Mechanisms of cell specification and differentiation in vertebrate cranial sensory systems

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Abstract

The number of sensory stimuli that are sensed are large and varied, it ranges from temperature, volatile and non-volatile chemicals, touch, pain, light, sound and gravity. These external and internal inputs are sensed in vertebrates by specialized cells present in sensory organs and cranial ganglia. Much of our understanding of the transcription factors and mechanisms responsible for sensory cell specification comes from cell-lineage tracing and genetic experiments in different species, but recent advances in single cell transcriptomics, high-resolution imaging and systems biology approaches have allowed to study these processes in an unprecedented resolution. Here I will compare the transcription factor networks driving cell diversity in the different sensory organs of vertebrates to then discuss *in vivo* data of how cell specification is coupled with tissue morphogenesis.

Introduction

The extraordinary capacity of vertebrates to respond to the environment relies on three specialized paired sensory organs (olfactory epithelium, inner ear and lens) and several cranial ganglia. All of them derive from ectodermal thickenings named cranial placodes arising in the head region during embryogenesis (Fig1. lens, adenohypophyseal, olfactory, trigeminal, lateral line, otic, epibranchial). Commonalities of all placodes are their organization into an apico-basally polarized columnar epithelium that undergoes interkinetic nuclear migration, initial expression of members of Pax transcription factors (TF), some kind of morphogenetic process and the production of neuronal derivatives (except the lens and adenohypophyseal placode) [1–3]. The olfactory and otic placodes, in addition to sensory neurons, diversify to other cells types and, thus possess a higher degree of complexity.

I summarize first the distinct cell types deriving from each placode to concentrate then on the molecular mechanisms responsible of this cell diversity. The olfactory epithelium of the nasal cavity contains an enormous large number of different olfactory sensory neurons (ORNs) that enable them to discriminate among the wide range odorant molecules. Distinct from the cranial sensory neurons originated from other placodes, each mature ORN expresses a single olfactory receptor (OR) that is selected from the more than a thousand OR genes (OR numbers are specie specific) through specific inter-chromosomal contacts between OR genes and their large set of enhancers [4]. In addition to mature and immature ORNs, the olfactory epithelium is also constituted by sustentacular cells (SusC)

with microvilli and two population of stem cells (horizontal basal cells (HBCs) and the globose basal cells (GBCs)) that continuously replenish the olfactory epithelium with new ORN (Fig. 1) [5]. In parallel, the inner ear contains hair cells (HCs) and supporting cells (SC) in sensory patches, non-sensory cells and auditory and vestibular sensory neurons (SN) of the VIII cranial ganglia, all of them of placodal-origin [6,7]. In contrast, the trigeminal and epibranchial (geniculate, petrosal and nodose) placodes develop only somatosensory and vicerosensory neurons of the V, VII, IX and X cranial ganglia (Fig. 1) [1]. Some cranial ganglia mix with SNand glia from neural crest origin, but these will not be reviewed here.

Proneural factors as pioneer factors in sensory cell specification: lessons extracted from reprogramming studies and chromatin dynamics

Specification of placodal sensory cells is ridden by proneural TF, homologous to the ones firstly identified in *Drosophila* participating in the development of the peripheral nervous system: *achaete* and *scute* complex (AS-C) genes specify external mechanosensory cells and *atonal/amos* genes stretch receptors and olfactory sensory cells. *Ascl1 or Ash1*, homologous to *AS-C* genes, specifies multipotent progenitors in the olfactory epithelium, while *Atoh1*, the vertebrate *atonal* homolog, specifies inner ear HC. In addition, two newly evolved family of proneural genes, *Neurogenin* and *NeuroD*, act sequentially to imprint a neuronal character to all SN (Fig. 2). Experimental data suggest that several proneural factors such as *Ascl1*, *Neurogenin2* and *NeuroD1* act as pioneer transcription factors [8–11]. Differently from other transcription factors, pioneer factors can target sequences of nucleosomal DNA and locally modify the epigenetic landscape into an active or repressed state [12–14]. Not all TF are pioneer factors, as studies indicate that pioneer factor function is linked to the ability of these TF to bind histones along with DNA and to the recognition of a reduced motif on the nucleosome surface [15,16]. As the study of chromatin organization *in vivo* is still challenging, most works exploring the ability of TF to reorganize chromatin have been undertaken in defined cell culture systems. When fibroblasts were infected with *Ascl1* or *Neurog2*, cells suffered major transcriptional changes, concomitant with a massive occupancy of these transcription factors in many loci and, finally reprogramming into glutaminergic neurons [10,17]. The binding loci primarily coincided with nucleosome DNA fragments that upon binding remodelled into euchromatin. Another question raised is how these two pioneer factors, which are on top of the cell specification cascade, can confer subtype identity together with pan-neural characteristics. Overexpression of *Ascl1* or *Neurog2* in embryonic stem cells (ESC) caused initial activation of pan-neuronal genes in both conditions, but

