic mechanisms involved in the chemical drive to breath is uncovered, it may be possible to treat the aforementioned pathologies with the newly developed purinergic agents. Recent work by Marina and colleagues (2013) demonstrates the utility of targeting purinergic mechanisms by transducing an ectonucleotidase, which breaks down ATP, in the RVLM to treat a rat model of heart failure (Marina *et al.* 2013). Based on our recent data, we have proposed that P2Y1 receptors could represent a therapeutic target for the treatment of cardiorespiratory diseases in which the peripheral chemoreflex is sensitized (Wenker *et al.* 2013). Most of the new purinergic pharmacological agents are ATP analogues and do not cross the blood–brain barrier, making them less practical for use in the central nervous system. It is therefore imperative that brain-permeable agents are developed (Burnstock, 2008). Better pharmacology, combined with further understanding of the specific purinergic receptor subtypes and signalling pathways involved in chemoreflex

control by RTN and C1 neurons, may allow for novel therapeutic strategies for cardiorespiratory diseases.

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Additional information

Competing interests

None declared.

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