after 48 h, the transcriptomes induced for each TF clustered separately. Comparison of the chromatin binding regions showed that both pioneer factors, already at initial timepoints, had very little overlap on the genomic binding loci and recognized distinct E-boxes motives. This resulted in different open chromatin landscapes at later timepoints that conditioned the binding patterns of two shared early-induced TF, such as *POU3f2* or *Ebf2*. Thus, the activity of widely expressed TF is not functionally equivalent in all neurons and will lean on the previous proneural gene activity [18]. Nevertheless, these works did not explore in detail whether the reprogrammed neurons had a sensory character or not.

The cell lineage trajectories in sensory cell specification in placodes have been summarized in Fig.2. Strikingly, *Ascl1* is only expressed in the olfactory lineage, suggesting that the activity of this pioneer factor might be underlying the differences between olfactory progenitors and the neuronal progenitors derived from the other placodes, such as their multipotency or maintenance of stem cell properties. Comparative studies of the chromatin landscapes in *Ascl1* and *Neurog1/2* -expressing sensory progenitors in their in vivo contexts are needed to provide insights in this regard. In the other cranial placodes, progenitors initially express *Neurog1/2* and then, upon *NeuroD* expression, delaminate from placodes, migrate and coalesce into cranial ganglia (Fig. 2). Interestingly, the morphogenetic activity of *NeuroD* has been linked molecularly with the epithelial-mesenchymal transition process by showing that *NeuroD* opens chromatin of epithelial-mesenchymal transition related loci [8]. One of the lessons extracted from the reprogramming studies is that pioneer factors poise chromatin for induced pan-neuronal TF factors to bind to newly opened chromatin in combination with pioneer factors. Therefore, the sensory subtype identities (auditory, vestibular, somatosensory, viscerosensory) must emerge by the combinatory action of other TF with *Neurog1/2* and *NeuroD*. The revolution of transcriptional analysis at single cell level (scRNA-seq), coupled with cell lineage tracing and epigenetic information is shedding light on the TF network operating in developmental lineages [19]. These new technologies are also quantifying the intrinsic heterogeneity and transcriptomic fluctuations within tissues. Only a handful of reports using single cell transcriptome analysis have been conducted in the cranial sensory organs. We find examples exploring the heterogeneity of transcriptomic programs in the olfactory lineage, auditory ganglion, cochlear and vestibular epithelia, nodose and geniculate progenitors or somatosensory neurons [20–28]. Some of the relevant subsets of TF found in all, some or individual SN have been compiled in Fig. 2 completed with other TF identified in cell-type specific bulk transcriptomics [29–31].

Together with SN, other sensory cells are generated in vertebrates. Due to the interest in identifying the molecular genes implicated in deafness and the development of therapies for HC regeneration, much work has focused on uncovering the molecular mechanisms of HC specification and differentiation. *Atoh1* specifies HCs that are secondary sensory cells (without axon), while in *Drosophila atonal* specifies primary mechanosensory cells (with an axon) and promotes both neuronal and mechanosensory programs[32] (Fig. 2). This raised the question whether *Atoh1* kept its ancestral activity and could activate neuronal genes in addition to HC genes. Indeed, *Atoh1* overexpression in vitro favours a neuronal program and binds to neuronal loci but, its activity is biased to HC fate cooperating with two other TF, *Gfi1* and *POU4f3*. CHIP-seq data indicate that *Pou4f3* recruits *Atoh1* to new HC loci and *Gfi1* on one hand represses neuronal gene transcription and, on the other, acts as a co-activator of *Atoh1* in HC loci [33,34]. The investigation of the dynamics of chromatin reorganization upon *Atoh1*, *Pou4f3* and *Gfi1* induction, suggests that *Pou4f3* but not *Atoh1* might be working as a pioneer factor in HC specification.

Putative terminal selectors in vertebrate sensory lineages

After cell specification, sensory progenitors exit the cell cycle and begin a differentiation program. Beautiful works in *C. elegans* and *Drosophila* have identified a handful of TF, termed terminal selectors, whose continuous expression is required for maintenance of fully differentiated characteristics such as the expression of neurotransmitters, ion channels and neuropeptides [35]. While pioneer TF dynamically reorganize the chromatin to allow cellular plasticity, terminal selectors of postmitotic neurons lock chromatin to allow the continued maintenance of terminal differentiation features. Some terminal selector TF identified in invertebrates belong to the *Lim*, *Lhx*, *POU* gene families, among others (Fig2)[36]. Vertebrate *Lhx2* has been shown to be a terminal selector in ORNs [37] and, although not tested, *Lhx3* and *Pou4f1* might have similar terminal selector functions in HC and auditory SN, respectively. *Rfx1/3* might also be a terminal selector in HC because in *C. elegans*, the phylogenetically conserved RFX-type TF *daf-19*, is a terminal selector in ciliated sensory neurons [38]. Interestingly, mice deficient for *Rfx1/3* are deaf, HC do not complete full maturation and die by apoptosis [29]. Of note, *Islet1*, *POU4f1* and *Runx1* are initially present in all early postmitotic SN of ganglia (not olfactory), thus some of these TF might be required for cell cycle exit without providing specific terminal differentiation features. A study in the neural crest derived somatosensory neurons, has found unexpectedly that subtype-restricted TF (*Runx1/3*, *POU4f2/3*, *Onecut2*, *Bhlha9*, among others) are initially co-expressed in an unspecialized postmitotic neuronal state. Progressively, these

TFs restrict to individual sub-types of somatosensory neurons. Mutants for either *POU4f2* or *POU4f3* confirm that they confer subtype identity, required for neurons with longitudinal lanceolate or circumferential axonal endings, respectively [39]. Thus, in cranial ganglia SN, some of the early expressed TF (*POU4f1* or *Runx1*) might have a dual temporal function and also confer sub-type identities and stabilize terminal differentiation programs when their restrict their expression.

Coupling of cell specification with morphogenetic mechanisms

When considering cell specification mechanisms, one should not forget the influence of extrinsic signals in the activation of the TF networks described above. These might be derived from the tissue itself or others from the niche. In comparison with what has been described for some niches, very little is known yet on the organization and cellular composition of cranial sensory organs niches. Recently, it has been reported that, similarly to the adult brain neurogenic niches, blood vessels surrounding cranial ganglia regulate the balance between sensory neuron proliferation and differentiation [40]. Initial communication between endothelial cells and cranial neuronal progenitors are mediated by directed and dynamical filopodia (cytoneme) contacts, highlighting the relevance of understanding the 3D organization of niches and of performing high spatiotemporal life imaging analysis to uncover the dynamics of developing systems. This is relevant if considering that, not only signals, but also morphogenetic mechanisms can feedback onto cell specification processes [41]. Several works in different models and sensory organs point in this direction. In the zebrafish lateral line, a mechanosensory organ present in anamniote vertebrates, imaging of the migrating lateral line showed that the formation of a microlumen in epithelized rosettes sequestered FGF ligands and by dampening FGF signalling, cell differentiation was potentiated [42]. Also in zebrafish, it was reported that ingression of *Neurog1* cells into the otic placode while is epithelizing favours otic neuronal specification within the placode [43]. In another study in *Drosophila* and zebrafish, epithelial ensheatment of trigeminal-derived somatosensory neurons was shown to feedback onto neurons by modulating their nociceptive sensitivities [44]. Some of the morphogenetic inputs into organ development can be mediated by biophysical forces as observed in ORN development. Interestingly, imaging and also microsurgery experiments indicated that axon elongation in first born ORNs is driven by mechanical forces generated by the lateral displacement of cell bodies in the placodal epithelium [45]. To date, cell specification and differentiation mechanisms have primarily focused on the action of cell specific TFs but the advent of new imaging techniques and quantitative analysis of forces *in*

vivo will shed light in new non-transcriptional mechanisms participating in sensory development and function.

Conclusions

The relevance of sensory organs for our daily life is unquestionable and, thus, the full understanding of how they work and are constructed is essential for developing proper curative strategies in sensory diseases. The molecular understanding of the TFs networks involved in sensory neuron specification and differentiation is becoming a reality thanks to the revolution of single cell genome-wide approaches. Other layers of complexity need to be incorporated nowadays, in vivo models, quantitative analysis and 3D spatial reconstructions and biophysical properties. With a holistic view, we will be in a good position to direct stem cell differentiation to proper cell types in a spatiotemporal manner or to recreate sensory organs in vitro. To date, inner ear organoids have successfully been set up that recapitulate the differentiation of HC and vestibuloacoustic neurons [46–48] but still require the incorporation of bioengineering tools for a correct maturation of sensory cells and spatial patterning.

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Conflict of Interests

The authors declare no conflict of interest.

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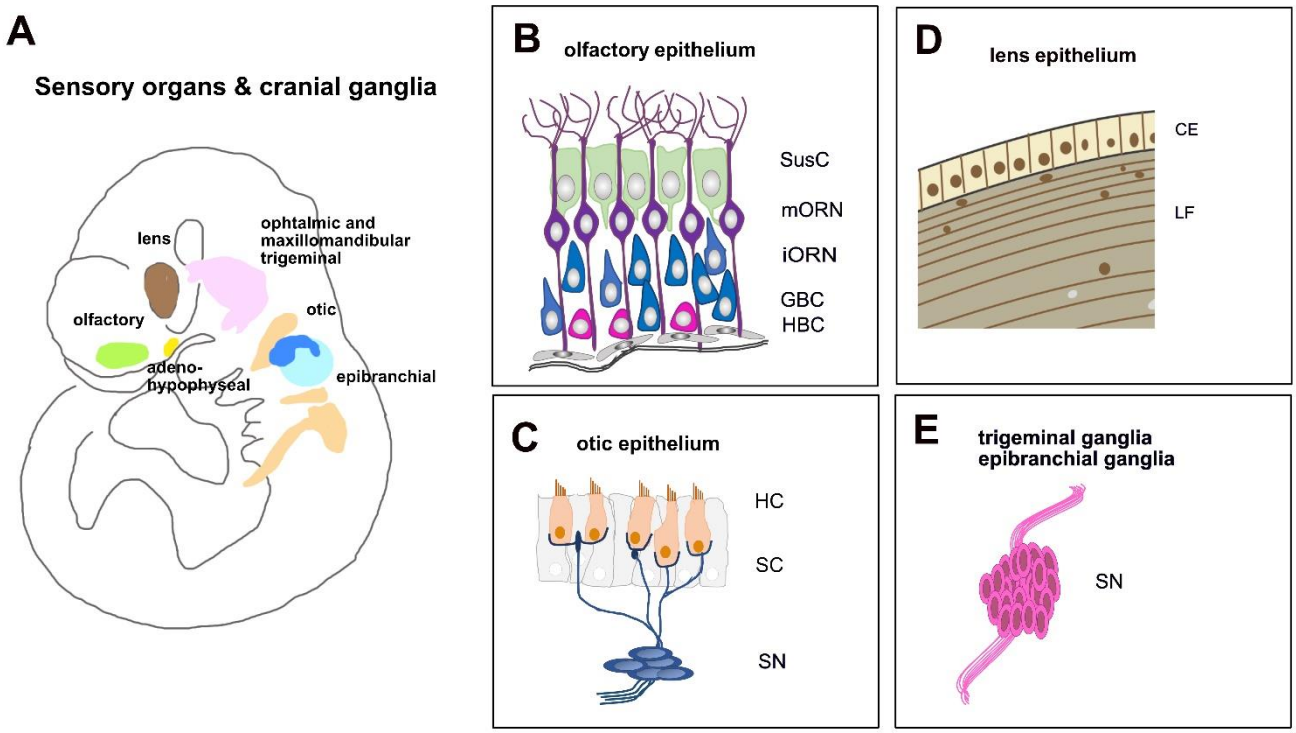
Figure 1



Schematic representation of sensory organs and cranial placodes in a mouse embryo.

A. Mouse develop three paired sensory organs that derive from the lens (brown) olfactory (green) and inner ear (blue) placodes, respectively. The lens helps the eye to focus. The olfactory epithelium and the inner ear sense olfactory, auditory and balance sensory information. The unpaired adenohypophyseal placode (yellow) is found underneath the eye and gives rise to the adenohypophysis with endocrine derivatives but no sensory neurons. The ophthalmic and maxillomandibular domains of the trigeminal placode (pink) produce sensory neurons of the ophtalmic and maxillomandibular ganglia than innervate much of the head to detect mechanical, thermal and chemical stimuli. Finally, the three mouse epibranchial placodes (orange; geniculate, petrosal and nodose) derive gustatory and general vicerosensory neurons[1–3].

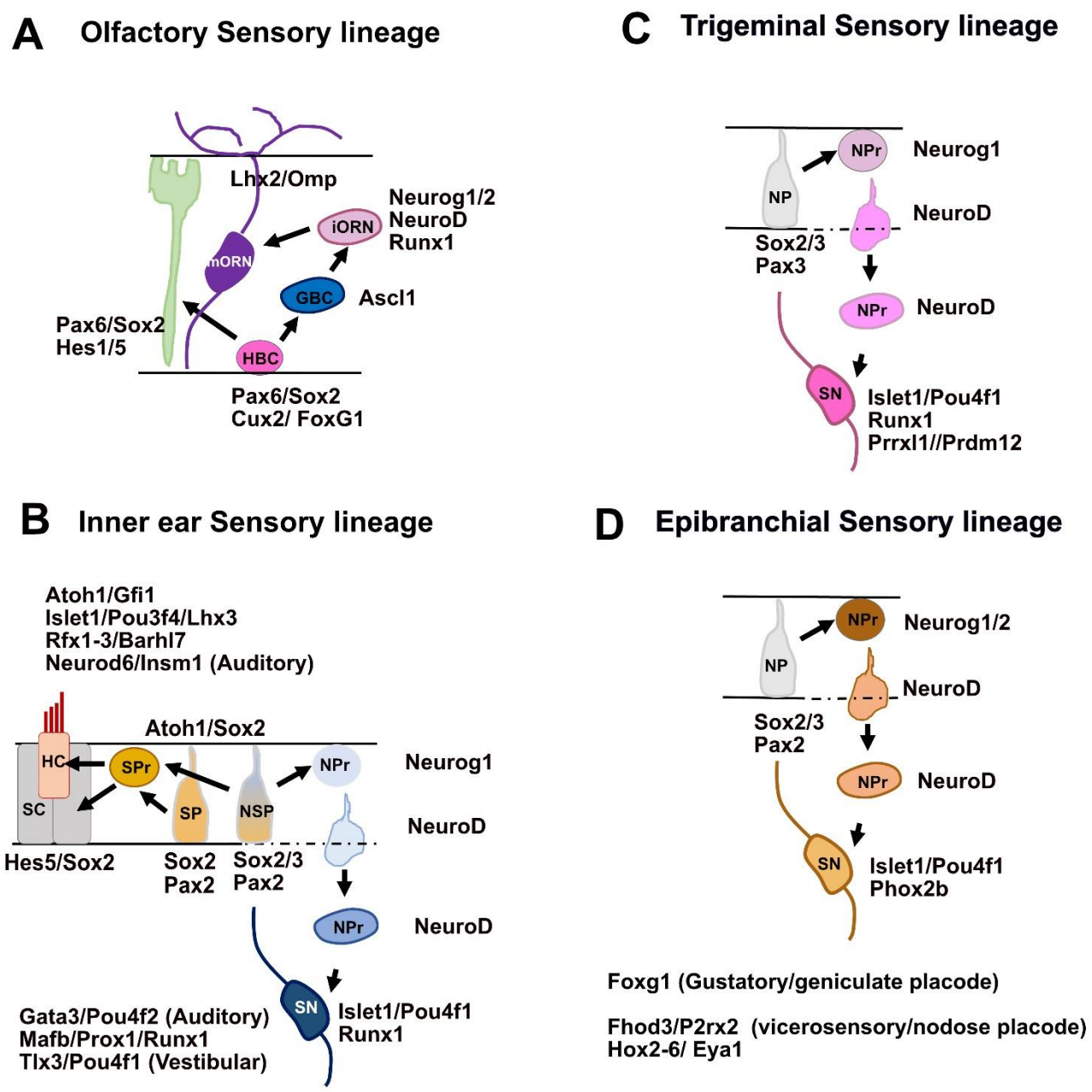
B. The olfactory epithelium is a pseudostratified epithelium containing two types of stem cells, supporting cells and olfactory receptor neurons (ORNs). Horizonal Basal Cells (HBC) are flat cells located adjacent to the basal lamina, kept in a quiescent state. In top, the Globose Basal Cells (BSC) continuously generate immature ORN (iORN). Mature ORNs (mORN) are bipolar neurons with specialized dendrite arbours facing the apical lumen and a neurite extending to

the olfactory bulb. Sustentacular Cells (SusC), the most numbered supporting cells, are apically located and provide physical and nutritional support.

C. The sensory epithelium of the inner ear contains mechanosensory cells (HC) and supporting cells (SC). HC are innervated by bipolar sensory neurons (SN) laying outside the epithelium that transmit auditory and vestibular information to the brainstem. Glial cells from the VIII ganglion derive from neural crest, while all SN are of placodal origin.
D. The lens is constituted by anucleated lens fibers (LF) mostly, that become transparent by the accumulation of crystallin proteins. On top, cells of the cuboidal epithelium (CE) perform absorption and secretion functions.
E. Trigeminal and epibranchial placodes only produce sensory neurons (SN) that coalesce in the V, VII, IX and X ganglia. These cranial ganglia have glial cells and SN from neural crest origin (not depicted here).

HBC: Horizontal Basal Cells; GBS: Globose Basal Cells; iORN: immature Olfactory Receptor Neurons; mORN: mature Olfactory receptor Neurons; SusC: Sustentancular Cells; HC: Hair Cells; SC: Supporting Cells, SN: Sensory Neurons; LF: Lens Fibers; CE: Cuboidal Epithelium

Figure 2.



Schematic representation of Placodal-derived Sensory Lineages

A. In the olfactory placode, HBC are multipotent progenitors that give rise to SusC and ORN. GBC are committed *Ascl1*$^{+}$ neuronal progenitors. *Neurog1/2* and *NeuroD* genes are activated in iORN. Lhx2 is a Lim-domain TF involved in ORN terminal differentiation [5,21,23,37].

B. In the otic placode, two types of otic progenitors exist, a NSP and a SP. NSPs give rise to NPr and SPr, while SPs give rise to SPr only. NPr express *Neurog1* that after activation of *NeuroD*, delaminate out of the epithelium. First post-mitotic SN are *Islet1*$^{+}$, *POU4f1/2* and *Runx1*. SN

further differentiate into Auditory and Vestibular SN by expressing a combination terminally differentiation factors. *Atoh1*, together with *POU3f4* and *Gfi1*, commit SP into HC fate. SC remain *Sox2*$^{+}$ [20,25,28,49].

C. In the trigeminal placode, NP (*Sox2/3*$^{+}$ and *Pax3*$^{+}$) commit to NPr by the activation of *Neurog1*. *NeuroD*$^{+}$ expression induces delamination and activation of *Islet1*, *Pou4f1* and *Runx1*. Postmitotic SN differentiate into specialized somatosensory SN of the Vth ganglion by the action of subtype differentiation factors [22,27].
D. In the epibranchial placodes, NP (*Sox2/3*$^{+}$, *Pax2*$^{+}$ and *Phox2b*$^{+}$) commit to NPr by the activation of *Neurog1/2*. *NeuroD*$^{+}$ NPr delaminate, express Islet1, Pou4f1, Phox2b and differentiate into viscerosensory and gustatory SN by the action of terminal differentiation factors [24].

HBC: Horizontal Basal Cells; GBS: Globose Basal Cells; iORN: immature Olfactory Receptor Neurons; mORN: mature Olfactory receptor Neurons; SusC: Sustentancular Cells;NSP: neurosensory progenitor, SP: sensory progenitor; NPr: neuronal precursor; SPr: sensory precursor; SN: sensory neuron; HC: Hair Cells; SC: Supporting Cells; NP: neural progenitors

Figure 1

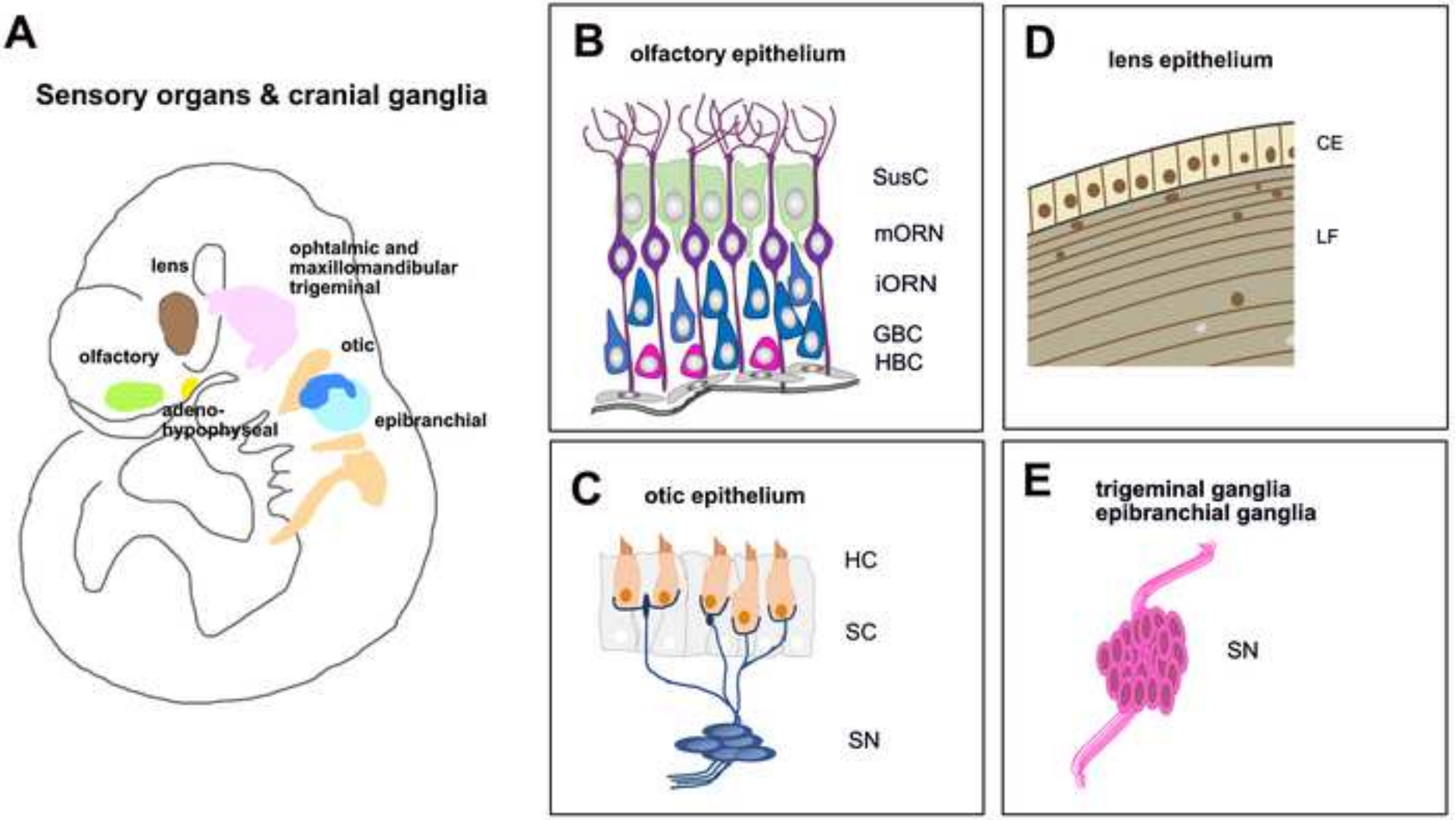
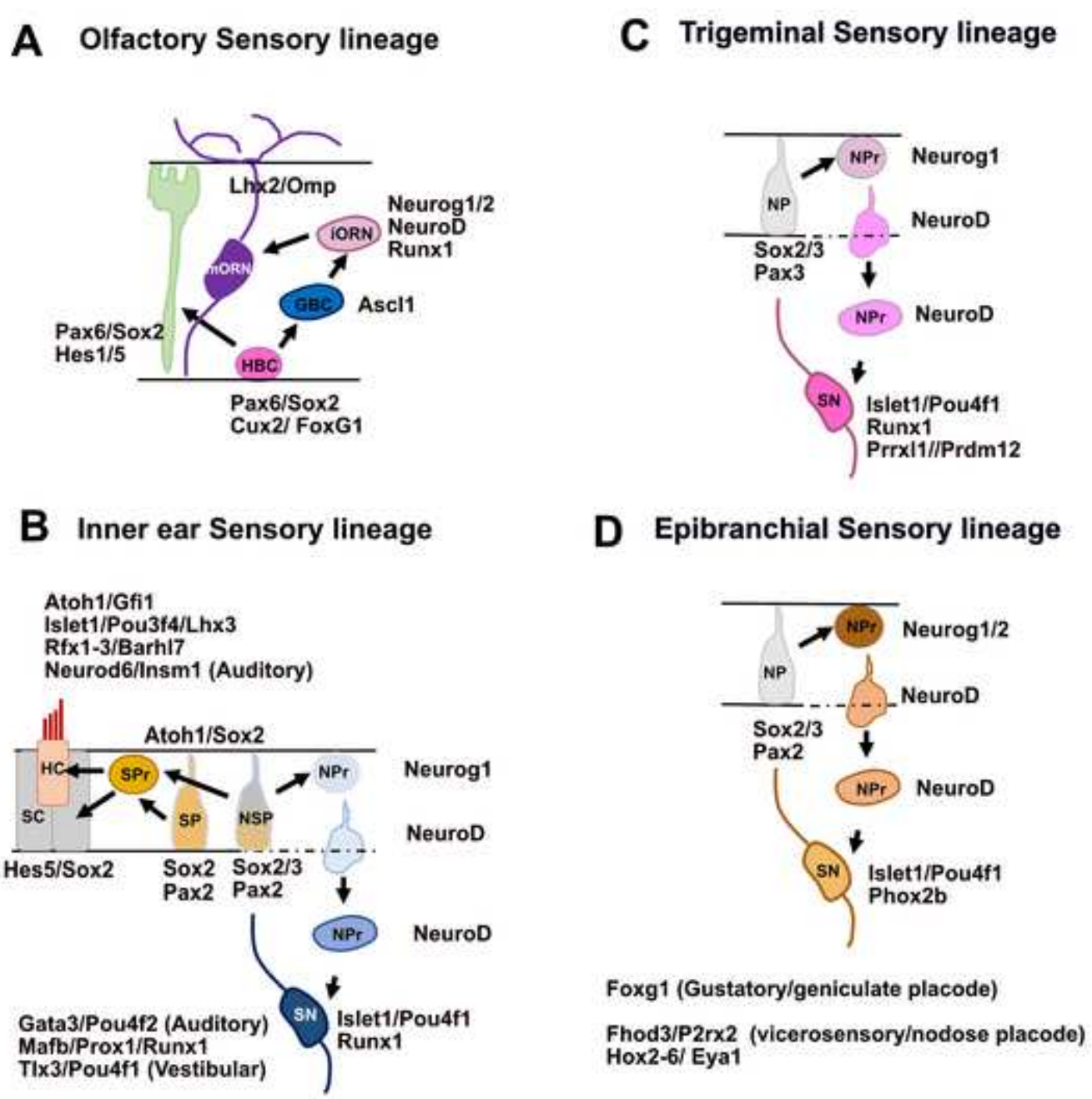


Figure 2



Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: