Mechanisms responsible of sensory afferent projections into the hindbrain

Andrea Zecca

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THESIS DIRECTOR

Cristina Pujades Corbi

Department of Experimental and Health Sciences
Universitat Pompeu Fabra

CO-DIRECTOR

Hernan Lopez-Schier

Research Unit Sensory Biology and Organogenesis
Helmholtz Zentrum München
Deutsches Forschungszentrum für Gesundheit
und Umwelt (GmbH)

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Ai miei genitori

La Scienza è uno sforzo collettivo dell'umanità verso la conoscenza.

J. A. Scrofani

La biología è la scienza che studia le forme di vita,

Essa ci insegna che le forme di vita sono in continuo cambiamento.

Non abbiate paura di cambiare perchè il cambiamento è sintomo di vita.

A. Bonfitto

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Hasta Siempre.

ABSTRACT

Animals perceive the external world through their sensory systems, which consists in: i) sensory receptors that detect external stimuli; ii) neuronal pathways that convey the sensory information to the brain; and iii) central neurons organized in relay nuclei that process this information. To study the selective innervation of hindbrain regions by sensory afferents, we mapped the fine-grained topographical representation of sensory projections at the central level: sensory ganglia located more anteriorly project more medially than do ganglia located more posteriorly, and this relates to the time of sensory ganglia differentiation. This somatotopic arrangement is laid out very early, prompting the question of the origin of the signals involved in the induction and maintenance of this patterning. Up to date, several studies have tried to unveil how peripheral ganglia "send" afferent projections to "reach" their entry points in the hindbrain exploring whether these neurons search for axon guidance cues coming from the vicinity tissues or whether they intrinsically know where to go. This view seems to consider differentiated neurons as a population of cells arising in the middle of nowhere that should cross through axonal navigation, a mesenchyme sort of dark forest. By SPIM in vivo imaging we demonstrate that once placodal-derived neurons of dorsal posterior cranial ganglia differentiate, they never lose contact with neural ectoderm. First, delaminated neuroblasts differentiate in close contact with the neural tube, and afferents entrance points are established by plasma membrane interactions between primary differentiated peripheral sensory neurons and neural tube border cells, with the cooperation of neural crest cells. Then, neural crest cells and repulsive slit1/robo2 guidance cues guide later-differentiated axons and mediate sensory ganglion coalescence, axonal branching and fasciculation.

RESUMEN

Los animales perciben el mundo externo a través de los sistemas sensoriales. Estos consisten en: i) los receptores sensoriales que detectan estímulos externos; ii) las vías neuronales que transmiten la información sensorial al cerebro; y iii) las neuronas centrales organizadas en núcleos que procesan la información. Con el objetivo de estudiar la inervación selectiva de las regiones del rombencéfalo por las aferentes sensoriales, hemos mapado la representación topográfica de las proyecciones a nivel central: los ganglios situados más anterior proyectan más medialmente que los situados más posterior, y esto depende del momento de diferenciación de estos ganglios. Esta organización somatotópica se establece muy tempranamente, lo que comporta la pregunta del origen de las señales implicadas en la inducción y mantenimiento de este *patterning*. Hasta ahora, muchos estudios han tratado de desvelar como los ganglios periféricos "mandan" las proyecciones aferentes para "alcanzar" sus puntos de entrada en el rombencéfalo y explorado si estas neuronas buscan claves de guidaje axonal provenientes de los tejidos adyacentes o si saben intrínsecamente dónde deben ir. Esta visión parece considerar las neuronas diferenciadas como una población de células que se originan en el medio de la nada y que deben navegar gracias a sus axones un mesénquima como si fuera un inhóspito bosque. Gracias a los métodos de imagen in vivo por SPIM hemos demostrado que una vez que las neuronas de los ganglios craneales posteriores han delaminado y diferenciado, nunca pierden contacto con el ectodermo neural. Primero, los neuroblastos se diferencian en íntimo contacto con el tubo neural, estableciéndose los puntos de entrada aferentes gracias a las interacciones entre membranas plasmáticas de las neuronas sensoriales y de las células del borde del tubo neural, con la cooperación de las células de la cresta neural. Luego, las células de la cresta neural y las señales de repulsión slit1/robo2 guían los axones que se han diferenciados más tarde y median la coalescencia de los ganglios, las bifurcaciones axonales y la fasciculación.

PREFACE

The ultimate goal of developmental neuroscience is to understand which steps bring to the correct formation of neural tissues and brain circuits that eventually, through a flow of electrical signals, give rise to the mind.

The way sensory systems develop have a great impact on how organisms interpret the world that surround them and how they interact with it. The plasticity of brain circuits permits us to remember and learn through the usage of our senses and on the bases of these perceptions we think and act. Every human being has daily life experience based evidences of the impact that sensations has on the interpretation of his environment.

Music or noise, landscapes or desolated scenarios, flavors or fuol odors, peppery or sweet tastes, cuddles or slaps. Everything influences in a short or long term the way we interpret the world, and in turn we interpret everything based on what we sensed in the past. Sensations are relatives and unique as the baggage of experiences that anyone carries with him and that shaped his characteristics brains circuits from a common basic plan. In turn, memories that we associate to a physical sensation lead us to feel differently the emotions, to react distinctly to inputs, even with the tiniest variation, and eventually to produce different thoughts, when compared with each other. This fact gives rise in principle, even if continually assaulted by huge mass homogenization tendencies, to an almost infinite number of variations populating the social biodiversity spectrum. To understand how this complicate experience based interconnection between sensations and reactions shape brain networks require huge studies, but deciphering the basic rules about brain networks construction during first steps of organism development could help in the task. In the field of sensory networks formation during embryonic stages is becoming clearer that circuits are shaped through neuronal activity and that this happen only after the establishment of the foundations of the sensory networks by intrinsic cellular mechanisms. Once the foundations are built, the whole structure of experience can be implemented above it. Therefore, to understand the mechanisms used by an organism to form the foundation of sensory networks in a correct and precise manner is of great

importance to understand how the remaining floors of consciousness are implemented above them.

Hence, to understand a little part of how neural networks are built in the first place has been the interest of the work I have performed during these last four years and brought me to develop the thesis you are going to read.

The usage of different model organisms, huge improvements in the possibilities of their genetic manipulation and high resolving optical visualization during the genomic era made real that our possibilities of studying, hence comprehending, biological processes would enhance in an exponential manner. Unluckily, in the same exponential manner the complexity of the global picture that our eyes have in front has been enhanced due to higher comprehension. Nevertheless, through the fundamental, because used by evolution, trial and error method of problem solving and our capabilities of anticipation, that is huge in our species compared to all the others we have knowledge about, we could be able to clarify and resolve the complicated global picture. We anyway do not need to hurry up, since even evolution took quite little time to give us these highly refined sensory systems and enormous cerebral capacity. The question would be then if evolution gave us enough of the latters to be able to understand the global picture before erasing the questioner itself.
In this case, and thanks again to evolution, the problem will be solved anyway and maybe, or maybe not, passed to another questioner, hopefully luckier, that is better adapted, than us.

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INTRODUCTION

First of all I will summarize the knowledge about sensory placodes and sensory ganglia formation, and neural crest cells (NCC) development will be introduced. This chapter is intended to highlight the close relationship existing between these two cell populations already at the moment of their specification from the initial unspecified ectoderm.

State of art knowledge on the reciprocity of interactions between the two populations needed to form sensory systems correctly will precede concepts about topographical mapping of sensory systems at central level, examples of different types of sensory central representations and their establishment during embryonic development.

This will lead me to introduce the reader to the hypothesis, and subsequent discussion, on how the first order of central connections is established during firsts steps of embryonic development, where time and place of sensory neurons differentiation play roles of great importance.

1.1 Sensory organs, Placodes and Neural Crest Cells (NCC)

Sensory organs are built up starting from two cell populations, placodal and neural crest cells, whose specification process starts early during embryo development, at gastrula stage, at the border between neural and non-neural ectoderm. Placodal cells are grouped in different placodes, visually distinguishable as a thickened portion of the ectodermal tissue, which are going to contribute to the development of different sensory organs, both to their structures and to their neurosensory lineages. Placodes form by the subdivision of the so called pre-placodal region (PPR), a horseshoe shaped region above the mesoderm, arising at the frontier between the non-neural ectoderm that becomes epidermis, and a neural ectoderm that is eventually specified in neural plate, further developing in central nervous system, and neural crest. Neural crest initially forms as a lateral stripe of cells at the border between the very same PPR and the neural plate, those latter remaining in contact at the rostral region. As the neural plate invaginates to form the neural tube, neural crest cell population undergoes epithelial to mesenchymal transition and becomes a highly migratory cell population involved in the development of the cranial skeleton, endocrine cells, smooth muscle cells and tendons, and most important for our matter, to gliogenic and part of neurogenic lineages of sensory organs. Neural crest delamination is followed by their peripheral migration along three paths named branchial, hyloid and mandibular stream, the latter populating also the frontal nasal region (for summary see Fig. 1).

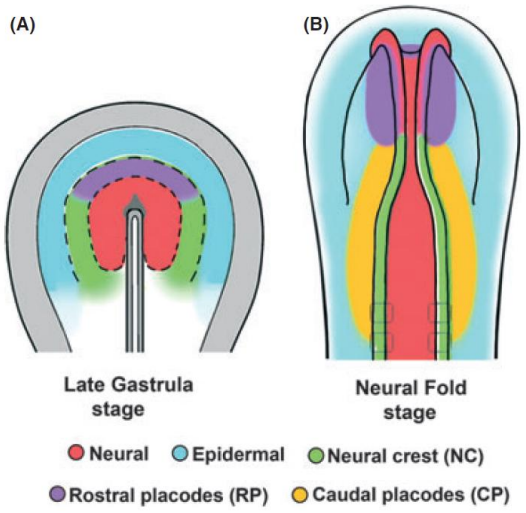


Fig. 1: Location of placodal progenitors and neural crest. (A) At the late gastrula stage, rostral placodal progenitors and caudal neural crest precursors are located between the neural and epidermal ectoderm. (B) At neurula stages, rostral placodal progenitors are located between the neural and epidermal ectoderm. Caudal placodal progenitors are located between the neural crest and the epidermal ectoderm. From (Jidigam & Gunhaga 2013b).

1.1.1 Placodes and derivatives

Once the pre-placodal region has been specified, placodes need to singularize and generate neurosensory lineages (Fig. 2).

The only sensory organs whose neurosensory structures seem to derive completely from placodal cells, having no contribution from neural crest cells, are the inner ear and the olfactory organs. The otic placode will give raise not only to sensory bipolar neurons and hair cells but also to secretory and supporting cells, while the olfactory placode derivatives are odorant and pheromone chemoreceptors as well as supporting and stem cells that have the ability to regenerate entirely, throughout the life, the pool of olfactory sensory neurons.

The lens is the only non-neurogenic placode, giving raise to lens fibres and lens epithelial cells, and it is considered the groundstate of placode development whose fate needs to be repressed for the ectoderm to acquire the ability to specify other kinds of placodes (Bailey et al. 2006). The hypophiseal placode develops into the anterior pituitary gland or adenohipophysis, the major control organ of vertebrates with six classes of endocrine cells. The invaginated hypophyseal pit, normally referred in amniotes as Rathke`s pouch, detaches from the roof of the oral cavity, ventral to the posterior diencephalon from which posterior portion of the pituitary gland, the neurohypophysis, is derived (Grocott et al. 2012; Jidigam & Gunhaga 2013a).

The trigeminal and epibranchial are strictly neurogenic placodes that undergo delamination, epithelial to mesenchymal transition, in order to form the distal part, and neural crest cells contribute in forming the proximal part of the respective ganglia that will innervate the epidermis and the viscerae. Cranial nerve V (Trigeminal) provides also information concerning the general texture of food as well as the taste-related sensations of peppery or hot, while the facial nerve VII (epibranchial Geniculate) carries taste sensations from the anterior two thirds of the tongue, the nerve IX (epibranchial Glossopharyngeal) carries taste sensations from the posterior one third of the tongue while a branch of the nerve X (epibranchial Vagus) carries some taste sensations from the back of the oral cavity. Fish and amphibians developed the so called lateral line organs, used to sense water velocity and acceleration, and electro-sensory organs, used to sense nearby electric fields fluctuations. Recently, a new placode has been discovered in chick, named the paratympanic placode (O'Neill 2014).

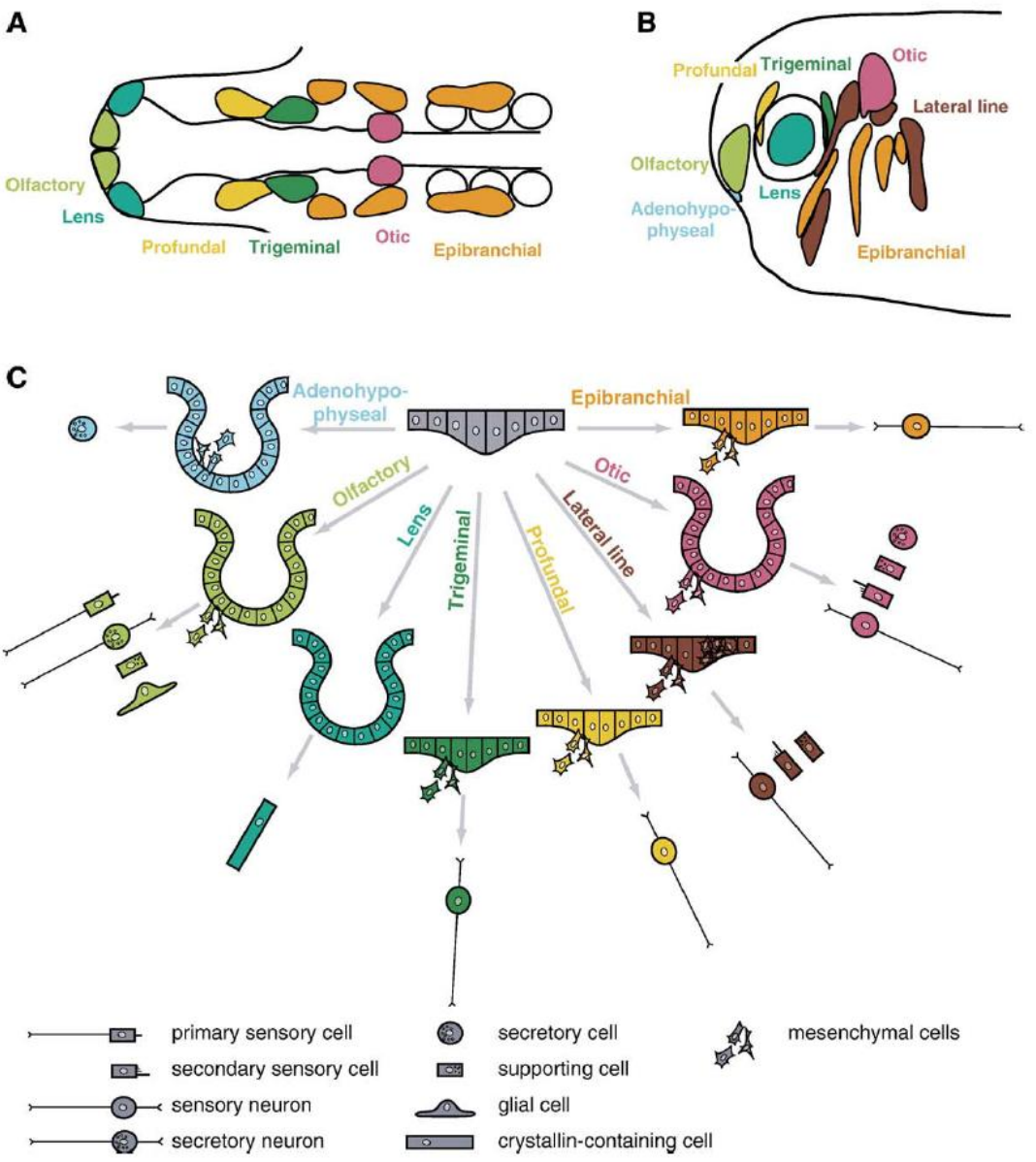


Fig. 2: Cranial placodes in vertebrates. (A) Cranial placodes in a 10- to 13-somite-stage chick embryo (modified from Streit, 2004; based on D'Amico-Martel and Noden, 1983; Bhattacharyya et al., 2004). In amniotes, profundal and trigeminal placodes are commonly referred to as ophthalmic and maxillomandibular placode of the trigeminal nerve, respectively. (B) Cranial placodes in a tailbud stage Xenopus embryo (modified from Schlosser and Northcutt, 2000). (C) Schematic summary of morphogenesis and cellular derivatives of various cranial placodes. Invagination occurs in adenohypophyseal, olfactory, lens, and otic placodes. Moreover, in all placodes except the lens placode, some cells migrate away from the placodal epithelium as mesenchymal cells to form sensory neurons, secretory cells, or glial cells. In lateral line placodes, another subset of cells migrates along the basement membrane and forms the lateral line primordial (from Schlosser 2006).

1.1.2 Neural Crest Cells and derivatives

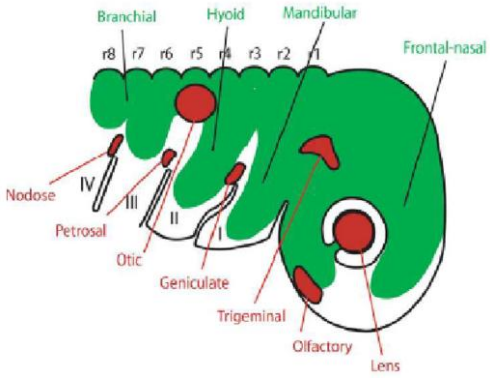


Fig. 3: The neural crest (green) migrates in three main streams: the branchial, hyloid and mandibular streams. In addition a fourth population migrates into the frontal nasal region of the head. Subsets of cranial placodes are shown in red (from Steventon et al. 2014a).

NCCs that originate from the diencephalon and anterior mesencephalon migrate into frontonasal process and extensively contribute to the frontonasal skeleton and the membranous bones of the skull. Whereas cranial NCC from the posterior mesencephalon and hindbrain, which is transiently subdivided into neuroepithelial segments, called rhombomeres, fill the pharyngeal or branchial arches (PA/BA) that will form the jaw, middle ear, hyloid and thyroid cartilages.

The NCC subpopulations that target individual PAs migrate in stereotypical streams dictated by the segmented organization of the hindbrain. Environmental signals force each stream into well-defined pathways and in most vertebrates hindbrain NCC migratory streams are separated by crest free regions lateral to rhombomere 3 and 5 where mesenchyme derived signals act to inhibit their migration (Fig. 3). Involved in sculpting the NCC migratory streams are the repulsive interactions between the Eph tyrosine kinase receptors and their ephrin ligands, and between the transmembrane neuropilin (Nrp) receptors and their secreted semaphorine (Sema) ligands.

NCC anteroposterior (AP) positional identity seems to be acquired at the pre-migratory stage and the progenitors' identity established by the same mechanism that controls segmentation and patterning of the rhombomeres from which they delaminate: the nested and combinatorial expression of transcription factors of the Homeobox gene family (*Hox* genes) (Minoux & Rijli 2010).

In addition to anteroposterior differential *Hox* code that distinguishes the segmental identity of each arch from that of its neighbours, NCCs need also to be provided with dorsoventral (DV) positional information to establish intra-arch identity, and this is achieved through a *distal-less* (*Dlx*) gene family expression code (Minoux & Rijli 2010). Thus, the molecular information provided at the intersection of AP and DV positions is

converted into NCC identity and in differentiation programs involved in the formation of structures of the appropriate shape, size and orientation.

NCC development in close relation with placode development has been shown to be of great importance in the placodes singularization process from the broad preplacodal region, and in migration and coalescence of placodal derived sensory ganglia. About the latter, Bronne-Fraser suggested that NCC may guide sensory neurons afferent projections through Robo/Slit signalling and increase in N-chaderin expression (Shiau et al. 2008; Shiau & Bronner-Fraser 2009), and Mayor showed that between placodal cell and NCCs there is a Chase-and-Run attraction/repulsion involving Planar Cell Polarity and N-chaderin signalling for what concerns the coordinated migration of those two populations (Theveneau et al. 2013a). On the other hand, Nechiporuk proposed that NCCs are involved in sensory neuron coalescence into sensory ganglia (Culbertson et al. 2011, see chapter 3).

The NCC involvement in the singularization of each placode will be further discussed in following specific paragraphs (see chapter 1.3).

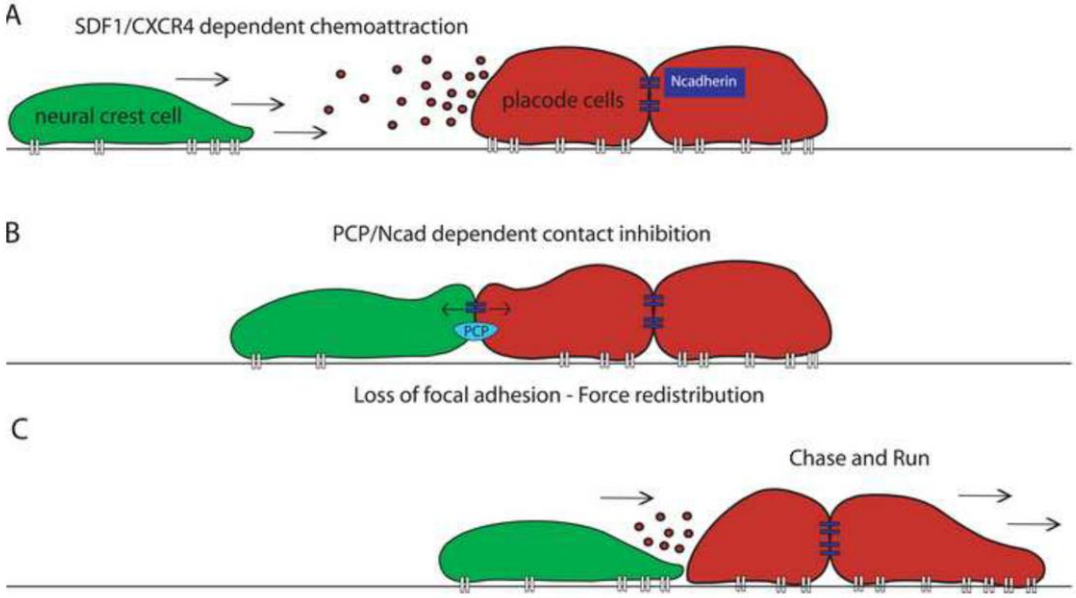


Fig. 4: Chase and run between neural crest and placode cells drives coordinated migration of adjacent cell populations. (A) The placode cells (red) release the chemoattractant Sdf-1 and attract the CXCR-4 expressing neural crest cells (green). (B) Upon contact between the two cell types, N-cadherin dependent junctions form together with an activation of the PCP pathway. This leads to a loss of focal adhesions in the region of contact and a redistribution of forces. (C) As a consequence the placode cells migrate away from the neural crest, which is in turn continually attracted by SDF1. (From Steventon et al. 2014a)

1.2 Model for the emergence of Pre-Placodal Region and Neural Crest Cells

The specification of the two borders between epidermis and neural plate, and preplacodal region and neural crest, is a fundamental process that allows the development of complex structures. This process eventually marked the evolutive step to a vertebrate "new head", making them much more adapted to a more active and predatory lifestyle through the development of highly specialised musculoskeletal and neuro-sensory structures (Gans & Northcutt 1983). As the structures form once they differentiate, the way those borderline progenitor populations are specified in the first beginning is not less intricate.

Placodal precursors dispersed in the ectoderm and intermingled with neural, neural crest and epidermal cells coalesce in the PPR during gastrulation. Shortly after, neural plate specification and the actual segregation from other ectodermal derivatives happen during neural fold formation.

Following the so called "binary competence model", during gastrulation various signalling molecules including FGFs, Wnt antagonist and BMP antagonists emanating from the organizer (prospective axial mesoderm) establish a dorsal ectodermal domain competent for adopting neural or neural crest fates, whereas the remaining ventral ectoderm is competent to adopt epidermal or placodal fates (Schlosser 2006).

Another model proposes that neural crest cells and cells populating the horse-shoe shaped preplacodal region (PPR) arise from a common neural plate border region specified by neural plate border specifier genes (Streit & Stern 1999, Pieper and Schlosser 2012).

Without favour one of the other of the two models I here try to describe in more detail the process and the temporal hierarchy events that bring to the formation of the placodal and neural crest cell populations.

At blastula stage, the embryonic ectoderm expresses pre-neural genes medially (*Sox3, Otx2, Geminin, ERNI*) and non-neural genes laterally (*Gata2/3, Dlxs, Msx1, Foxi1/3* and *Ap2*), with an overlap at the expression borders (Saint-Jeannet & Moody 2014). Pre-neural expression initiates prior to gastrulation as a consequence of mesodermal FGF, with the contribution of BMP and WNT antagonists, among others, for *Otx2 expression* (Papanayotou et al. 2008; Streit et al. 2000)

On the other hand, non-neural genes expression largely depends on BMP activity before gastrulation, with WNT positively regulating *Gata2* and *Msx1* and negatively regulating *Foxi1* and *Dlx3*.

Thus, a first subdivision between FGF receiving neuroectoderm and BMP/WNT expressing non-neural ectoderm is established (Fig. 5).

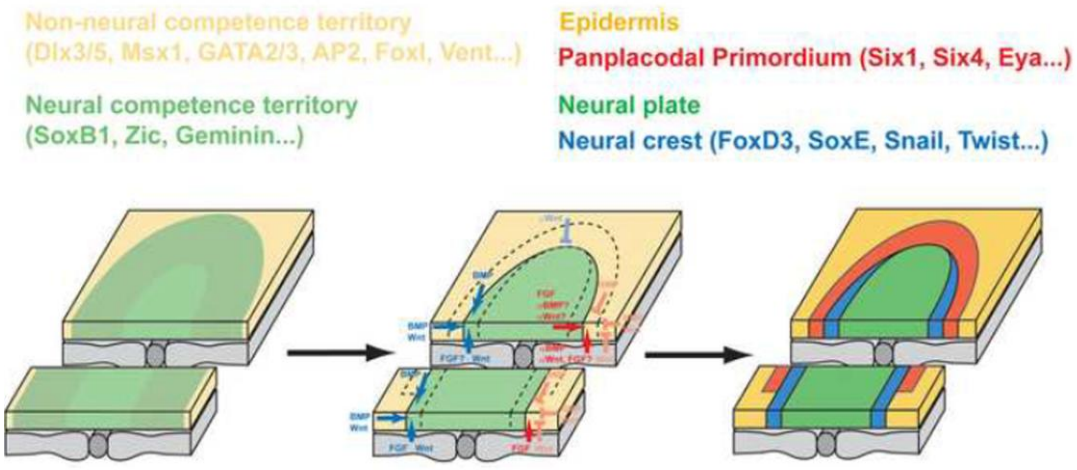


Fig. 5: Dorsoventral ectodermal patterning and induction of panplacodal primordium. Time course of induction according to the binary competence model. During gastrulation dorsally enriched neural competence factors (green) and ventrally enriched non-neural competence factors (yellow) are overlapping (left panel), but distinct competence territories are established at the end of gastrulation (middle panel). Neural crest inducing signals (FGF, Wnt, BMP; blue) then induce neural crest at the border of the neural territory, while other signals (FGF, BMP- and Wnt-inhibitors; red) induce the panplacodal primordium at the border of the non-neural ectoderm (right panel) (from Schlosser et al. 2014).

With the onset of gastrulation expression of new genes further defines borders between neural and non-neural ectoderm: non-neural *Gata2/3* and *Foxi1* expression is confined laterally while *Ap2* and *Dlx* genes more medially where they abut the neural plate (Kwon et al. 2010). *FoxD3* and *N-myc* are transiently expressed in the neural plate territory before moving in the neural crest domain, together with *Zic1-5* (McCabe & Bronner-Fraser 2009).

At the late gastrula *Sox2* expression is initiated in the neural plate in response to signals from the organiser (Uchikawa et al. 2003). Nevertheless, at the neural plate border, neural and non-neural transcripts overlap and neural and non-neural derivatives still intermingle. AP patterning is established by the differential expression of *Otx2* in the anterior and *Gbx2* in the posterior PPR (Steventon et al. 2012), while *Irx1* expression mediated by FGF and BMP signalling, sign the starting of borders subdivision being an

upstream effectors of placode bias activating *Six/Eya* families, which are positive regulators of preplacodal fate (Fig. 6; (McCabe & Bronner-Fraser 2009).

Msx1, *Pax3* and *Pax7* expression in the posterior-lateral neural plate and subsequent anterior expansion is determinant for later NCC fate specification and negative regulation of PPR specific genes: at early gastrula stages, when *Pax3* and *Msx1* are present in the posterior non-neural ectoderm, they restrict *Six1* expression to the head ectoderm; on the other hand, at neurula stages, when both genes are expressed in the neural folds, where neural crest cell are located, they prevent *Six1* expansion into the crest territory (Hong 2007). *Ap2* from the non-neural side and *cMyc* from the neural one, seem to be required for both placodal and neural crest specification, while *Foxi1* and *Gata2/3* only for placodal fate (Kwon et al. 2010, Bellmeyer et al. 2003).

Initiation of *Six* and *Eya* expression at neurula stage in the PPR domain, together with their upstream regulators *Irx1* and *Dlx3/5*, is necessary to promote PPR and antagonize neural specification. Through a positive feedback loop, *Six* and *Eya*, promote sensory progenitor fates and repress non-placodal character as well as their own competence factors *Gata3* and *Dlx5*, thus stabilizing placode progenitor identity (Pieper et al. 2012, Kwon et al. 2010, Christophorou et al. 2009).

Thus, at the end of gastrulation, signals including Wnts, FGFs and BMPs, from the prospective epidermis, neural plate border region and the paraxial mesoderm induce neural crest specification genes at the border of the neural/neural crest competence region, while FGFs together with BMP and Wnt antagonists from the anterior neural plate and the cranial dorsolateral endomesoderm induce generic placodal markers such as *Six1* and *Eya1* at the border of the epidermal/placodal competence region in the head at neural plate and neural fold stages (see summary in Fig. 6; (Schlosser 2006).

In summary, the subdivision of the ectoderm into different domains occurs sequentially from pre-gastrula stages. PPR is first identified at neural plate stages, shortly after induction of the central nervous system and concomitant with neural crest specification.

Neural plate border and Binary Competence models have been discussed as two opposing models, however, as reviewed by Streit in 2012, taking in consideration the temporal hierarchy of events those might be unified (Grocott et al. 2012).

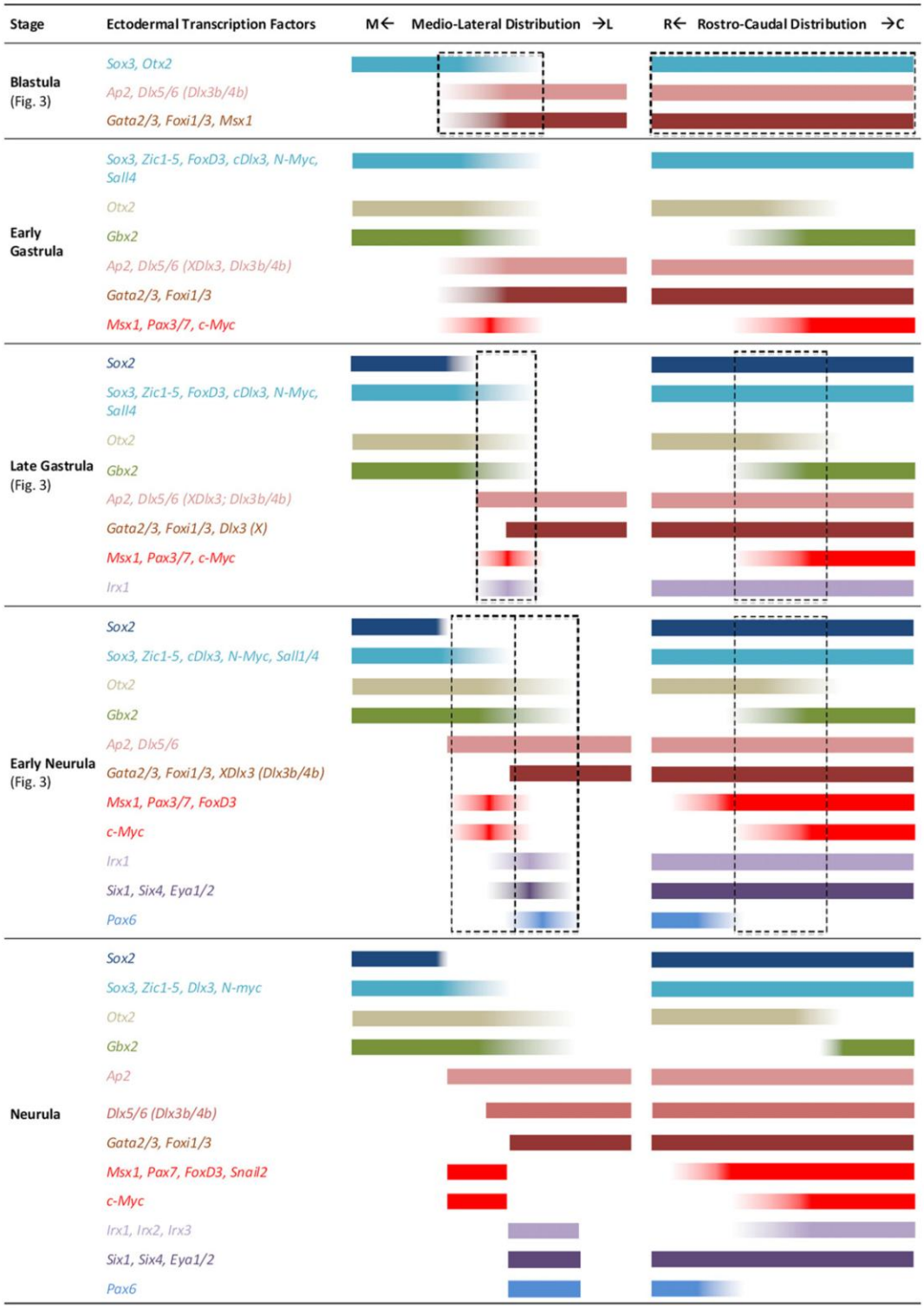


Fig. 6: Distinct regulatory states as ectodermal cells progress towards pre-placodal progenitors. The medio-lateral and rostro-caudal distributions of different ectodermal transcription factors are represented schematically, from pre-gastrula to neurula stages (from Grocott et al. 2012).

1.3 Anterior to posterior Preplacodal subdivision

The process of sensory organ formation passes through many subsequent steps of tissue specification from more large multipotent domains of cells. Through the interaction of different signalling molecules by tissues that are in close contact, a combination of different gene expression domains are created and this -partly overlapping and partly nested- expression of transcription factors permits the formation of specific placodal domains inside the panplacodal ectoderm.

As we have already seen, during embryonic development the ectoderm is divided first in neural versus non-neural tissues, then those two domains further specify in epidermis, panplacodal territory, neural crest cells zone and neural plate. Placodally expressed transcription factors are not restricted to a single prospective placode but extend through larger areas from which multiple placodes originate. The majority of these transcription factors, which are not panplacodally expressed, have expression domains centered on anterior or posterior areas inside the preplacodal ectoderm. Thus, this combinatorial model of nested and overlapping expression of transcription factors eventually subdivides the panplacodal ectoderm in anterior, intermediate and posterior placodal areas and the same combinatorial mechanism of transcription factors expression further specifies each of those areas, resulting in placode singularization and the subsequent sensory organs formation (see summary in Fig. 7).

As I will point out in the next paragraphs, the process of placodal domain specification is paralleled in time with vast morphogenetic movements and NCC specification and migration around the forming placodes. The result is a highly dynamic situation where combinatorial expression of transcription factors, NCC migration and tissue rearrangements are all essential processes that synergize in the correct formation of sensory organs.

"As two spots drawn close together on a balloon may end up far apart when the balloon is inflated, intermingled precursor cells within PPR that appear as random local mixed in particular positions, would segregate to distinct regions as the balloon inflates" (Bailey & Streit 2006).

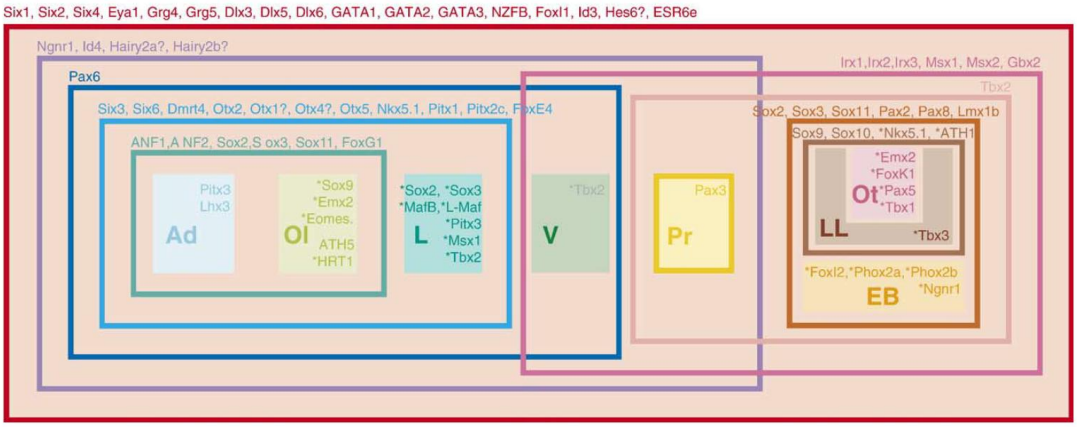


Fig. 7: Schematic summary of transcription factor expression domains in the placodal ectoderm of neural plate stage Xenopus embryos. Listing of transcription factors without asterisk refers to their expression domains established at neural plate stages, while listing of transcription factors preceded by asterisks refers to expression domains established at later stages. Question marks indicate tentative assignments because precise domain boundaries cannot be determined unambiguously from published information. Note that there are two foci of transcription factor expression, one centered on an anterior placodal area, the other centered on a posterior placodal area. However, the spatial extension of various expression domains differs resulting in two nested hierarchies, which are overlapping in the regions of profundal and trigeminal placodes. Abbreviations: Ad: adenohypophyseal placode; EB: epibranchial placodes; L: lens placode; LL: lateral line placodes; Ol: olfactory placode; Ot: otic placode; Pr: profundal placode; V: trigeminal placode (from Schlosser 2006).

1.3.1 Anterior placodes specification: Hypophyseal, Lens and Olfactory placodes

The anterior placodal area is defined where the domain of influence of *Six1* at neurula stage overlaps with *Otx2*, *Pitx3, Six3/6* and *Pax6* expression domains. This territory is able to give rise to any anterior placode, adenohypophyseal, olfactory or lens precursors, in combination with the different grades of Shh, BMP and FGF signals coming from surrounding tissues.

FGF from the anterior neural ridge promotes olfactory identity repressing lens fate and Shh from the midline promotes anterior pituitary character repressing lens and olfactory state. *Shh* expressed in the mesoderm underlying the prospective adenohypophyseal placode, and the placode itself expressing *Ptc2*, make this signalling pathway critical for specification of adenohypophyseal placode cell identity, in contrast to prospective lens and olfactory placodes whose underlying mesoderm lack *Shh* expression (Sjödal & Gunhaga 2008).

The *pitx3* positive domain of the anterior PPE may demarcate an equivalence field, a PPR subdomain from which more than one placode can emerge, which develop into lens cells unless adenohypophysys fates are induced by Shh signals. In fact, lens precursors in the more lateral *pitx3*-expressing region remain at some distance from the Shh source. At the same time *Dlx3/4* expression restricts the area within the anterior PPE that is competent to respond to those signals leading to olfactory fates, thus patterning it along the medio-lateral axis (Dutta et al. 2005).

In recent years Paired homeobox gene family arose as fundamental in the subdivision of the pan-placodal ectoderm, since they interact in a regulatory network with the pan-placodally expressed *Eya*, *Six* and *Dach* but in a more restricted manner (Schlosser 2006). The differential expression of *Pax6*, *Pax2/8* and *Pax3* in the rostral, medial and posterior domains respectively, parallel their requirements in specifying anterior medial and posterior placodes. The anterior PPR strongly expresses *Pax6* that is necessary for anterior placodes formation, being the lens fate a sort of groundstate. *Pax6* is the first gene of this family to be expressed and its downregulation by Wnt and FGF is crucial for the formation of all other placodes, even in the very same region. BMP from the ectoderm seems to be required to maintain the lens groundstate of PPR in combination, later in development, with FGF and BMP from the optic vescicle. BMP is needed in

lens specification by inhibiting olfactory fates and inducing *L-Maf* that in turn, together with *Sox2* and *Pax6*, induces the transcription on the *δ-cristallin* promoter as first step for the occurrence of primary lens fibre differentiation (Fig. 8; (Bhattacharyya & Bronner-Fraser 2004). The olfactory placode region uses FGF coming from the neural ridge to transiently downregulate the expression of *Pax6* while promoting *Dlx3/5* expression. Thus lack of Dlx proteins is essential for lens and endocrine cells specification, while Dlx are necessary for olfactory fates. Pax6 is needed for lens cells specification but devoid of Shh signal that, instead, is necessary for adenohypophysys cells to specify.

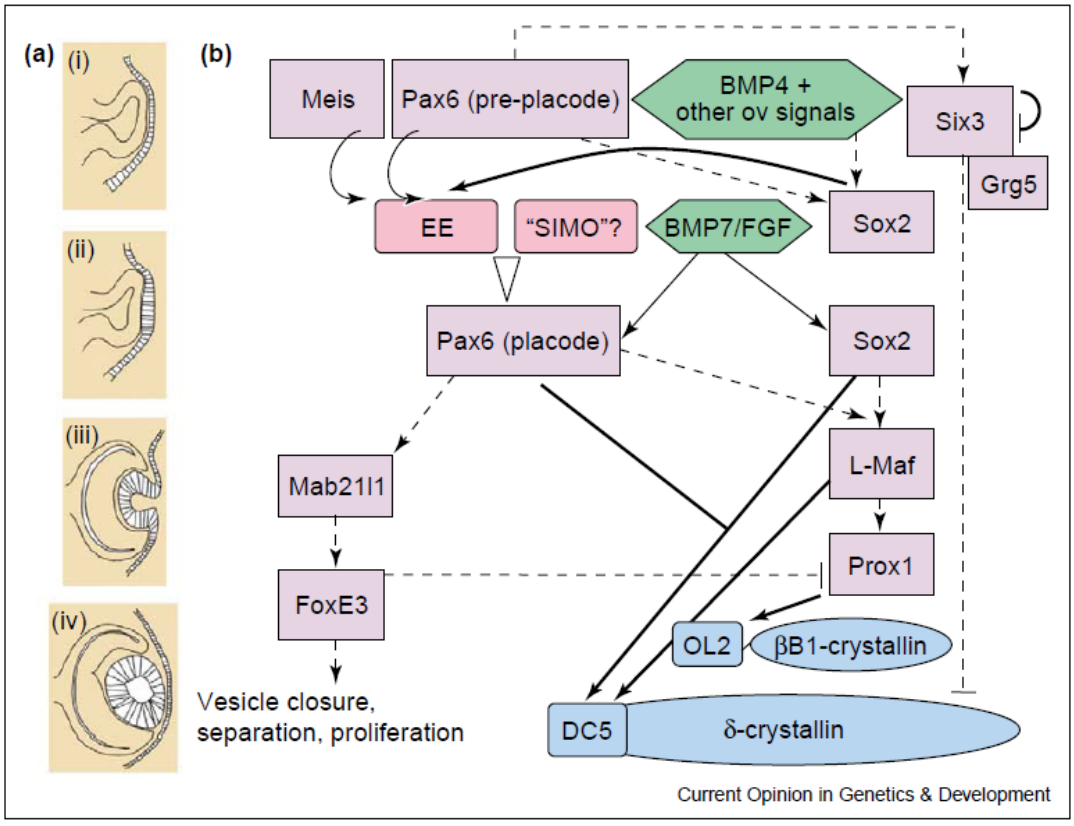
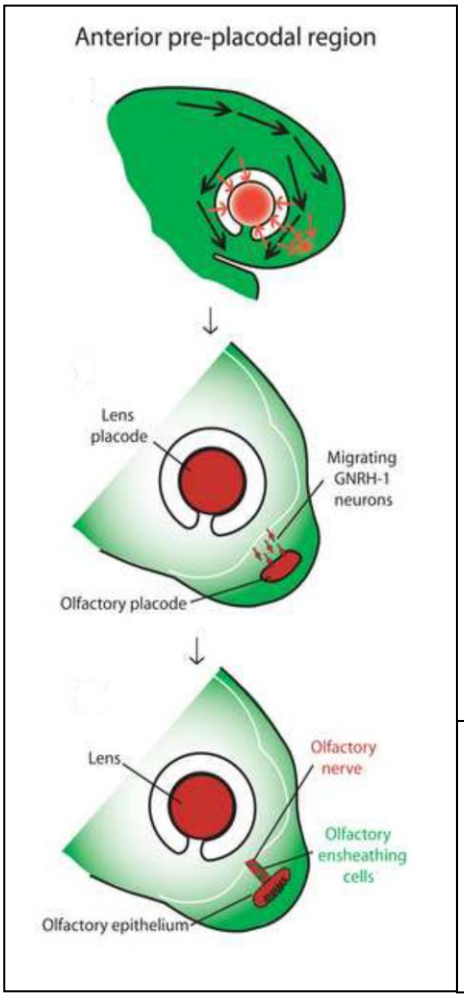


Fig. 8: Outline of the gene regulatory network involved in lens formation. (a) Schematic diagrams illustrating the morphological development of the lens at times that correspond to gene deployment in (b). (i) Presumptive lens placode ectoderm shown in white overlies and abuts the optic vesicle. (ii) The ectoderm thickens to form the lens placode and is in direct contact with the optic vesicle. (iii) The lens placode invaginates to form a pit, and finally (iv) the mature lens vesicle buds off and is covered by a sheet of corneal epithelium. The lens is housed within the cup-shaped structure of the retina. (b) Both FGF and BMP7 signaling are required to maintain placodal expression of Pax6, suggesting that it is required for the later placodal stages of Pax6 expression. On the other hand, BMP4 and other optic vesicle (ov) derived signals act upstream of Sox2 expression. Pax6 and Sox2 cooperate toinduce L-Maf and δ-crystallin expression. Pax6, Sox2 and L-Maf all bind the enhancer region of the δ-crystallin gene (from Bhattacharyya & Bronner-Fraser 2004).



The process of anterior placodes singularization is paralleled with the migration of the first NCC stream that emerges between the diencephalon and the rhombomere 2.Neural crest cells from the posterior mesencephalon together with those from rhombomere 1 and 2 populate the first pharyngeal arch, while the ones from the anterior mesencephalon and diencephalon migrate into the frontal nasal region to surround the optic vesicles, downregulating δ-cristallin expression outside the optic field, but also the olfactory placode (Fig. 9; (Patthey et al. 2008; Grocott et al. 2012; Bhattacharyya et al. 2004).

Fig. 9: Neural crest migrating into the frontal-nasal region migrates around the developing eye and olfactory placode. GnRH-1 cells delaminate from the olfactory placodes. Neural crest cells provide olfactory ensheathing cells to the placode-derived olfactory nerve (from Steventon et al. 2014b).

During this process, while the presumptive olfactory placode is expressing the chemochine receptor CXCR4, the abutting neural plate expresses the chemochine CXCL12. CXCR4/CXCL12 have been proposed to induce the convergence, and to retain olfactory placodal cells in their position during extensive anterior directed tissue rearrangements that happens due to morphogenetic movements and to NCC migration from their initial position posterior to the olfactory placode (Fig. 10, reviewed in Miyasaka et al. 2012).

Thus, along with the action of the first neural crest stream, also the morphogenetic movements seem to play important roles in the further separation of multiplacodal region in specific placodes.

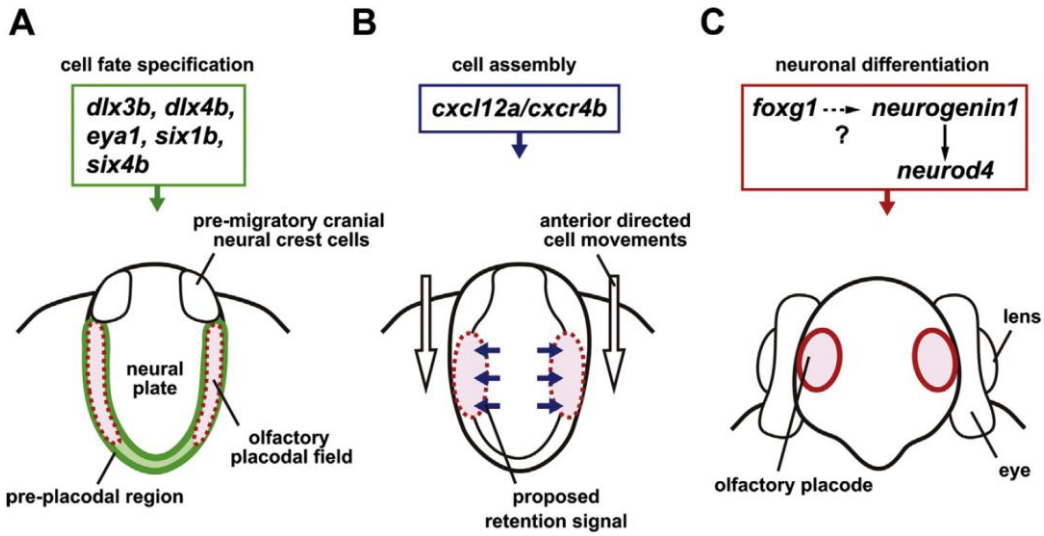


Fig. 10: Schematic summary of olfactory placode development. (A) The cranial sensory organs develop from a common territory at the border of neural plate, the pre-placodal region (area with a green border), which is specified by the expression of several transcription factors (green box). The olfactory placode arises from a large cellular field (olfactory placodal field; area with a red dashed border) within the pre-placodal region. (B) The neural plate-derived Cxcl12a provides the Cxcr4b-expressing olfactory placodal precursors (area with a red dashed border) with a retention signal (blue arrows) to withstand the anterior-directed movement of neighboring cells such as cranial neural crest cells. (C) Differentiation of OSNs within the olfactory placode (area with a red border) is regulated by forkhead family and bHLH family transcription factors (red box), (from Miyasaka et al. 2012).

As stated by Schlosser in 2006, the separation of the adenohypophyseal and olfactory placodes from the remaining placodes seem to rely on the elevation of neural folds during neurulation, with rostralmost placodes displaced more dorsally due to the extension of the anterior most part of the preplacodal ectoderm until the tip of the outer neural folds, while more posterior parts of the preplacodal ectoderm are positioned more laterally. Those would then be further separated by expansion of the anterior non-neural ectoderm associated with bulging of the retina and apoptotic and proliferative events that enhance placodal separation and morphological appearance, respectively.

Further specification of adenohypophysys, for example, requires Shh signals from the oral ectoderm and WNT, BMP4 and FGF signals from the prospective diencephalon from which the neurohypophysys is derived, and put in contact with, by the very same anterior directed morphogenetic movements (Fig. 11, Pogoda & Hammerschmidt 2009).

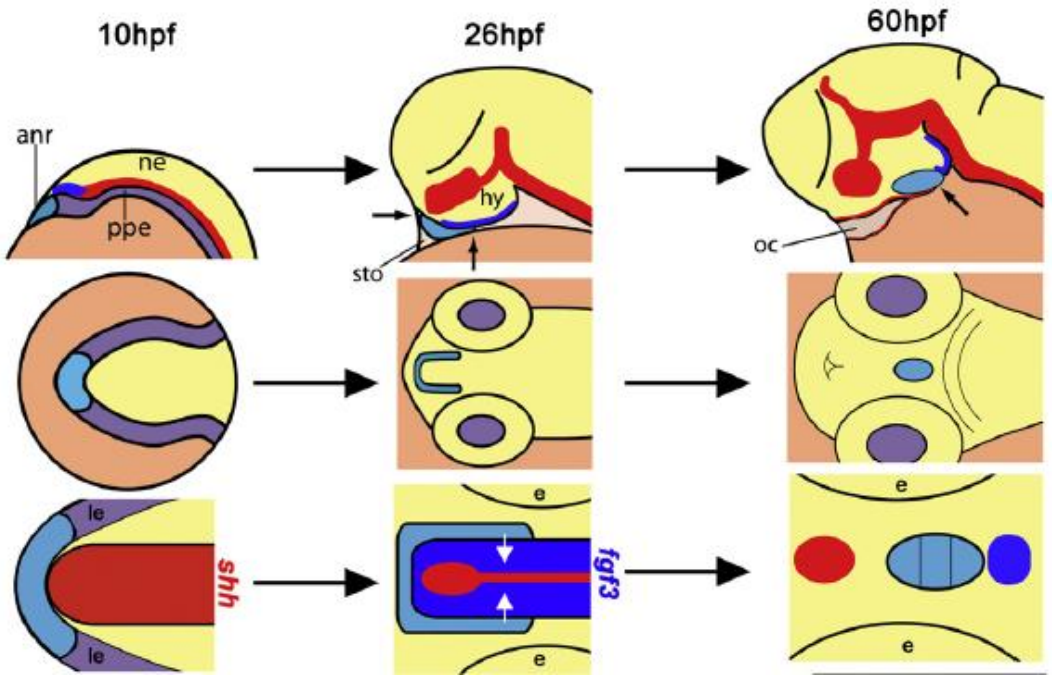


Fig. 11: Simplified cartoons of zebrafish pituitary development at 10, 26 and 60 hpf. The adenohypophisis (AH) is in light blue, the neural ectoderm (ne) in yellow, and the pre-placodal ectoderm (ppe) in purple. Other abbreviations: anr, anterior neural ridge; e, eye; h, hypothalamus; le, lens; oc, oral cavity; sto, stomodeal ectoderm. At the end of gastrulation (10 hpf), AH precursors are located in the anr, the most anterior part of the ppe. The ventral neuroectoderm expresses hedgehog genes (*shh* and *twhh*), with the rostral tip of their expression domains extending anteriorly up to the anr. At these early stages, Hedgehog signals are essential for the general induction of AH specificity. During further development, ppe cells at the anr aggregate to form the AH placode, which becomes visible as a distinct structure at the anterior edge of the head by 19 hpf. At this stage, the hypothalamic hedgehog expression domain is located at the anterior aspect of the AH anlage. At the same time, *fgf3* expression comes up in the ventral diencephalon, in close dorsal proximity of AH anlage. The f*gf3* expression domain progressively shifts towards posterior regions of the gland. Diencephalic Fgf3 signals are required for AH specification and survival between 18 and 22 hpf. In addition, opposing Hedgehog and Fgf3 signaling gradients pattern the pituitary placode along its anterior–posterior axis during mid-segmentation stages, with Hh specifying anterior and Fgf3 specifying posterior fates. At 26 hpf, the AH placode gets internalized into the head, coinciding with the formation of the oral cavity, and reaches its final position between 48 and 72 hpf. With the onset of oral cavity formation, sonic hedgehog (*shh*) also starts to be expressed in the oral ectoderm, establishing a second source of Hh signals with probable impact on AH patterning along its dorsal–ventral axis (from Pogoda & Hammerschmidt 2009).

1.3.2 Intermediate placode specification: The trigeminal placode

As seen before, already at early gastrula stages a first anteromedial to posterior subdivision of the embryo is present based on the differential expression of *Otx2* and *Gbx2*, being the first confined in the rostromedial and the second in the posterior ectoderm. The mutual repression *Otx2/Gbx2* seems to be a general mechanism to define rostrocaudal embryo identity: in the neural plate it is involved in the formation of mid/hindbrain boundary, while in the PPR the cell sorting that happens at their overlapping limit of expression is involved in the formation of a boundary that once sharpened eventually serves for the segregation of the medial ophtalmic/maxillomandibular (opV/mmV) placode from the more posterior otic progenitors region (Steventon et al. 2012).

WNT signalling within the neural plate border is known to establish posterior identity activating *Gbx2*, *Irx1-3* and *Pax3* expression as well as regulating *Fgf8* expression in the isthmic region (Jidigam & Gunhaga 2013a; Grocott et al. 2012). As seen in the previous paragraph, FGF signaling seems to be involved in the anterior restriction of *Pax6* expression and its later confinement in the lens territory. *Irxs* mediate *Pax2* downregulation confining its expression in the posterior placodal domain while WNT antagonists protect anterior placodal region from WNT influence (Wakamatsu 2011; Dude et al. 2009). In this way, an intermediate domain is defined that separates anterior and posterior placodal areas: following *Pax6* restriction, in the region where *Gbx2* and *Irx* expression overlaps, *Pax3* starts to delineate the ophthalmic/trigeminal intermediate placodal domain, with some contribution, of anterior *Pax6*- and posterior *Pax2*-expressing cells in the population of the profundal and trigeminal ganglion respectively.

Pax3 is required for the expression of FGFR4 that in turn activates *ngn2* (*ngn1* is expressed in mmV), *Pax3* expression itself in the opV (Begbie 2002) and the expression of an early marker of trigeminal placodes derived sensory neuron lineage named Brn3a (Artinger et al. 1998). Together with FGF8 signals from the isthmic region that act via MAPK pathway and TGF-β signalling activated by migrating NCC, neurogenins (*ngn*) are needed to differentiate *Pax3*-positive placodal cells in post mitotic Isl1-positive neurons eventually populating the distal portion of the corresponding cranial ganglia and, in combination with r2-derived neural crest cells populating its proximal portion,

they mediate touch, pain and temperature sensation from the skin of the face, jaws and teeth (Grocott et al. 2012; Blentic et al. 2011; Canning et al. 2008).

The same set of brain derived signals from the isthmus may simultaneously establish early positional differences in both the rostral hindbrain and in trigeminal ganglion neural progenitors: an FGF8 source at the rostral margin of the telencephalon is also involved in the early spatial patterning and positioning of the progenitor area which generates the cortical facial map, thus an important parallelism arise in both time of development and signalling pathway used to establish primary neurons to second order nuclei connection in the central neural network that will be introduced later (Erzurumlu et al. 2010; Fukuchi-Shimogori & Grove 2001).

1.3.3 Posterior Placodes specification: Otic, epibranchial and lateral line placodes

Already before *Six/Eya* network is established, *Gbx2* expression starts in the posterior ectoderm. Shortly after its localization to the posterior placode territory, at neurula stages, members of the *Irx* family become confined in the posterior PPR with their anterior limit rostral to *Gbx2* defining, as we have seen before, the intermediate placodal area. At this stage, members from *Dlx* and *Foxi* family (*Dlx3/4* and *Foxi1*), that are normally expressed in the non-neural ectoderm, quickly shift to the posterior placodal area forming a network of interactions that regulates, in response to FGF3/8 from the underlying mesoderm and hindbrain, the onset of *Pax2* and *Pax8*, known to be both critical in otic specification from the PPR and later ear development, and *Sox3* expression. Those in turn regulate otic, lateral line or epibranchial placodes fate (Christophorou et al. 2010; Mackereth et al. 2005).

Thus, *Foxi1* and *Dlx3/4* seem to behave as competence factors for the OEPD domain establishment. Activated independently of FGF signalling, they cooperate with it to initiate first *Pax8* downstream of *Foxi1* followed by *Pax2*, downstream of *Dlx3/4* and *Pax8*, involved in controlling proliferation, and hence the size of the progenitor pool of those placodes. The upregulation of *Pax2* and *Pax8* in the otic field eventually leads, respectively, to *Foxi1* and *Sox3* downregulation, whose expression is instead maintained in the epibranchial domain pointing to a role in the division in the otic-epibranchial competence field.

As seen for the specification of the other placodes, signalling molecules from tissues next to the forming-placodes ectoderm or, like in the case of adenohypophysys formation, to which placodes are brought in contact due to morphogenetic movements, are involved in further commitment.

The very same mesodermal FGF that mediates posterior placodal area (PPA) induction establishes the expression of Wnt8a from the hindbrain, and then its expression falls. Being the PPA closer to the hindbrain, in contrast to the more lateral positioned epibranchial one, the presumptive otic field gets in contact with the Wnt8a signals responsible for otic specification. High levels of WNT seem to upregulate components of the Notch signalling pathway, such as *Jag1* that feeds back augmenting WNT signalling in this region but not in the lateral OEPD where WNT signalling is too low and hence *Jag1* is not activated (reviewed in Ladher et al. 2010).

WNT activation also promotes *Pax2/8* expression whose high levels confer a bias toward otic fate instead of EB fate (McCarroll et al. 2012). At the same time FGF signalling inhibitors such as *Sprouty* are upregulated in the differentiating otic placodes and are required for its subsequent differentiation. By contrast, the more lateral region of the EOPD does not express FGF inhibitors and sustained FGF signalling in this region is compatible with differentiation into epidermis and epibranchial ganglia (Freter et al. 2012; Freter et al. 2008; Chen & Streit 2013; Mahoney Rogers et al. 2011). In fact, PPA seems to signal to the epidermis, through FGF24 in zebrafish, to recruit EB placodal cells from the non-neural ectoderm (McCarroll et al. 2012). Furthermore morphogenetic movements bring the lateral PPA region in close proximity with the FGF expressing paraxial mesoderm and *Bmp7* expressing pharyngeal pouches under formation. This endodermal tissue, that later form species-specific number of pharyngeal arches, is involved in the induction of foci of epibranchial neurogenesis in the correct position. That is, every pharyngeal arch induces the neurogenesis and delamination of its own epibranchial ganglia *Phox2b*-positive neurons inside the broader epibranchial placodal field (Fig. 12).

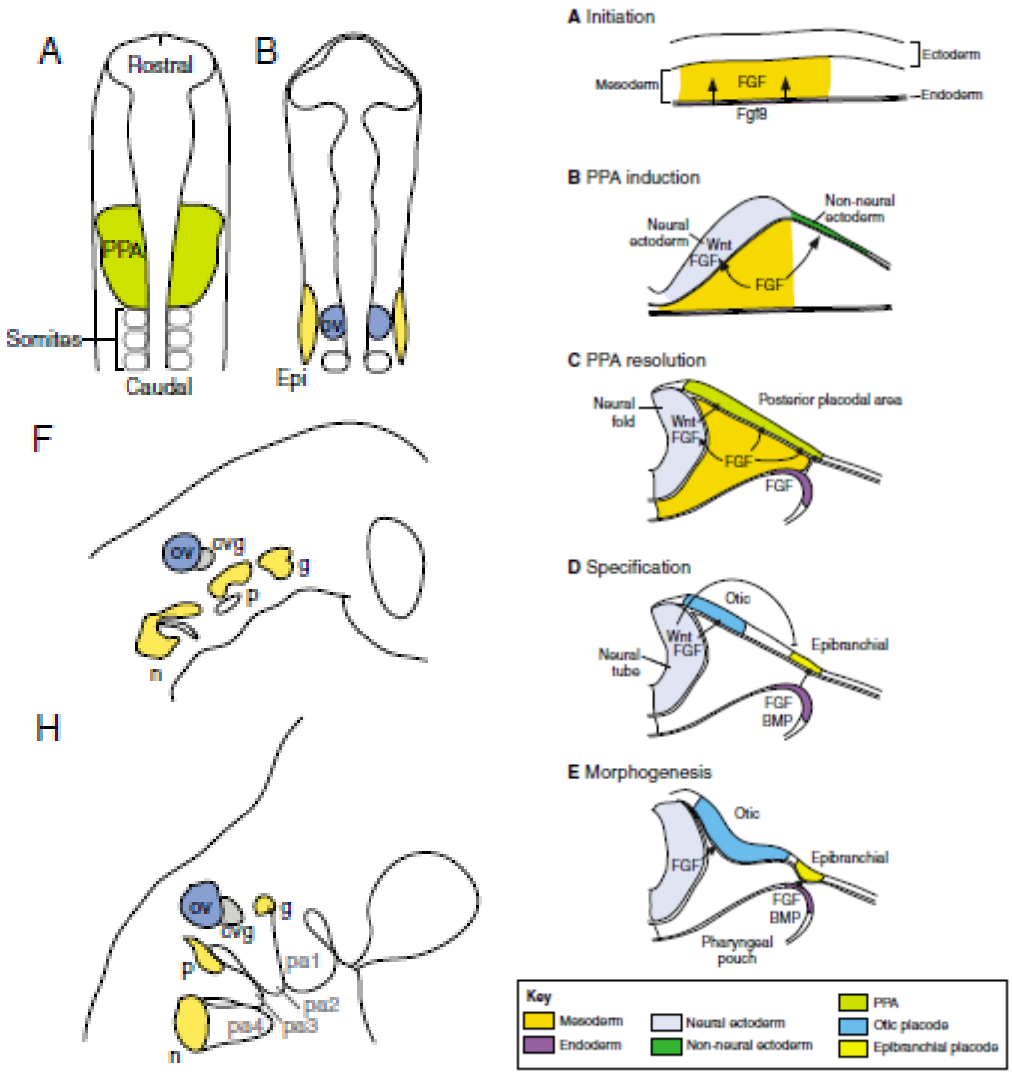


Fig. 12: Left panel: Schematic of the posterior placodal area (PPA, green) at stage HH8 of chick development. (B) Schematic, showing specification of otic (blue) and epibranchial (yellow) territories. (F) Schematic showing the resolution of the epibranchial domain into individual neurogenic foci in a chick embryo at HH14. At this stage, the first neuroblasts delaminate from the otocyst to form the CVG (grey). (H) Schematic of the final positions of the otic (blue) and epibranchial (yellow) placodes. Rostral is to the upper right. Epi, epibranchial domain; g, geniculate placode; p, petrosal placode; n, nodose placode; ov, otic vesicle; cvg, cochleovestibular ganglion; pa, pharyngeal arch. Right panel: Model of the cellular and molecular interactions involved in PPA induction and in otic and epibranchial placode specification. A scheme for PPA induction and inner ear and epibranchial development synthesised from mouse, chick and zebrafish embryonic data. Dorsal is uppermost. (A) Initiation occurs during early neurula stages in the hindbrain when Fgf8 secreted by endoderm induces FGF expression in overlying mesoderm. (B) During mid-neurula stages, mesodermal FGF acts on overlying non-neural ectoderm to induce the PPA and acts on neural ectoderm to induce FGF and Wnt8a expression. (C) The PPA domain expands and is sharpened by FGF emanating from neural ectoderm, mesoderm and endoderm. (D) During late neurula stages, mesodermal FGF expression is attenuated. This allows neural Wnt8a and endodermal FGF to act. Wnt8a acts positively to regulate otic fate, while negatively influencing epibranchial fate; FGF specifies epibranchial fate. (E) In early pharyngula stages, neural FGF expression acts on the basal side of the newly induced otic placode to induce its invagination. Epibranchial placodes are resolved into individual foci through the action of BMP and FGF (from Ladher et al. 2010).

Interestingly, Wnt signalling is involved in the placodal area in the downregulation of *Six/Eya* expression and Wnt inhibitors are needed to avoid that neural border cells acquire a NCC fate. At the same time high doses of *Six/Eya* induce *SoxB1* family genes expression and, as a consequence, a proliferative neural progenitor state. In contrast low doses of *Six/Eya* genes promote neuronal differentiation.

Six/Eya dosage dependent effects parallel with the dosage dependent effects of *SoxB1* family genes: high doses of *SoxB1* genes are essential for maintaining proliferative neural progenitors, while low doses are needed for placodal cells to acquire their path through prosensory specification and differentiation (Schlosser et al. 2009). One may wonder if FGF induced Wnt signals from the hindbrain are the ones to implement these changes in *Eya/Six* and *SoxB1* genes expression eventually leading to neurosensory specification.

A special case is represented by the otic placode that differently from the trigeminal and epibranchial placodes gives rise not only to neurons but also to the sensory cell population and to all the morphogenetic complicated non-sensory structures of the inner ear. Here, once the neurosensory field in the forming placode has been established, the choice for the progenitor cell population toward specification as neurogenic or sensory fate involves Notch signalling lateral inhibition. Notch expression is found in the entire otic epithelium and one of its modulators, *LFng*, only in the proneural domain. The effector Delta is found in a salt and pepper pattern inside this domain (Alsina et al. 2004) with complementary expression of *Hes5* (Abelló et al. 2007). In the non-neural region of the otic epithelium the other Notch ligands *Jag1* and *Hes1* are expressed and involved in early otic patterning and formation of non-neural structures (Abelló et al. 2007). Thus the Delta positive cell will be specified as *ngn1*-positive neurogenic population repressing at the same time this path of specification in the neighbouring cells that will end up to constitute the *Atoh1*-positive sensorigenic population (Fig. 13).

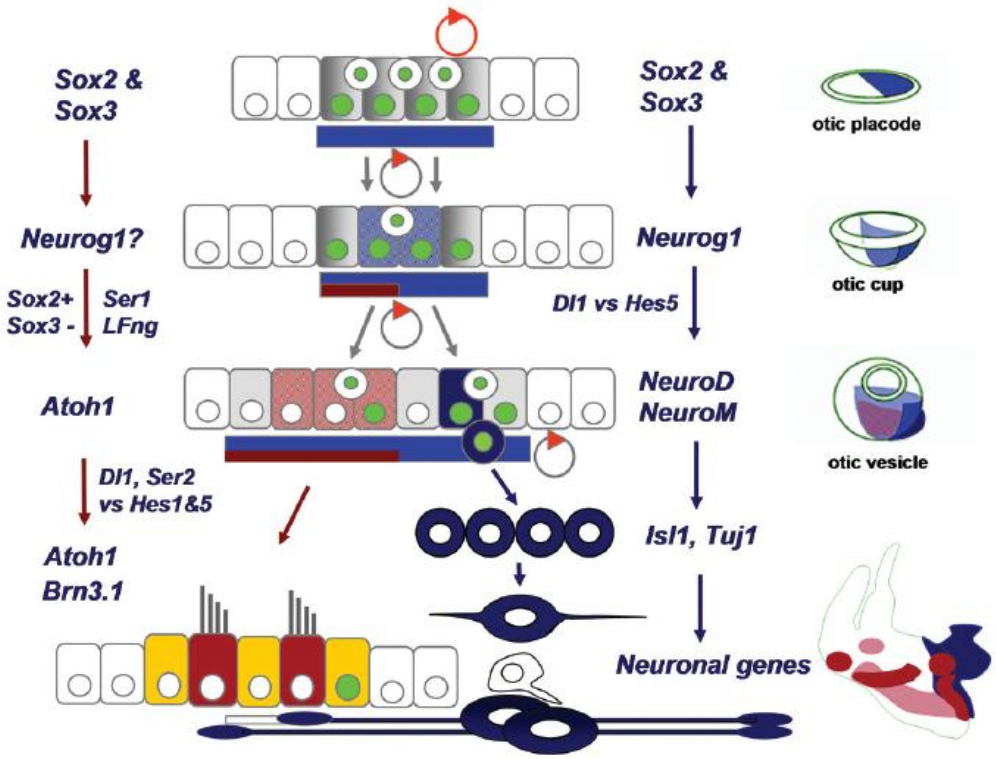
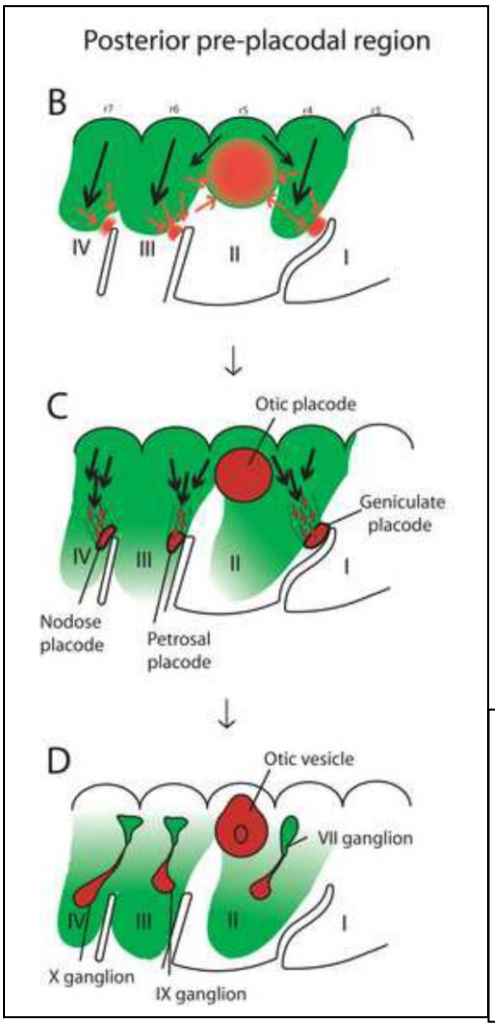


Fig. 13: Cell fate specification in the inner ear. The diagram shows a model of hair cell and neuron specification during ear development in amniotes. The sequence of gene expression for sensory (left) and neuron development (right), is indicated. The neural competent domain is common for the two lineages and expresses genes of the *SoxB1* group, which maintain the cell renewal state and commit progenitors to neural fate. Those are probably multipotent progenitors that generate all cell lineages (Satoh and Fekete 2005). This domain is specified either by temporal and/or spatial cues to give rise the two main lineages: sensory and neuronal. The bars under the epithelia indicate neural competence (blue) and prosensory specification (brown). *Ser1* is necessary for sensory specification as is *Sox2,* and probably the down-regulation of *Sox3*. *Neurog1* expression is required for both lineages in macula, but not for crista or auditory epithelium. Neuronal specification then takes place by the enhanced expression of Neurog1 via the Delta-Notch pathway, and the subsequent expression of *NeuroD* and *NeuroM* proneural genes. The latter allow delamination and transient amplification of neuronal precursors within the ganglion. Hair cells are singled out within *Atoh1* clusters, under the sustained expression of *Dl1/Ser2*, in a positive feed-back loop (from Alsina et al. 2004).

Interestingly the formation of an *Eya/Six/Sox2* complex is known to induce sensory or neuronal specification depending on the cellular context through chromatin remodelling complex of the SWI/SNF family, allowing the expression of *Ngn1* or *Atoh1* thus specification in delaminating neuroblast or sensory progenitors, respectively (Yang et al. 2013). *Sox2*, but not *Sox3*, expression is maintained in the sensory cell lineage and is only lost after hair cell maturation, but maintained in supporting cells in line with a regenerative potential of supporting cell by transdifferentiation. On the other hand for the delamination process that lead to the final maturation of neuroblasts, *SoxB1* family genes and *Ngn1* need to be downregulated in order to allow *NeuroD* gene expression.

This gene as stated above is involved in the delamination of neuroblast from the otic placode/vescicle and in the further maturation of neuroblasts until the expression of *Islet1*, marker of differentiated neurons, is established.

Like otic and epibranchial placodes, lateral line placodes arise from the *Pax2/8*-positive, *Sox2/3*-positive posterior placodal area induced by FGF signalling, but to date anything else is known about the molecules involved in the early steps of lateral line placode induction except of an involvement of further signals from both mesoderm and neural plate that would establish the *Tbx3* specific marker expression (Piotrowski & Baker 2014).



During the subdivision of the posterior placodal territory neural crest migrating from r4 (hyloid stream) and r6 to r8 (branchial stream) together with NCC from r5, that migrate around the otic placode (and lateral line placodes if present) to join the streams coming from the adjacent rhombomeres, travel from the neuroectoderm to sit next to coalescing facial and glossopharyngeal-vagal placodes and follow migrating ventrally to populate the pharyngeal arches (Fig. 14; (Culbertson et al. 2011; Theveneau et al. 2013b).

Fig. 14: (B) Neural crest cells of the hyoid and branchial streams migrate around the otic placode and in between the epibranchial placodes as they invade pharyngeal arches II-IV. (C) Neuroblasts from the epibranchial placodes delaminate and migrate along the neural crest to form the cranial ganglia. (D) Joint contribution of neural crest and placodal cells to the epibranchial ganglia (from Steventon et al. 2014b).

1.4 Neurogenesis

As seen before, placode neurogenesis begins within the epithelial proneural niche, through a mechanism involving Delta-Notch pathway. This happens before cells delaminate and migrate, hence coupling neuronal cell selection and changes in cell adhesion leading to delamination. This is evidenced by the consecutive non-concomitant spatio temporal expression of early specification neuronal markers such as *Ngn1/2*, *NeuroD/M* (markers of singled out delaminating neurons) and markers of differentiated neurons such as *Islet2b* and *Phox2b*, that eventually populate the sensory ganglia, and by a significant reduction in cycling cells, although a fase of transit amplification of IGF-1 positive neuroblast population happens after delamination (Camarero et al. 2003; Vemaraju et al. 2012, reviewed in Lassiter et al. 2013).

The timing of terminal differentiation occurs within the epithelium prior to delamination in the ophtalmic portion of trigeminal neurons (McCabe et al. 2009), while it happens after delamination and transit amplifying step, for otic and epibranchial ganglia and maxillomandibular portion of trigeminal ganglia (Begbie 2002).

1.5 Sensory neural networks

Once neurogenesis has occurred, and delaminated neuroblasts underwent the maturation process to differentiated neurons, they need to innervate the cells that are able to receive the external stimuli. This is achieved by connections to mechanosensitive, proprioceptive, chemosensitive sensory cells or sensory free ending nerves connected to the epithelia. The information from the external world of the organism is thus relayed to a corresponding central nervous system nucleus in the brainstem. There, sensory stimuli from the periphery are processed and compared with the previous stimuli, in order to have a behavioural response.

The work presented in this thesis tries to question, thanks to recent improvements in optical microscopy and live imaging, the concept of afferent innervations "sent" to the hindbrain by sensory neurons. Observation of peripheral neuronal central afferents suggest that the first ones are actually "left" at the entry point of the neural tube by membrane contacts established before growth of any axonal projections (see Results). These observations have been done on three peripheral neuronal populations, two sensory organs arising in the posterior placodal area, stato-acoustic and lateral line ganglia, and one from the intermediate trigeminal placodal zone. Hence, the question of innervation of central brain targets by peripheral sensory systems should (in my opinion) be treated as an actual central problem with few, if any, needs of peripheral neurons struggling in the search for a safe path through the mesenchyme. For this reason I would like to give a deeper introduction about sensory central circuits and the formation of sensory maps.

1.5.1 Concepts of neural circuits and topographical representation

In general a sensory stimulus is sent from the primary peripheral neurons to a central nucleus of interneurons, or second order neurons, where first steps of processing are established. Those interneurons send their output to the forebrain where, with its high calculation power due to the vastness of interconnection among processing areas, the stimulus is transformed in its internal representation and is compared with the representation that had just been acquired before. The forebrain reacts to the changes in the internal representations sending back through sensory efferents and motor nerves a response, an order, to the periphery of the organism to be accomplished in the form of a sensory modulation or muscular reaction respectively.

The first sensory stimuli relay and processing areas are found in the brainstem. Individual rhombomers give rise to distinct portion of sensory and motor columns depending on the position of progenitors along the dorsoventral axis, mainly through gradients of morphogenes (FGF, BMP and WNT) coming from the roofplate, floorplate and notochord, generating nuclei of multi-segmental origin and topographic patterns of connectivity. Thus, the formation of second order neurons, whose patterning along the anteriorposterior and dorsoventral axes intersect to determine subcircuits connectivity with functionally related longitudinal neuronal columns and the parallel development of the central projections of primary neurons, is essential for the establishment of a functional sensory neural circuit (Fritzsch 2003; Di Bonito et al. 2013).

To note is that in the case of the olfactory system the information is sent directly to relay nuclei present in the higher brain areas while the visual system has its layer of first order interneurons already present at the level of the retina.

1.5.1.1 Somatosensory circuit

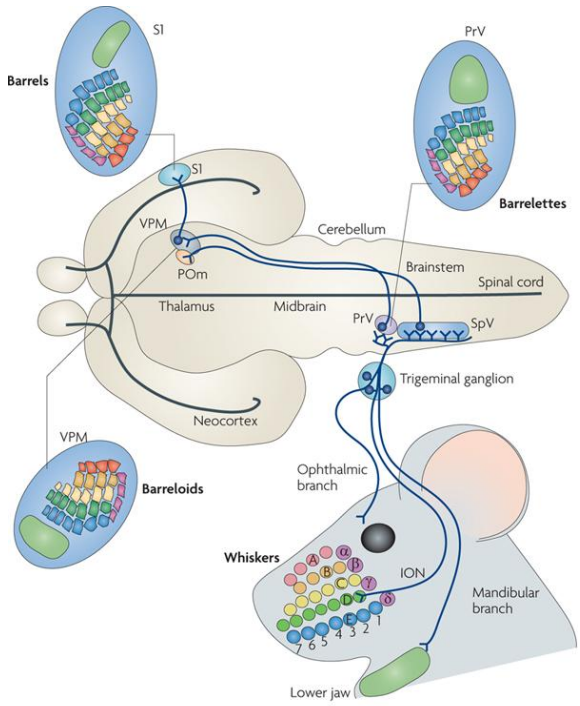


Fig. 16: Trigeminal circuit and face maps in the mouse brain. The ophthalmic (supplying the skin above the eye and forehead), maxillary (supplying the whiskers, upper jaw and lip) and mandibular (supplying the lower jaw and lip) branches of the trigeminal ganglion convey an inverted face map to the brainstem trigeminal nuclei —the rostral principal nucleus (PrV) and the caudal spinal nucleus (SpV) (from Erzurumlu et al. 2010).

The somatosensory system of rodents, through afferent innervation from maxillo/mandibular or facial/ophtalmic dermatomes, targets the rhombomere 2 or 3 derived components of the principal trigeminal sensory nucleus respectively. At the hindbrain entrance point, the afferent projections actually split in two main branches: the ascending contact, the principal trigeminal sensory nucleus (prV), and the descending one, which innervates the caudal spinal nucleus (spV) and contacts the ascending Lissauer's tract from dorsal root ganglion of the spinal cord. The information collected by the barrelets in both the prV and spV is then relayed to the barreloids in the ventroposteromedial (VPM) nucleus of the thalamus, with the spV also projecting to posteromedial nucleus (POm) and from here to the layers of the S1 somatosensory cortex devoted to the representation of the orofacial structures (barrels) in a topographical manner. Sensory inputs are in this way relayed and somatotopically mapped at each level of the pathway as spatially ordered sets of neuronal modules. In this way a facial somatosensory map, which is the central representation of the position of the peripheral sensory receptors, is built and used to allocate from which point of the face a proprioceptive stimulus arise (Erzurumlu et al. 2010).

1.5.1.2 Auditory and vestibular circuit

Topographic connectivity and employment of sensory and motor nuclei are also described during the formation of auditory and vestibular circuits. Vestibular nuclei in the hindbrain also originate from different rhombomeres and display specific sets of axonal trajectories with distinct forebrain targets. The vestibular ganglion afferents projection build a network with those nuclei to relay the information of changes in angular and linear acceleration detected by the hair cells of the cristae that sense fluid motion in the semicircular canals, and the maculae that sense linear acceleration due to gravity (Fekete 2012).

In mice, the specialized hearing organ is the cochlea, which harbours the organ of Corti. Two kind of receptors are found here: the inner hair cells (IHC) are the major detector of auditory stimuli, while the outer hair cells (OHC) enhance low level sounds by increasing the amplitude and frequency selectivity of basilar membrane vibrations (cochlear amplification). From these peripheral auditory organs the sound information travels through primary neurons of the spiral ganglion to the cochlear nucleus (CN) complex in the brainstem (MON in fish) that originate from different portions of the rhombomeres 2 to 5 and is subdivided in anteroventral (AVCN) posteroventral (PVCN) and dorsal (DCN) cochlear nuclei. Processed sound related signal leading to sound perception, travels from the cochlear nucleus through the lateral lemniscus complex to the inferior colliculus (IC) in the midbrain and medial geniculate nucleus (MG) of the thalamus, that sends auditory information to the cortex. Temporal and spatial information of the auditory stimulus travels in a parallel pathway in the ventral brainstem through the superior olivary complex (SOC) that is mostly derived from rhombomere 5 (Di Bonito et al. 2013).

1.5.1.3 Lateral line circuit

The lateral line sensory system found in fish and amphibians is used to sense water motions occurring in the surroundings of the organism through arrays of ciliated organs called neuromasts. They are left in a spatially ordinate manner by the migrating portion of the lateral lines placode, the primordium, along the body of the fish. Up to six lateral lines can be developed by the same organism but the most studied, because better recognizable, are the anterior and posterior lateral line.

At the level of rhombere 4 and 6 the sensory axons, from anterior and posterior lateral line ganglia respectively, enter the brainstem and bifurcate in two branches extending rostrocaudally in the neuropil region ventral to the medial octavolateralis nucleus in the hindbrain (MON), where also nerve bundles from auditory and trigeminal organs project mediolaterally in an exquisite spatially ordered fashion. Lateral line second order connections from MON reach the optic tectum and the torus semicircularis (inferior colliculus in mammals) setting the final innervation with the telencephalon (Piotrowski & Baker 2014).

Before introducing what is known about neural maps formation I would like to stress that visual and olfactory system, and more general, the sensory systems that arise from the anterior placodal area, build their maps connecting directly to the higher processing area of the central nervous system without joining the brainstem. On the other hand, the intermediate and posterior placodal sensory systems connect first to nuclei of the lower rhomboencephalon that then relay the information to the higher brain areas in the diencephalon and telencephalon. This concept creates, in my opinion, a substantial difference on the map formation needs: anterior sensory systems should use laws of the higher brain to contact their targets, while posterior sensory systems would need an ancestral way to contact the lower brain, which then would use the connecting rules of the higher brain to relay information to the areas of third level of processing (as has been shown for auditory ephrins expression, see discussion).

1.5.1.4 Visual circuit

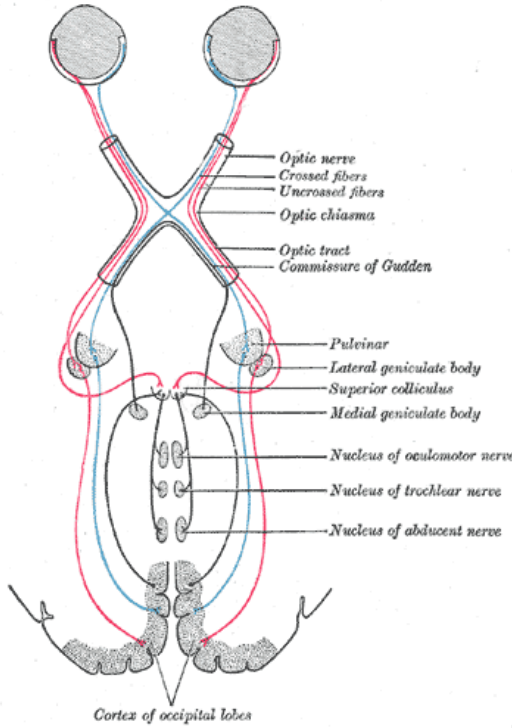


Fig. 17: The visual central system (Schiller 1986).

The information about the image via the eye is transmitted to the brain along the optic nerve. Different populations of ganglion cells in the retina send information to the brain through the optic nerve. About 90% of the axons in the optic nerve go to the lateral geniculate thalamic nucleus. Another population sends information to the superior colliculus in the midbrain, which assists in controlling eye movements. The optic nerves from both eyes meet and cross at the optic chiasm, at the base of the hypothalamus of the brain. At this point the information coming from both eyes is combined and then splits according to the visual field. Information from the right visual field (now on the left side of the brain) travels in the left optic tract. Information from the left visual field travels in the right optic tract. Each optic tract terminates in the lateral geniculate nucleus (LGN) in the thalamus. The LGN consists of six layers in humans. Layers 1, 4, and 6 correspond to information from the contralateral (crossed) fibers of the nasal retina (temporal visual field); layers 2, 3, and 5 correspond to information from the ipsilateral (uncrossed) fibers of the temporal retina (nasal visual field). The neurons of the LGN then relay the visual image to the primary visual cortex (V1), which is located at the back of the brain (caudal end) in the occipital lobe (Schiller 1986).

1.5.1.5 Olfactory circuit

In the olfactory system various odorants are detected by different odorant receptors (OR) expressed by the olfactory sensory neurons (OSN). Each OSN in the olfactory epithelium expresses only one functional OR gene. OSN expressing the same OR converge their axons to a specific pair of glomeruli at stereotyped location in the olfactory bulb (OB). Thus, the odorant information detected by the olfactory epithelium is topographically represented in the pattern of activated glomeruli in the OB. Inside a glomerulus, OSNs axons contact, among others, the dendrites of mitral cells. Mitral cells send their axons to a number of brain areas, including the anterior olfactory nucleus, piriform cortex, the medial amygdala, and the entorhinal cortex. The piriform cortex is probably the area most closely associated with identifying the odor. The medial amygdala is involved in social functions while the entorhinal cortex is associated with memory, e.g. to pair odors with proper memories (Doty 2009).

In the next section I will introduce the way the visual and olfactory maps are formed and the mechanisms they use to connect the right targets, and then the work I have done in collaboration with Pujol-Marti and Hernan Lopez-Schier (Pujol-Marti et al. 2012 in Annex) on the posterior lateral line map formation (a good example of posterior/ancient way of map formation).

1.5.2 Sensory map formation

In general terms, a topographic map is a projection from one set of neurons to another, wherein the receiving set of cells reflects the neighbouring relationship of the projecting set. In the nervous system of higher vertebrates topographic maps are common and include sensory maps of the body, tonotopic maps for auditory stimuli, and maps of the visual field. Furthermore, topographic maps persist in some form throughout the circuitry from first-order to higher-order connections.

From studies on retinotopy and olfactory map formation, two principal classifications for the formation of groups of maps have been proposed, based on the attribute encoded by the map: i) continuous (or topographic) maps, that represents positional information connecting nearby neurons in the input region to nearby neurons in the target region through a point to point map of connection whose coarse initial architecture needs little or no refinements, and ii) discrete maps that, like in the case of the olfactory system, represent the identity of spatially dispersed odorant receptive related neurons whose input converges in the same neuronal cluster in the target region that undergo huge wiring rearrangements. The rest of the sensory maps seem to fall in the grey zone between those two groups.

Keeping this classification as good, for example the tonotopy or the somatotopy of the lateral line, established by the auditory or somatosensory system would be ascribed to a continuous map. However, in my opinion another classification based on evolutionary, comparative and complexity features is needed. This classification should take into account both the cytoarchitecture of the sensory systems and the level of processing complexity of the neuronal population they connect with. This means that to build connections between higher orders of network complexity, where for example integration of different layers of inputs are needed, would require the usage of more sophisticated strategies than the establishment of connections between lower order of processing complexity.

1.5.2.1 Visual map

Vertebrates use graded labels in the retina and its targets to specify synaptic partners: complementary gradients of Eph receptors and ephrin ligands have been observed along the mediolateral axis of the visual system such that areas of high EphA expression project to areas with low ephrinA expression, and viceversa, EphB/ephrinB signalling connect ventral high EphB expressing neurons with medial high ephrinB expressing areas in the tectum. Thus creating a point-to-point inverted map between the retina and superior colliculus (optic tectum in lower vertebrates), namely a molecular gradient dependent retinotopy (Luo & Flanagan 2007; Clandinin & Feldheim 2009; Fig. 18).

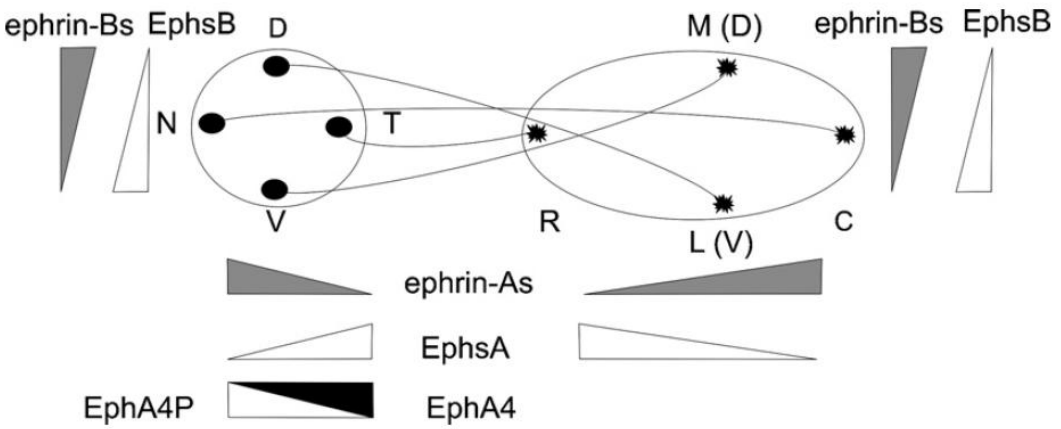


Fig. 18: Schematic representation of retinotectal/collicular projections and the expression patterns of EphAs and ephrin-As along its rostrocaudal axis and the EphBs and ephrin-Bs along its dorsoventral axis. Retinal ganglion cells (RGCs) project to contralateral tectum in chicks whereas they project bilaterally to colliculus in mice. Nasal (N) RGCs axons project to caudal (C) tectum/colliculus whereas temporal (T) RGCs axons project to rostral (R) tectum/colliculus. Dorsal (D) RGCs axons project to lateral (L)–ventral (V) tectum/colliculus meanwhile ventral (V) RGCs axons project to medial (M)–dorsal (D) tectum/colliculus. Ephs and ephrins are expressed in gradients both in the retina and the tectum/colliculus (from Scicolone et al. 2009).

However, the description of the retinotopy map formation of Drosophila (Huang & Kunes 1996) and crustacean's retina (Flaster and Macagno 1984) are different. Receptive neurons in the ommatidias project in a time of differentiation ordered fashion to neurons in the lamina where the anterior to posterior retinotopy is conserved by the order of axons arrival. Dorsal to ventral retinotopy map maintenance instead seems to be based on molecular cues such as Wnt4. The lamina cells, then, send their projections to the following order of processing in the medulla. Interestingly medullas R7 and R8 position of projections seem to be dependent on axonal interactions and repulsion and in extrinsic positioning factor and the mechanisms used to set up this insect map are formally similar to strategies used by vertebrates (Ashley & Katz 1994).

The differences between invertebrates and vertebrates retinotopy and the contradictions between Katz and Macagno and Kunes, are actually only apparent, as Cajal already showed in 1915 (Sanchez and Cajal 1915), since they consider different levels of maps formation and signal processing. Taking the retina cytoarchitecture without changing the order of processing, Cajal moved ommatidia cell bodies to the vertebrate bipolar layer and Drosophila lamina neurons to the retina ganglion cell layer (Panel B in Figure 19). In this way the Drosophila cytorachitecture (Panel A in Figure 19) resembles exactly the vertebrate one (Panel C in Figure 19), even in respect to the order of processing: vertebrate bipolar and Drosophila ommatidia are first order neurons while Drosophila lamina neurons and vertebrate retina ganglion cells are second order.

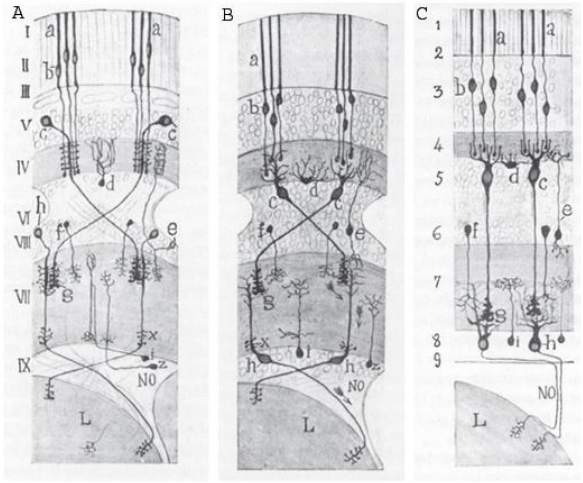


Fig. 19: Similarities in the structure of the fly and vertebrates visual system: these are reproduction of three panels (81,82 and 83 from Cajal and Sanchez, 1915) showing the morphology of individual cell with the fly and vertebrate system (A and C, respectevely). The left panel shows the fly visual system. Note photoreceptors (b) making connections with lamina neurons (c) in the lamina. Lamina neurons in turn make connections with Tm neurons (h) in the medulla. The right panel shows the vertebrate retina. The photoreceptor neurons connect to the bipolar neurons (c) and the bipolar neurons, in turn, make connections with the dendrites of retinal ganglion cells (h). Cajal speculated that the neural pathways for processing visual information, at least near the periphery, were surprisingly similar between the fly and vertebrate. Indeed, as shown in the middle panel Cajal simply move the cell body positions of neurons in the fly to be similar to their corresponding positions in the vertebrate (modified from Sanes & Zipursky 2010).

Thus, to consider retinal ganglion cells as first order neurons as has been done in recent years when speaking about the comparison between time of differentiation based maps of Drosophila eye and molecular gradient map in vertebrate retina is misleading because it compares maps formation of two different order of processing: first to second order of processing in Drosophila retina and second to third order of processing in the vertebrate studies.

1.5.2.2 Olfactory map

Recent works by Sakano and colleagues showed that the olfactory map might be first laid down through a combination of retinal system molecular gradient, somatosensory system temporal gradient and local adhesive and repulsive axonal interactions. Subsequently refinement to a precise discrete glomeruli map would be an activity dependent process.

Dorsoventral position of the projections in the olfactory bulb is regulated by the sequential dorsomedial to ventrolateral differentiation of olfactory sensory neurons in the olfactory epithelium, thus the first OSN differentiate in a dorsomedial position in the olfactory epithelium and project their axon toward the dorsal part of the olfactory bulb (Fig. 20). At early embryonic stages the ventral domain of the olfactory bulb expresses Slit1 axon repelling molecule while olfactory neurons express the receptor Robo2, so they can navigate towards the dorsal olfactory bulb, avoiding ventral non-bulb regions. As development proceedes, the olfactory bulb expands ventrally and as a consequence also does the slit1 signal domain of influence. In this way, later ventrolateral forming OSN are able to navigate more ventrally. At the same time early differentiating OSN express the axon-repelling molecule Sema3F, and do this at a higher concentration than late differentiating OSN. The expression of semaphoring receptor Nrp2 is complementary higher expressed by late ventrolateral differentiating neurons. In this way, Sema3f secreted by the dorsal projecting neurons prevents late arriving Nrp2 axons from invading the dorsal region of the OB (Fig. 21).

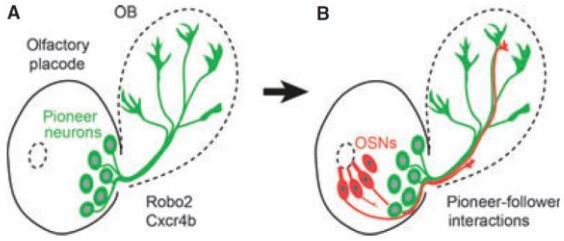


Fig. 20: Pioneer–follower interaction in the olfactory axon guidance. (A) In the zebrafish olfactory system, axonal projection is initiated by pioneer neurons (green), which are unipolar in shape and histochemically distinct from olfactory sensory neurons (OSNs). (B) Bipolar OSNs (orange) extend axons along the trajectories of pioneer axons. Laser ablation of pioneer neurons results in mistargeting of following OSN axons, suggesting that pioneer axons serve as cues or scaffolding for following OSN axons. Robo2 and Cxcr4b are required for pioneer axon projection. OB, olfactory bulb (from Imai & Sakano 2011).

Anteroposterior positioning of projections is instead due to odorant receptor specific agonist independent baseline activity on the expression of cAMP levels and in turn of Nrp1 and Plexin1a expression within OSN. Following this model the OSN that have an OR that induce a high baseline of cAMP, in the absence of their odour ligand, express

more Nrp1 and thus would be sent to the posterior region of the bulb repelled by the high concentration of Sema3f present in the dorsal region. The low level cAMP OSN instead expresses high levels of PlexinA1 and low levels of Nrp1 and would then project to the anterior region (Fig. 21). By this strategy, an initial coarse dorsoventral and anteriorposterior map would be created based on both spatiotemporal gradient of differentiation (like the anteroposterior axis of Drosophila retinal system map) and molecular uniqueness of odorant receptor expression.

However, the fact that both Nrp1 and Nrp2 bind to PlexinA1, that Sema3F functions only with Nrp1/PlexinA1 complexes, while Nrp2/Plexin1A function needs Sema4 and that this is expressed as well in the OE between maybe others (Ronnet 2000), suggest that the mechanism is a bit more complex.

At later stages of development, an activity dependent refinement segregation of OSN projection into specific glomeruli is achieved by two methods: i) Eph/ephrin levels of expression, to repel axons that are expressing a different OR, ii) expression of kirrel homophilic adhesive molecules to group to the same glomeruli axons that come from OSN expressing the same OR. Thus, neurons carring the same receptor, following activation by their odour ligand will recognize each other through expression of molecules of the kirrel family and, at the same time, will repel the neurons that do not carry the same OR through Eph/ephrin signalling (see summary in Fig. 21; Takeuchi et al. 2010; Imai & Sakano 2011; Imai et al. 2010; Takeuchi & Sakano 2014).

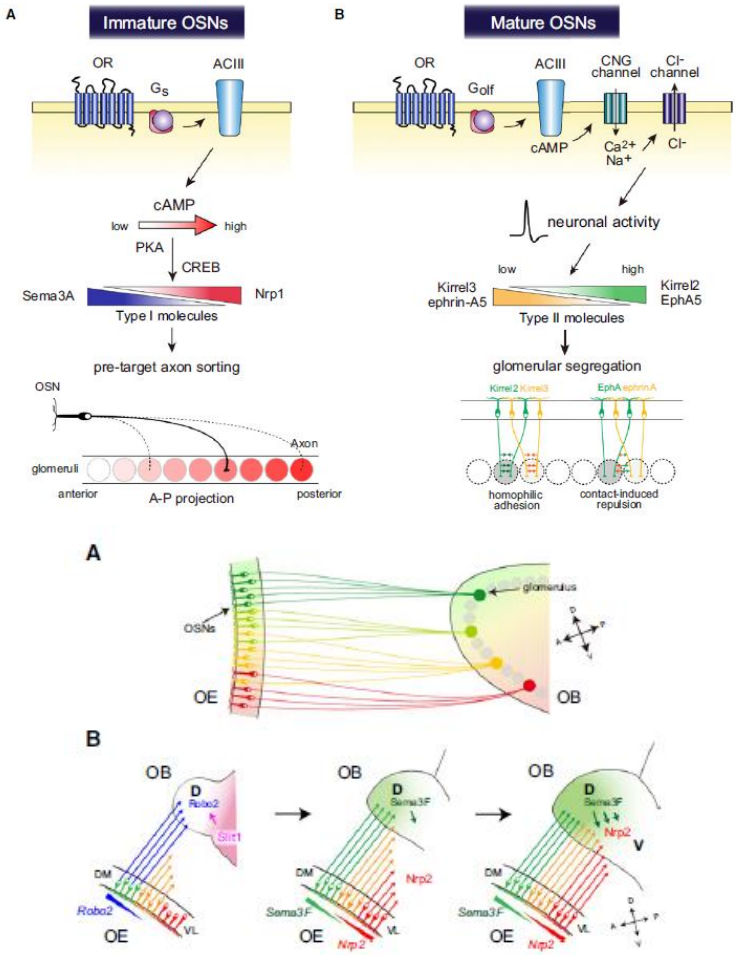


Fig. 21: Panel 1: In the mouse olfactory system, axon-sorting molecules can be categorized into two different types, type I and type II. Type I is expressed at axon termini of OSNs in a graded manner along the AP axis in the OB and regulate AP positioning of glomeruli. In contrast, type II is expressed at axon termini of OSNs, showing a mosaic pattern in the OB, and is involved in glomerular segregation. Expression of both, type I and type II genes, is regulated by OR-derived cAMP signals. (A) Type I molecules expressed in immature OSNs. Each OR generates a unique level of cAMP with the aid of G proteins and ACIII. The level of cAMP signals is converted to a relative expression level of type I molecules, e.g., Nrp1 and Sema3A, via cAMP-dependent PKA and CREB. (B) Type II molecules expressed in mature OSNs. Different ORs generate different neuronal activities through the CNG channel, which determine the expression levels of axon sorting molecules for glomerular segregation. Panel 2: (A) The DV arrangement of glomeruli in the OB is correlated with the expression areas of corresponding ORs along the DM-VL axis in the OE. (B) A model for the DV projection of OSN axons. In the OE, D-zone OSNs mature earlier and reach the OB earlier than V-zone OSNs. D-zone OSNs express Robo2 and project their axons to the prospective dorsal domain of the embryonic OB (left). The Robo2 ligand, Slit1, is expressed in the septum and ventral OB during early development. Repulsive interactions between Robo2 and Slit1 are probably needed to restrict early OSN projection to the embryonic OB. In the OE, the Nrp2 and Sema3F genes are expressed in a complementary and graded manner. Sema3F is deposited at the anterodorsal region of the OB by early-arriving D-zone axons (middle). Axonal extension of OSNs occurs sequentially along the DM-VL axis of the OE as the OB grows ventrally during development. This may help to maintain the topographic order during the process of axonal projection. Sema3F secreted by the D-zone axons in the OB prevents the late-arriving Nrp2+ axons from invading the dorsal region of the OB (right). DM, dorsomedial; VL, ventrolateral; D, dorsal; V, ventral; A, anterior; P, posterior (from Takeuchi & Sakano 2014).

1.5.2.3 Posterior Lateral Line map

In the lateral line system, in collaboration with J Pujol-Marti (see article in the Annex section), we have shown that two populations of neurons arise consecutively in the posterior lateral line ganglion over time: one population that innervates the Mauthner cell and the second population non-innervating it, thus representing distinct modality and/or functions. In the first population, neurons that differentiate first send the projection together with the migrating primordium innervating the most caudally laid neuromast, and project more dorso-laterally in the hindbrain. The last neuron of this population, that differentiates too late to send the axon together with the primordia, innervates the first neuromast and projects more ventromedially. Even if no such precise collection of data is available for the second population, we know that they differentiate after the population that contact the Mauthner cell and project in an even more lateral position (Fig. 22; Pujol-Marti et al. 2012 in Annex).

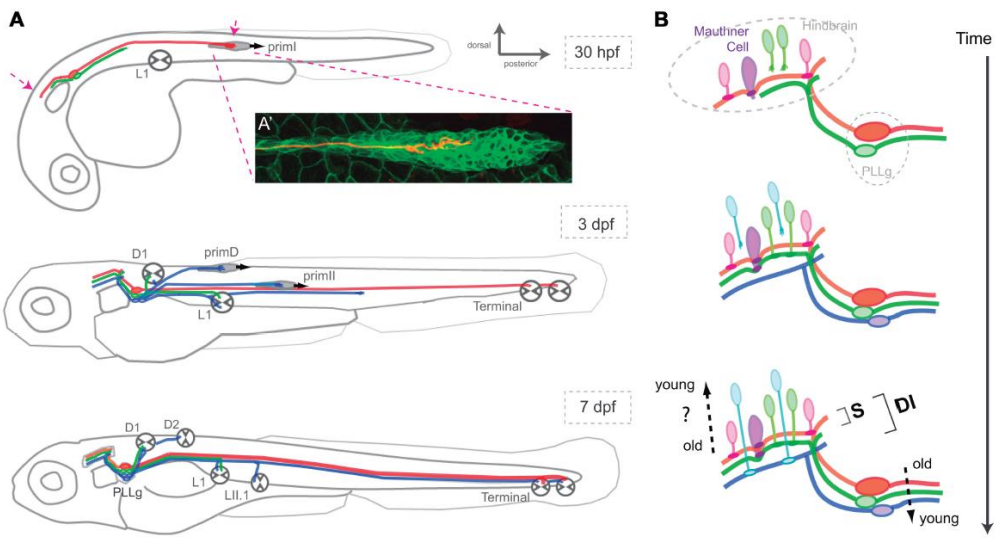


Fig. 22: Assembly of the posterior lateral line neural map. (A) Lateral view of a developing zebrafish showing the coincident progressive development of the lateralis afferents and their peripheral targets. Arrows at 30 hpf indicate growing lateralis afferent's central and peripheral axons. PLLg: posterior lateralis ganglion. (A') A single lateralis afferent neuron is labeled in red in a transgenic zebrafish embryo expressing GFP in primI. The peripheral axonal growth cone can be observed within the migrating primordium. (B) Lateral view of the developing hindbrain and lateralis afferent's central axons depicting an hypothetical temporal code that matches lateralis afferents with second-order neurons that are born at similar times. Both in (A) and (B), red and green lateralis afferents belong to the first neuronal subclass, which projects dorsal axons that contact the Mauthner cell. Red and green neurons innervate posterior and anterior primary neuromasts, respectively. Blue lateralis afferents belong to the second neuronal subclass which projects ventrolateral axons that do not contact the Mauthner cell. The two neuronal subclasses form a dimorphic neural map (DI) whereas only the neurons of the first subclass shape the somatotopic map (S). Neurons in red are the first-born neurons whereas neurons in blue are the latest-born neurons. primI, first primordium; primD, dorsal primordium; primII, second primordium. L1. Terminal and D1 neuromasts are primary and parallel (>). LII.1 and D2 neuromasts are secondary and perpendicular (⋀).

AIMS

Several studies have shown that: i) anterior and posterior lateral line ganglia project somatotopically from a medioventral to a dorsolateral position (Ghysen & Dambly-Chaudière 2004), ii) this is due to the time of innervations (Kimmel et al. 1990), and iii) the same time dependent topographical innervations of a common brainstem target, the Mauthner cell, is maintained for other posterior sensory nerve bundles.

With Pujol-Martí and Hernan López-Schier (Pujol-Marti et al. 2012 in Annex) we have shown that the position of posterior lateral line (PLL) central afferents correlates with timing of differentiation of neurons and their position within the ganglion. Moreover, the order of differentiation creates distinct kind of networks (i.e.: neurons connecting or no to the Mauthner's cell escape response network), due to the different connections established at different time of development or to different neuronal populations arising in a same ganglion during development.

Thus, the main questions of my interest are the following:

1. To understand how first order neurons are able to relay a physical stimulus to higher brain processing centers in form of an electric impulse without losing information such as modality, position, intensity and timing of the stimulus. In other words, how they build up a somatotopy, a retinotopy, a tonotopy or an olfactory map? How neural maps are established?

2. To unveil how the primary neuronal axons from the periphery of the organism know where to enter the brainstem to contact with the nuclei of second order neurons.

3. To seek how primary sensory neurons are able to establish a functional sensory circuit in the first beginning.

As we have seen in the previous chapter the way the first question is approached by the developmental biologists is based on two well studied mechanisms used to create neural maps: the retinal map and the olfactory maps, both developing from the anterior placodal area. In my opinion, the establishment of these maps is quite different in respect to the intermediate and posterior placodal sensory organ maps.

For the second question, during the last years several hypothesis have aroused based either on the knowledge of axonal guidance in the CNS, or in the information from the primary sensory neurons to their peripheral targets, which actually share similar, if not the same, strategies used by visual and olfactory maps formation. Nevertheless, the results we obtained studying central afferents projection and NCC involvement in posterior sensory system central projections propose another explanation on the specific topic of afferent axonal projections formation (see Results and Discussion).

To answer the third more conceptual question, I think it is fundamental to compare how the first two problems are solved in distinct sensory systems and different organisms. A comparison among these different modalities is needed to find similarities and differences along the development and evolution of the sensory systems that may clarify what happens.

To tackle these questions my aims were the following:

- First, to study the correlation between the medioventral to laterodorsal topography of sensory afferents projection at the level of the hindbrain and the time of differentiation of the sensory systems through high-resolution confocal imaging and 3D reconstruction.

- Second, to unveil the mechanism through which firsts differentiating sensory neurons reach their entry point into the brainstem, by through the usage of time-lapse SPIM imaging.

- Third, to decipher the contribution of the NCC in sensory ganglia formation and maintainance, by loss-of-fucntion experiments using pharmacological agents.

- Fourth, to dissect the role of Robo/Slit signaling axon guidance cues in the formation of the network that sensory neurons create at the central level. For this, we used gene expression studies and loss-of-function experiments with morpholino oligomers.

RESULTS

Cooperation between pioneer axonal contacts and neural crest cells is crucial in the establishment of cranial sensory neuron afferent central connections

Andrea Zecca, Sylvia Dyballa, Roger Bradley* and Cristina Pujades[+]
Department of Experimental and Health Sciences, Universitat Pompeu Fabra
Parc de Recerca Biomèdica de Barcelona, PRBB, 08003 Barcelona, Spain
*Department of Cell Biology and Neuroscience, Montana State University, Bozeman, US

[+] Corresponding author: cristina.pujades@upf.edu

Corresponding author:
Cristina Pujades, PhD
Department of Experimental and Health Sciences
Universitat Pompeu Fabra
PRBB, Dr Aiguader 88
08003 Barcelona, Spain
Tel. +34.933160839
cristina.pujades@upf.edu

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Conflicts of interest: The authors declare no conflicts of interests.

SUMMARY 150w

Establishing topographical maps of the external world is an important but still poorly understood feature of the vertebrate sensory system. To study the selective innervation of hindbrain regions by sensory afferents, we mapped the fine-grained topographical representation of sensory projections at the central level: sensory ganglia located anteriorly project more medially than do ganglia located posteriorly, and this relates to the time of sensory ganglia differentiation. By SPIM in vivo imaging we demonstrate that once placodal-derived neurons of dorsal posterior cranial ganglia differentiate, they never loose contact with neural ectoderm. First, delaminated neuroblasts differentiate in close contact with the neural tube, and afferents entrance points are established by plasma membrane interactions between primary differentiated peripheral sensory neurons and neural tube border cells, with the cooperation of neural crest cells. Then, neural crest cells and repulsive slit1/robo2 guidance cues guide later-differentiating axons and mediate sensory ganglion coalescence, axonal branching and fasciculation.

Running title: Cell contact and neural crest control sensory innervation

HIGHLIGHTS

- Topographical organization of cranial sensory afferents is a multistep process.
- Establishing the CNS entry point requires sensory neuron-hindbrain contact and cNCC.
- As sensory neurons migrate, trailing axons are maintained to form pioneer axons.
- cNCC and slit1/robo2 signaling use this initial scaffold to guide later migrating axons.

INTRODUCTION

A fundamental organizational principle in neural development is the ability of the brain to build an internal representation of the external world based on sensory information. This internal depiction of the outside world relies on the establishment of topographic projections, whereby the relative positions of sensory cells in the receptive field are represented in the brain by the relative positions of the corresponding axon terminals. Higher-order processing of sensory information largely relies on the accurate construction of spatially arranged neuronal projections, known as neural maps. This spatial distribution is essential for the accurate transmission of environmental stimuli to processing centers in the brain (for review see Luo and Flanagan, 2007). For instance, in the inner ear, vestibular or acoustic signals acquired by the mechanosensory transducers, the hair cells, are transmitted to bipolar afferent neurons that project central axons to the corresponding nucleus in the hindbrain. This first mechanosensory relay contains a topographic neural map, in which the afferent central projections are stratified along the dorsoventral (DV) and mediolateral (ML) axes reflecting the spatial distribution of the sensory patches (Sapède and Pujades, 2010).

During development, specialized sensory organs in the vertebrate head, the cranial sensory placodes, originate from thickenings in the embryonic ectoderm. The placodes give rise to two key cell types that underlie the function of sensory systems: the cells that receive the stimuli –such as the hair cells in the inner ear or the lateral line-, and the sensory afferent neurons, which conduct the extracted information to the brainstem (for review see Patthey et al., 2014). Several studies have tried to unveil how peripheral ganglia “send” afferent projections to “reach” their entry points in the hindbrain. The mechanisms proposed include: i) cranial neural crest cells (cNCC) form corridors, which provide a passive mechanism for sensory axons to migrate towards the Central Nervous System (CNS) (Freter et al., 2013) ; and ii) guidance molecules such *robo/slit* signaling, which direct axons towards the hindbrain. Chemorepulsion has previously been shown to play roles in organizing sensory systems, such as maintenance of the spatial restriction of distinct sensory neuron assemblies (Wang et al., 2013), control of axonal arborization (Campbell et al., 2007), formation of placode-derived ganglia (Shiau et al., 2008; Shiau and Bronner-Fraser, 2009), and as key

regulator of specific afferent projection patterns (Pan et al., 2012). However, both views seem to consider differentiated sensory neurons as a population of cells arising far from the neural tube that must then extend their axons through the mesenchyme towards the hindbrain, and do not take into account the extensive morphogenetic changes that occur during the formation of the neural system.

In order to elucidate the mechanism by which external stimuli are relayed to the brain, we have mapped the topographical organization of the sensory axonal projections of the trigeminal, statoacoustic and lateral line ganglia within the hindbrain. We show that there is a fine-grained somatosensory representation map at the central level: the anterior sensory ganglia project more medially than those located more posteriorly, and this recapitulates the time of ganglia differentiation. We unveil the importance of sequential spatiotemporal sensory differentiation in the positioning of the sensory entry points into the hindbrain: placodal-derived neurons of dorsal posterior cranial ganglia differentiate in close apposition with the neural ectoderm and their afferent entrance points are established by membrane interactions between the pioneer sensory neurons and cells at the border of neural tube, prior to afferent sensory axon formation. This first contact of sensory axons with the central system, together with the input from NCCs, is indispensable for establishing the afferent entry points. Once the entry point is established, NCC and guidance cues such as *slit1/robo2* play important roles in organizing the architecture of the sensory system, by maintaining ganglion coalescence and by guiding the later-differentiated sensory neurons, thereby controlling axonal branching and fasciculation of the nerve bundle.

RESULTS

Mapping the neurosensory network

Spatial and temporal differences in axonal projections can provide clues as to the connectivity patterns of the overall neural circuits. Thus, to understand the early differences among sensory cranial ganglia projections we first explored their topographical organization in the hindbrain using double transgenic Tg[hspGFF53A]Tg[UAS:KAEDE] embryos, which express photoconvertible KAEDE^{Green} early in the developing sensory ganglia. Distinct sensory ganglia neurons were photoconverted at 48hpf and the expression of KAEDE^{Red} in the sensory projection towards the hindbrain was assessed (Figure 1A). We found that when the photoconversion was performed in the trigeminal ganglion neurons (TGg), photoconverted axonal projections were located very ventral within the neural tube (Figure 1B-B'). In fact, the KAEDE^{Red} bundle was the most ventrally located when compared with all KAEDE^{Green} projections (Figure 1B'). When the analysis was performed along the mediolateral (ML) axis, TGg projections were positioned completely medial when compared with the rest of sensory projections (Figure 1C). Secondly, we photoconverted the anterior and posterior lateral line ganglia neurons (ALLg/PLLg) and observed that KAEDE^{Red}-ALLg projections were in an intermediate position along the dorsoventral (DV) axis: they are more dorsal to the non-photoconverted axons of the statoacoustic ganglion (SAg) but more ventral to another non-photoconverted sensory bundle (Figure 1D-D'). Similar results were obtained along the ML axis: ALLp is allocated in a middle position (Figure 1E). On the other hand, the PLLg projection is the most dorsal and lateral of the sensory projections (Figure 1F-F',G). These observations support previous studies describing that PLLg neurons projected more dorsally than ALLg neurons, prior to sensory organ innervation (Gompel et al., 2001) ,and are located more dorsal and lateral to the TGg projection.

We next investigated the position of the two different neuronal populations of the SAg (Anterior SAg, A-SAg; Posterior-SAg, P-SAg). The fact that Tg[hspGFF53A]Tg[UAS:KAEDE] embryos exhibit KAEDE mosaicism expression in the sensory ganglia was advantageous to discerning the two very close SAg neuronal populations. Thus, we first photoconverted the A-SAg neurons and observed that this

population projected quite ventral and medial (Figure 2A-A',B). However, it is not the most ventrally positioned, considering the allocation of the TGp (compare with Figure 1B-B'). When we followed the KAEDE^{Red}-P-SAg projections, we observed that they were more dorsal and lateral than the A-SAp (Figure C-C',D). These results suggest that the TGg projection is the most ventral and medially located, while the PLLg projection the most dorsal and lateral positioned. However, to decipher the relative position of the projections of the distinct SAg neuronal populations and the ALLg, we photoconverted two different ganglia at once (Figure 2E-E',F-F'). We observed that the ALLg neurons project more dorsally than the A-SAg neurons (Figure 2E-E'); on the other hand, the PLLg neurons constitute the most dorsal projections while the P-SAg is allocated ventral to them and separated by projections from the ALLp (Figure 2F-F'). A summary of the fine-mapping of the neurosensory network is depicted in Figure 2G, which reveals a highly ordered connectivity map with the dorsal and lateral to ventral and medial organization as follows: PLLg/ALLg/P-SAg/A-SAg/TGg. This brought us to the next questions: how is the entrance point into the hindbrain chosen and how is this topography maintained?

Ordered differentiation of sensory neurons prefigures the entry point at the central level

Previous works studying how early axonal contacts from cranial sensory systems were established during embryonic development observed that inputs from the separate cranial systems arrive sequentially in the order trigeminal-statoacoustic-lateral line (Kimmel et al., 1990). Our next thought was that this sequence of arrival could determine the entrance points in the hindbrain. With this in mind, we first investigated how pioneer sensory axons navigate to the entrance point by in vivo imaging studies using the SPIM system (for review see Weber and Huisken, 2011). Embryos expressing GFP in differentiating sensory neurons were injected with lyn-TdTomato mRNA to label the plasma membranes (in order to see cell-cell interactions) and live-imaged for several hours (Figure 3, Movies S1-S3). As expected, the timing of neuronal differentiation is sequential: first the TGg differentiates and then the LLg/SAg (Movies S1-S3); but most interestingly, the first neurons of each ganglion to differentiate do so in close contact with the hindbrain cells and at the same anteroposterior (AP) level

where the entrance point will be (see white arrowheads in Figure 3A, D,G). Specifically, the plasma membrane of the sensory neurons establishes close interactions with the plasma membranes of the neural tube border cells at the level of the future nerve entry point (see inserts in Figure 3A,D,G), and this happens prior to formation of afferent sensory axonal processes. Primary neurons maintain these established contacts with the neural tube, even when they are pushed away due to morphogenetic growth (Figure 3B,E,H), so that although the sensory neuron is pushed towards the periphery it leaves a trailing axon and later differentiating neurons reach the same entrance point (see white arrows in Figure 3C,F,I). For a better understanding, movies showing this cell behaviour are included (Movie S1 for TGg, Movie S2 for SAg, and Movie S3 for PLLg). We mapped the position of the entrance points along the AP axis by analysing transgenic embryos that expressed mCherry in rhombomeres 3 and 5. We allocated the entry points in r2 for the TGg, in r4 for ALLg and SAg, and in r6 for the PLLg (Figure S4), as suggested by the in vivo imaging experiments.

To investigate whether the original plasma membrane contact was sufficient for the establishment of the entry points, we carried out ablation experiments where the pioneer axon was ablated using multiphoton microscopy. Ablation of the first sensory axons did not result in alterations in the entry point (Figure 3J-M), suggesting that either they are not necessary or that our ablation system did not completely remove the axon. However, several defects in nerve bundle elongation were observed: otic axons enter the central system (Figure 3J,L), but once there they cannot elongate the nerve bundle projection leaving an empty space between the TGp and the PLLp (n=4/9; see asterisk in Figure 3K,M). These results indicate that while differentiating sensory neurons directly contact neural tube border cells at the site of the future entry point and this contact is maintained by pioneer axons as the ganglia move away, the pioneer axon contact alone is not sufficient for establishing the entry points.

Pioneer axons and neural crest cells cooperate in the establishment of the entry points

cNCC are specified in the dorsal part of the neural tube, undergo epithelium-mesenchymal transition and begin migration around 14-15hpf, segregating into three distinct streams lateral to rhombomeres 2, 4 and 6. Our aim was to explore whether cNCC were involved in instructing the sensory neurons to find the position of the central entry point. In order to do this, we first assessed the spatial relationship between cNCC and placodal derived-neurons, by in situ hybridization experiments with *crestin,* which labels early NCC derivatives, in Tg[Isl3:GFP] embryos, which express GFP in the sensory ganglia (Figure 4A-F). NCCs were observed primarily on the exterior surface of the aggregated ganglia, with *crestin*-positive cells usually adjacent to (or surrounding the) GFP-neurons in all sensory ganglia (Figure 4A-F). In order to understand the relationship between NCC and the centrally projecting axons, *crestin*-stained embryos were imaged by confocal microscopy, collecting complete *z*-stacks of images through the region of interest. The *z*-stack images were examined as individual slices or observed as Maximal Intensity Projections (MIP). No overlap between *crestin* and GFP was observed in single *z*-stacks (data not shown), and only some overlap was obtained when MIPs were analyzed (Figure 4G), indicating that cNCC cells envelop sensory ganglia in order to maintain coalescence as previously reported (Freter et al., 2013; Sandell et al., 2014) and suggesting that cNCC may help instruct sensory axons to reach the hindbrain. To determine whether cNCC play a role in defining/instructing sensory neurons to reach the right entry point at the central level, we blocked the migration of NCC precursors by using Leflunomide, an inhibitor of neural crest cell development (White et al., 2011). Embryos treated with Leflunomide do not display *crestin*-expression (Figure 4H-I), and when observed at 28hpf they exhibit defects in ganglia coalescence as expected, although no effect on the entry point position was observed (Figure 4H; n=0/4). When Leflunomide-treated embryos were analyzed at 32hpf, a lack of coalescence in the three ganglia was clearly observed (TGg, SAg, PLLg), as well as defects in axonal navigation and defasciculation (Figure 4H-I; n=4/4), when compared with control embryos (Figure 4G). However, neither the differentiation of the ganglia nor the position of the entry points was altered (Figure 4I). These results support the hypothesis that cNCC envelop the sensory ganglia in order to maintain

coalescence and may support and guide their late-differentiating axons as previously suggested (Freter et al., 2013; Sandell et al., 2014), although they are not sufficient in defining the central entrance points of the sensory axons or in guiding them there. Given that cNCC play a role in ganglia coalescence and axonal navigation, we next asked whether NCCs cooperated with the pioneer axon contact in the establishment of the afferent entry point. In fact, when the pioneer axon for ALLg/SAg is ablated in the absence of NCCs migration we did observe ectopic entry points into the hindbrain (Figure 4J-J'; n=3/6). This phenotype is accompanied by nerve bundle elongation defects (Figure 4K; n=6/6), as observed when the pioneer axon was ablated, and somatotopy problems since ALLg/SAg axons innervate the PLLp at central levels (Figure 4L-L'', n=5/6). Thus, these results indicate that pioneer axons are indispensable for the establishment of the coarse map of central projections, and in combination with NCC are required for the establishment of the entry point.

Robo2 and *slit1a/b* genes are expressed in SAg sensory neurons and afferent target field

Our next aim was to understand how the later-developing axons reach the proper entry site. To investigate whether *slit/robo* signaling guides later-differentiating sensory axons to the central entrance points, we studied the expression of *slit* and *robo* molecules in the hindbrain and in the cranial sensory placodes, focusing in the otic vesicle. The expression profile analysis of *slits* (the secreted ligands for *robos*) showed that in the hindbrain all four zebrafish *slits* were expressed (Figure 5A-F and data not shown; Pan et al., 2010); however, only *slit1a* and *slit1b* were expressed adjacent to the sensory afferent bundle (Figure 5A-F). Both *slit1s* are expressed in similar regions, although *slit1b* is expressed in a more restricted domain than *slit1a* (compare Figure 5A and D). Although the expression of *slits* is quite diffuse in the hindbrain, there are regions devoid of *slit*-expression (see white arrowheads in Figure 5A). Neither *slit1a* nor *slit1b* overlap with sensory axonal projections (Figure 5B-C,E-F). These results reveal that *slit1a* and *slit1b* are enriched in the vicinity of the sensory neuron afferents in the hindbrain, suggestive of a role in entry point selection and/or axon guidance.

We then analyzed the expression of the axon guidance receptors *robo* and found that only *robo2* and *robo3* members are expressed in the sensory neurons (Figure 5G-L, and data not shown). We found that *robo2* was dynamically expressed in otic sensory neurons, and became restricted to the Isl3:GFP-positive population (Figure 5G-G', see white arrows), although cells within the SAg not yet differentiated display *robo2* as well (Figure 5H'-I', see red arrows). On the other hand, *robo3* has a complementary expression profile to *robo2* within the SAg: it is mainly present in the non-differentiated SAg neuroblasts (Figure 5J-L', see red arrows), and in very few or no Isl3-positive differentiated neurons (Figure 5J-L', see red arrow). To better characterize the expression of *robo2/3* receptors in the SAg neuronal populations, we did a more thorough analysis using different neuronal markers (Figure 6). We took advantage of *neuroD*, which labels otic placode-delaminated neuroblasts but is downregulated in differentiated neurons (Figure 6A-A'), and the Tg[neuroD:GFP] fish line, where *neuroD*-positive cells display neuroD:GFP, although not all GFP-positive cells express *neuroD* due to a difference in the stability of the *neuroD* mRNA and GFP protein (see white arrow in Figure 6B'). The fact that *neuroD* expression and GFP do not fully overlap in differentiated neuroblasts provides a useful marker for the latest-differentiated neurons (*neuroD*-negative, neuroD:GFP-positive). *robo2* is expressed in a subpopulation of neuroD:GFP cells (see red arrow in Figure 6C-C'), supporting our previous observation that the *robo*2 receptor is present in differentiated and non-differentiated SAg populations. Triple staining for *robo2/neuroD* and Isl3:GFP shows three neuronal populations: one expressing Isl3:GPF/*robo2* (see white arrow in Figure 6E-E'), another that expresses *robo2/neuroD* (Figure 6E', see red arrow), and a third one which displays *neuroD* (Figure 6E-E'). *robo3* is expressed in most of the neuroD:GFP population, with few GFP-cells without *robo3* expression (Figure 6D-D', see red and white arrows respectively). A previous study illustrated the various stages of SAg development, which involves a sequential process of specification, delamination, proliferative expansion and differentiation of precursor cells to form the mature SAg (Vemaraju et al., 2012). When we combined the analysis of the *robo2/3* and *neuroD*/Isl3:GFP expression territories with SAg markers such *snail, cadh6* (data not shown) or *cadh10*, which label subpopulations of otic neurons, we could ascribe *robo2* and *robo3* expression to different SAg neurons according to their differentiation state

(Figure 6). Thus, we find: i) neuroblasts that just delaminated from the otic epithelium (*snail/neuroD*); ii) transit amplifying neuronal population (*snail/cadh6/cadh10/neuroD/robo3*), iii) neurons ready to undergo differentiation (*neuroD/robo2/robo3*), and iv) differentiated neurons (Isl3:GFP/neuroD:GFP/*robo2*).
In summary, *robo2/slit1* genes are expressed in SAg neurons and afferent target fields consistent with a role in guiding the later differentiated sensory neurons to the proper target site.

Slit1/robo2 signaling regulates the number of sensory branches and coalescence of the sensory bundle

In order to assess the effects of downregulation of *slit1/robo2* signaling we used translation-blocking morpholinos against the ligands (*slit1a/slit1b*, Barresi et al., 2005; Kastenhuber et al., 2009) and the receptor (*robo2*, Zhang et al., 2012). Tg[Isl3:GFP] embryos were injected at 1-2cell/stage and the effects were analyzed at 48hpf. We focused on three main phenotypes: the number/position of SAg nerve entry points at central levels, branching of the sensory nerves towards the hindbrain and the coalescence of the sensory nerves bundles along the AP path in the hindbrain (Figure 7). Interestingly, no effects were observed at 24hpf (data not shown), suggesting that *slit1/robo2* pathway plays a role only in late differentiated neurons. At 48hpf, morphants displayed ectopic entry points (Figure 7B), ectopic branches (Figure 7C) and defects in fasciculation (Figure 7D); in some cases, a mix of phenotypes was obtained (Figure 7E-F). In order to allow statistical treatment of phenotypes occurrence, score 0 or 1 was given respectively to the absence or presence of each phenotype for each injected embryo (Figure 7G). Negligible effects were observed when control morpholino was injected (Figure 7A, see MO-p53 in Figure 7G). Upon downregulation of slit1a, over 57% of embryos displayed ectopic entry points and half of the embryos showed defasciculation of the sensory nerves bundles compared with control embryos (Figure 7G). Only n=4/14 embryos displayed ectopic branches towards the hindbrain, and when compared with control embryos (n=4/26), this result was not statistically significant. No effects were observed in any of the analyzed phenotypes upon downregulation of slit1b by itself (Figure 7G). However, when the function of both ligands was inhibited (MO-slit1a/b), over 44% of embryos had ectopic entry points

(n=6/15), 69% displayed ectopic branches (n=11/15) and 75% had problems in nerve bundle fasciculation (n=12/15). Overall, these results suggest that slit1 repulsion signals are involved in keeping the coalescence of the sensory bundle, in accordance with the expression of *slit1a/b* around the sensory projection running along the AP of the hindbrain (Figure 5). To verify that slit1a function was executed through robo2 receptor, we knocked-down robo2 in the sensory neurons and analyzed the embryos for similar phenotypes as seen with the slit1a/b morphants. We observed a consistent increase in the number of embryos presenting ectopic entry points (n=18/52), 35% of the embryos had ectopic branches (n=29/52), and 75% displayed ML defasciculation of the sensory nerves bundle along the AP path through the hindbrain (n=39/52). No problems in the primary entry point were observed in any of the analyzed embryos (Figure 7A-F). These results support our hypothesis that slit1/robo2 pathway does not play a role in defining the central entrance points of the sensory axons, however it is important in guiding the late differentiated neurons to the proper site. In addition, slit1/robo2 signaling plays a crucial role in keeping the fasciculation of the bundle, most probably due to the repulsion cues sent by slit1s to sensory axons to avoid their expansion through ML axis. Interestingly, the observed phenotype of ectopic branches upon MO-slit1a/b might involve another receptor, since the effects of MO-robo2 are quite mild (MO-slit1a/b 69%, MO-robo2 37%).

In summary, we show that while *robo2/slit1* axon repulsion signaling is apparently not involved in establishing the pioneer axons, it does play an important role for later-differentiating sensory neurons to: i) reach the proper entry point established by the pioneer axons; ii) maintain fasciculation of nerve bundles to avoid bundle expansion, and iii) restrain sensory central afferents at the border of the neural tube to avoid branching into the hindbrain in incorrect places and moments.

DISCUSSION

We have used a combination of genetics, in vivo imaging and functional studies in zebrafish to investigate the establishment of cranial sensory neuron afferent central connections. Sensory neurons establish close interactions through plasma membranes with neural tube border cells and the contact between the pioneer sensory axon and the neural tube cells is maintained through cell-cell interactions. We think that the ordered differentiation of sensory neurons may prefigure the entry point at the central level**.** We show that the first contact of sensory axons with the central system relies on the differentiation site of pioneer sensory axons and requires the collaboration of NCCs. This is likely independent of guidance molecules, which are important in guiding late-differentiating neurons to the proper sites and together with NCCs to keep the overall architecture of the sensory system.

Our photoconversion studies demonstrated that the fine-mapping of the neurosensory network displays a highly ordered connectivity map at the central level: sensory ganglia located more anteriorly project more medially than the ones located more posteriorly, and this relates to the time of cranial sensory ganglia differentiation. This somatotopic arrangement is laid out very early. Studies in the PLLg suggested that somatotopy in this system is achieved in the absence of sensory input (Gompel et al., 2001) and that the order of afferents differentiation establishes the sequence of central projections (Pujol-Martí et al., 2012). This is consistent with our observations, trigeminal neurons differentiate earlier than posterior lateral line neurons, and therefore their projections are allocated more ventral and medial. However, we cannot rule out that genes expressed or active in the DV gradient in the hindbrain could act as molecular landmarks of somatotopy, and help to determine the molecular identity of the projecting sensory neurons as they do in other systems (Fariñas et al., 2001; Schuster et al., 2010; Wang et al., 2013). Indeed, Eph receptor tyrosine kinases and their ligands are proteins that regulate axon guidance and are known to contribute to the establishment of topographic projections in several areas of the nervous system. Eph proteins are extensively expressed in structures of the inner ear as well as in neurons in the peripheral and central components of the auditory system.

Furthermore, functional experiments demonstrated a role for Eph signaling in the formation of auditory system connections between the hindbrain and the diencephalon, and in the innervation of the SAg and the hair cells of the sensory patches; however, they do not instruct how sensory neurons find their target region in the hindbrain (for review see Cramer, 2005). We have observed that the sensory bundle is located within the *pax7a/pax6/pax3*-positive domain (Figure S6), which would suggest that a transcription factor code could play a role in placing the bundle along the DV axis. However, this could explain only early stages of bundle organization, since *pax* gene expression around the bundle is downregulated from 36hpf onwards (data not shown).

In vivo imaging confirmed that differentiation (and most probably cell-fate commitment) is a key aspect in the topographical organization. In addition, the differentiation date and position defines the location of the entry point of the sensory neurons into the CNS in collaboration with NCCs. First traced neurons differentiate in close apposition to the neural tube cells, establish a very robust membrane contact with the neural tube cells and change their shape. This first axonal contact remains even when challenged by morphogenetic growth, resulting in a trailing axon that likely can be used by late differentiating neurons to reach the same entrance point. This mechanism operates in the TGg, LLg and SAg, suggesting that it is a common strategy for posterior and dorsal cranial placodes, however opening the question about other placodes. What positions the neuroblasts in this specific Cartesian grid along the AP axis is not known. Embryos with gross defects in the AP patterning of the hindbrain, such as the *vhnf1* mutants (Hernandez, 2004) do not display any defects in the hindbrain position of the entry points of the sensory axons (Sapede and Pujades, unpublished results). Thus, sensory cells may sense positional information from the surrounding tissues, or may have intrinsic cues responsible for this. Although there are no available molecular markers for subsets of sensory neurons at this stage, their capacity to respond to external signals may differ. It would interesting to combine the search for signaling cues and SPIM imaging to trace the very first neuroblasts in order to see their dynamics for becoming differentiated neurons.

Recent experiments demonstrated that cNCC streams transform into corridors that are subsequently associated with migrating sensory neuroblasts in chick and mouse,

bridging the domain between the placodal epithelium and the CNS for neuroblasts to extend their axons (Freter et al., 2013). With this in mind, we addressed whether NCC could be involved in defining the entry points of the sensory ganglia into the CNS. Although there is an intimate relationship between NCC and sensory neurons and NCC envelop the sensory ganglia, in our system NCC are necessary to maintain coalescence of the ganglia, but by themselves are not sufficient to define the entry point since inhibition of NCC migration does not have any effect in the number or position of the pioneer axons. Although this mechanism could be used by epibranchial neurons residing far away from the neural tube, the fact that differentiating neurons of dorsolateral ganglia contact the neural tube borders before even sending their axons makes it less suitable. Importantly, we show here that the first axons do not migrate toward the CNS, rather they differentiate in close contact to it and with the cooperation of cNCC establish the entry point. As the ganglia are pushed away from the neural tube, they leave behind the pioneer axons that later-differentiating axons can use as a scaffold to migrate to the hindbrain. When first axonal contact is ablated and migration of NCCs inhibited, ectopic entry points are generated, strongly supporting an intimate collaboration between these two mechanisms to establish pioneer axons. To our knowledge this represents the first demonstration that the pioneer axon can form from direct sensory neuron contact with the CNS. The question remains as to what information the cNCC provide the sensory neurons as they differentiate? They may be involved in direct cell-cell contact with the first axon, or they may secrete some instructive signals that do not involve slit molecules; however further investigation will be needed to unveil these putative signals.

Global loss-of-function experiments show that *robo2* and *slit1a/b* have late effects in the control of entrance points into the hindbrain, in restraining sensory central afferents at the border of the neural tube, and are inhibitors of defasciculation of the sensory bundles. No effects were observed at 24hpf, in accordance with the observation that the first entrance points are established upon sensory neuron differentiation, with no need for other cues except the right positioning of the neuroblasts and the cooperation of NCCs. Thus, slit1 repulsion signals are probably involved in avoiding expansion of the sensory bundle, with the expression of *slit1a/b* around the sensory projection running along the AP axis of the hindbrain serving to

repulse stray axons and keep them in a compacted fascicle. This would be a mechanism to restrain the range of central neurons that the sensory bundle would contact, and consistent with this hypothesis, upon slit1 downregulation, the nerve bundle broadens. Previous work has nicely shown that the vertebrate hindbrain contains stripes of neurons with shared neurotransmitter phenotypes that extend throughout the hindbrain of young zebrafish, reflecting a structural and functional patterning (Kinkhabwala et al., 2011). Accordingly, the sensory bundle would contact a single neuronal stripe displaying the appropriate transcription factors and therefore with a given molecular identity.

Although slit1a/b controls arborization of sensory branches, our results suggest this effect may also be mediated through a robo2-independent pathway. As robo2 is the only roundabout family member expressed in differentiated SAg neurons, this indicates that slit1 may act through an additional, non-robo, receptor. Consistent with this observation, previous work in the retinotectal system elucidated the effects of slits inhibiting arborization and synaptogenesis in the CNS through a robo2-independent mechanism (Campbell et al., 2007). Previous work in the trigeminal ganglion has shown the effect of slit2/robo2 for promotion of the axonal elongation and branching of the sensory neurons (Yeo et al., 2004), and slit3/robo2 signaling has been proposed to prevent erroneous innervation of these neurons (Pan et al., 2012). However our results suggest that in the inner ear, the synergy between slit1a/b is the main regulator of sensory branch arborization.

Slit/Robo signaling has pleiotropic functions. For instance, in chick and mice perturbation of *slit1* or *robo2* disrupted proper ganglion formation (Shiau et al., 2008; Shiau and Bronner-Fraser, 2009). Interestingly, *slit1* is expressed in NCC in these species, which is not the case in zebrafish (data not shown). Thus, inhibition of NCC migration in zebrafish phenocopies the downregulation of *slit1/robo2* in chick or mice, namely lack of ganglia coalescence. Although slit/robo signaling plays a role in the establishment of the dorsoventral topology of the longitudinal tract of the forebrain (Devine and Key, 2008), it seems that it is not the case in the sensory bundle of the hindbrain. These results support a model in which robo2-dependent slit1a/b activity maintains the normal spread of fascicles, and that slit1a/b signaling controls the arborization of sensory branches.

Overall our data reveal that establishing proper topographical organization of the cranial sensory afferents is a multistep process. First, the entry point in the hindbrain is established by close apposition between sensory neuron and neural tube cell membranes, together with the cooperation of NCCs. Second, sensory neurons are pushed away leaving the pioneer axonal contact as a trailing cue for late differentiating neurons. Third, instructions from cNCC and slit1/robo2 signaling help maintain this topographical organization when challenged by morphogenetic growth. This third step is important for helping axons navigate and for building a more complex system upon the initial scaffold.

EXPERIMENTAL PROCEDURES

Zebrafish strains and maintenance

Zebrafish embryos were obtained by mating of adult fish by standard methods. All fish strains were maintained individually as inbred lines. All procedures used have been approved by the institutional animal care and use ethic committee (PRBB–IACUC), and implemented according to national rules and European regulations. The Tg[Isl3:GFP] (Islet3 also called Isl2b), expresses GFP in the afferent sensory neurons of the ear and lateral line system and in facial and trigeminal ganglia (Pittman et al., 2008). Tg[neuroD:GFP] expresses GFP in the neuronal progenitors (Obholzer et al., 2008). The Tg[hspGFF53A] line that carries the DNA-binding domain of Gal4 fused to two short transcriptional activation motifs of the VP16 designated Gal4FF, was generated by random integration of an enhancer-trap construct (Asakawa and Kawakami, 2008). It expresses the Gal4FF in afferent neurons of the trigeminal, inner ear and the lateral line, with background expression in axial muscle. It was crossed with the Tg[UAS:KAEDE] line for photoconversion experiments (Pujol-Martí et al., 2010), which allows the expression of KAEDE^{Green} in a subpopulation of these sensory neurons. The use of Tg[hspGFF53A]x Tg[UAS:KAEDE] crosses was very useful because due to the time needed for Gal4 to bind to UAS and activate KAEDE transcription, there is a delay in the expression of KAEDE in sensory neurons, resulting in a mosaic expression of KAEDE^{Green} at 48hpf. Mü4127 is an enhancer trap line in which the trap cassette containing a modified version of Gal4 (KalT4) and mCherry (KalTA4-UAS-mCherry cassette) was inserted in the 1.5Kb downstream of *krx20* gene, and therefore labeling r3 and r5 (Distel et al., 2009).

In situ hybridization and immunolabeling

Whole-mount *in situ* hybridization (ISH) was performed as described previously (Hauptmann and Gerster, 1994) with some modifications. For chromogenic ISH, FLUO- and DIG-labeled probes were detected with INT-BCIP and NBT/BCIP substrates respectively. For fluorescent in situ hybridization, embryos were first equilibrated in 0.1 M Tris-HCl pH 8.2, and then DIG-labeled probe was detected with Fast Red (Roche) dissolved in 0.1 M Tris-HCl pH 8.2.

Probes were as follows: *cadh6* and *cadh10* (Liu et al., 2006), *crestin* (Berndt and Halloran, 2006), *neuroD* (also called *neuroD1*, Itoh and Chitnis, 2001), *pax6* (Macdonald et al., 1994), *pax7a* and *pax7b* (Minchin and Hughes, 2008), *slit1a* and *slit1b* (Hutson et al., 2003), *snail2* (Thisse et al., 1995), *robo2* and *robo3* (Lee et al., 2001). *pax3a* probe was obtained by PCR-amplification of cDNA retrotranscribed from total RNA, with the following primers: forward primer: 5'-CCA AAC CGC TTT GAG ATA AA-3', reverse primer: 5'-ACT ATC TTG TGG CGG ATG TC-3' and cloned into pGEM vector.

For GFP immunolabeling, staged embryos were fixed in 4% paraformaldehyde (PFA) at room temperature for 20min, washed in PBST (0.1% Tween 20/PBS) and incubated overnight at 4°C with anti-GFP primary antibody (1:400, Clontech) in blocking solution. The day after embryos were washed in PBST and incubated 4 hours at room temperature with secondary antibodies conjugated with Alexa Fluor®488.

Cryostat sectioning

Embryos were fixed in 4% PFA, cryoprotected in 15% sucrose, and embedded in 7.5% gelatin/15% sucrose. Blocks were frozen in 2-methylbutane (Sigma) to improve tissue preservation, and then 20□m sections were cut on a LeicaCM1510-1 cryostat.

Leflunomide Treatment

Embryos at 50% epiboly were dechorionated and grown until the desired stage in 6.5□M Leflunomide (L5025, Sigma) in EB buffer solution. The EB buffer containing the drug was renewed every 12 hours. Leflunomide is a pharmacological agent that inhibits the transcriptional elongation of genes required for neural crest development (White et al., 2011).

Antisense morpholinos injections and analysis of the phenotype

For morpholino knockdowns, embryos were injected with translation-blocking morpholino oligomers (MOs) obtained from GeneTools. MO injections were as follows: 5ng/□l of MO-robo2, 5' –AAG GAC CCA TCC TGT CAT AGT CCA C- 3' (Zhang et al., 2012); 5ng/□l MO-slit1a, 5' –GAC AAC ATC CTC CTC TCG CAG GCA T- 3' (Barresi et al., 2005); 5ng/□l MO-slit1b 5' –GCT CGG TGT CCG GCA TCT CCA AAA G- 3' (Kastenhuber et al., 2009); 7.5ng/□l of MO-p53 as control, 5' -GCG CCA TTG CTT TGC AAG AAT TG-3'

(Langheinrich et al., 2002). MO-p53 was included in all MO injections. In the case of double MO-slit1a and MO-slit1b injection, 2ng/□l of each MO was used. In order to trace the injected embryos, they were co-injected at 1-cell stage with 80ng/□l of either *H2B–mCherry* mRNA (Olivier et al., 2010) or *lyn-TdTomato* mRNA (Ingham, 2009). They were left to develop at 28°C until desired stages.

Phenotype analysis

Three phenotypes were taken into account in order to describe the effects of morpholino injections and quantify their penetrance: i) ectopic SAg nerve entry points; ii) ectopic sensory nerves branches towards the hindbrain; iii) mediolateral defasciculation of the sensory nerve bundles along the anteroposterior path through the hindbrain. In order to allow statistical treatment of phenotypes occurrence, score 0 or 1 was given respectively to the absence or presence of each phenotype for each injected embryo. Defasciculation phenotype was further analyzed comparing nerve bundle width between control embryos and morphants. Nerve bundle width measurements were carried out taking the center of the otic vesicle as landmark along the AP axis, where the sensory nerve bundle seemed to be in general more compact and its width more robust in control embryos population. Values obtained from scores assignment and bundle width measurements were plotted, and corresponding statistical *p* values were determined.

Photoconversion experiments

Photoconversion of KAEDE protein was performed on a Leica SP5 inverted confocal microscope scanning one focal plane in a Region of Interest (ROI) centered on trigeminal, statoacoustic or lateral line ganglia, with 8 to 16 frame averages per image, under a 405nm laser excitation. Before photoconversion ganglia were visualized in the green emission wavelength under 488□ □ □ laser excitation. Proper photoconversion was monitored by the appearing in the red emission wavelength (550nm to 630nm) of strong photoconverted red KAEDE signal, under excitation with 543nm laser. As internal control we checked disappearance of $KAEDE^{Green}$ upon appearance of $KAEDE^{Red}$ (Figure 1A).

Ablation experiments

Tg[Isl3:GFP] and Tg[neuroD:GFP] embryos were used at 24hpf for ablation of first differentiated neurons from statoacoustic and anterior lateral line ganglia. For this purpose, the small region of interest (ROI) for each ganglion underwent high intensity irradiation (910nm) on a multiphoton laser (tunable Mai Tai broadband laser 710-990 nm) connected to an upright Leica TCS SP5 confocal microscope. Successful ablations were monitored for absence of the first differentiated neurons and axonal degradation during the following hour. Afterwards, embryos were incubated at 28C and imaged 24 hours later on a Leica SP5 inverted confocal microscope to monitor the reinnervation established by the late-differentiating neurons.

Imaging and image processing

Embryos were anesthetized in tricaine and mounted lateral on glass-bottomed Petri dishes (Mattek) in 1% LMP-agarose, or fixed and mounted in 100% glycerol. Photoconversion experiments, drug-treated embryos, morphant phenotypes analyses and bundle width measurements were done using Imaris software (Bitplane).

Confocal imaging

Confocal imaging was performed on a Leica TCS SP5 II CW-STED inverted confocal microscope system (without stimulated emission depletion) using hybrid detectors and 20x objective. In the case of x*yz* confocal cross-sections, *z*-stacks were acquired with a 1,5υm *z* distance.

SPIM Imaging

Anesthetized embryos were mounted in 0.75% agarose in glass capillaries size 2 (volume 20l, from BRAND GMBH). Imaging was performed on a Zeiss Lightsheet Z.1 microscope using a 20x objective. Image processing was done using the Zeiss ZEN software and involved dual (illumination) side fusion and Deconvolution (Regularized Inverse Method). Movies and stills were generated from the 4D datasets using FIJI.

Fluorescence Microscope Imaging

Cryostat sections were imaged on a Leica DM6000B fluorescence microscope with DFC300KX camera under the control of LAS-AF (Leica Application Suite Advanced Fluorescence 1.8) using 20x and 40x objectives. ISH and fluorescent images processing was done with FIJI (NIH ImageJ 1.46j).

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FIGURE LEGENDS

Figure 1: Photoconversion (PhC) of specific sensory neuronal pools in Tg[hspGFF53A]xTg[UAS:KAEDE] embryos.

(A) Depiction of the experiment. Embryos express KAEDE^{Green} in the sensory ganglia since very early stages and this persists several hours later. Distinct sensory ganglia neurons were photoconverted at 48hpf and the expression of KAEDE^{Red} in the sensory projection towards the hindbrain was assessed few hours later. (B-C) PhC of neurons from the TGg. (B') is an insert of the framed region in (B), and (C) is a dorsal view of (B'). Note that red projections run very ventral and medial. (D-E) PhC of neurons from the ALLg. (D') is an insert of the framed region in (D), and (E) is a dorsal viewof (D'). Note that KAEDE^{Red}-ALLp projections are in an intermediate position along the DV axis. (F-G) PhC of neurons from the PLLg. (F') is an insert of the framed region in (F), and (G) is a dorsal view of (F'). Note that KAEDE^{Red}-PLLg projection is allocated completely dorsal and lateral. Anterior is always to the left. Axes are indicated in the figures. TGg/p, trigeminal ganglion/projection; ALLg/p, anterior lateral line ganglion/projection; PLLg/p, posterior lateral line ganglion/projection; SAg/p, statoacoustic ganglion/projection. The contour of the otic vesicle is indicated in white circles.

Figure 2: Photoconversion (PhC) of specific sensory neuronal pools in Tg[hspGFF53A]xTg[UAS:KAEDE] embryos.

(A-B) PhC of neurons from the A-SAg. (A') is an insert of the framed region in (A), and (B) is the dorsal view of projections in (A'). Note that red projection runs very ventral and medial although it is not the most ventrally positioned, considering the allocation of the TGp . (C-D) PhC of neurons from the P-SAg. (C') is an insert of the framed region in (C), and (D) is the dorsal view of projections in (C'). Note that KAEDE^{Red}-P-SAg projections are more dorsal and lateral than the A-SAp. (E-F') Double PhC of neurons from the ALLg and A-SAg (E-E'), and the P-SAg and PLLg (F-F'). (E', F') are inserts of the framed regions in (E, F). (G) Scheme depicting the neurosensory network with the highly ordered connectivity map with DV/LM organization as follows: PLLg/ALLg/P-SAg/A-SAg/TGg. Right hand drawing represents a transverse section in (g). Anterior is always to the left. Axes are indicated in the figures. TGg/p, trigeminal

ganglion/projection; ALLg/p, anterior lateral line ganglion/projection; PLLg/p, posterior lateral line ganglion/projection; SAg/p, statoacoustic ganglion/projection. The contour of the otic vesicle is indicated in white circles.

Figure 3: Sensory neuron differentiation and establishment of hindbrain afferents entrance points. SPIM time-lapse analysis of Tg[neuroD:GFP] (A-C) or Tg[Isl3:GFP] (D-I) embryos injected with TdTomato mRNA to label cell membranes at 1cell-stage. First differentiated sensory neurons of the TGg (A), SAg (D) and PLLg (G) are in close contact with neural tube border cells through plasma membranes, at the level of the future nerve entry point (see white arrowheads). Inserts in (A,D,G) are *z*-resliced images of (A,D,G) to show as transverse views the contact point of the respective sensory axon with the border cells of the hindbrain; dorsal is to the top. Note that this happens already before afferent sensory axonal processes are formed. Primary sensory neurons maintain contacts with the neural tube, even when they are pushed away by morphogenetic growth (B, E, H). Note that they leave trailing axons (white arrows) that may be used by later differentiating neurons to reach the same entrance point (C,F,I). Images are Single Confocal Planes except for the PLLg that are MIP of few confocal planes. For more information about the settings of the movies see Experimental Procedures. (J-M) Tg[Isl3:GFP] embryos were used for pioneer axon ablation experiments; (J,L) lateral views showing no ectopic entry points after pioneer axon ablation, and (K,M) dorsal views of (J) and (L) respectively showing the sensory projections at the central levels and defects in otic nerve bundle elongation upon ablation (see asterisk in M). Orientation of the embryos is indicated in the figures. Anterior is always to the left. TGg, trigeminal ganglion; ALLg, anterior lateral line ganglion; PLLg, posterior lateral line ganglion; SAg, statoacoustic ganglion; nt, neural tube; ov, otic vesicle.

Figure 4: Cooperation of pioneer axonal contacts and NCC in the establishment of the entry points. (A-F) Tg[Isl3:GFP] embryos were assayed for *crestin* (blue) in situ hybridisation. (A-B) TGg, (C-D) ALLg/SAg, (E-F), PLLg. Note that GFP-cells in these cranial ganglia are usually surrounded by *crestin*-positive cells. (G-I) Tg[Isl3:GP] were treated with DMSO (G), or with Leflunomide (H-I), an inhibitor of *crestin* and NCC

migration, and hybridised with *crestin* probe (red). Note that *crestin* expression is abolished in Leflunomide-treated embryos, and no effects in the entry points are observed, although sensory ganglia present defects in coalescence (white arrows in H-I). (J-L'') Tg[neuroD:GFP] embryos were treated with Leflunomide and the pioneer axonal contacts of the ALLg/SAg were ablated using multiphoton microscopy. (J) Lateral view showing ectopic entry points (see white arrows); (J') Insert of framed region in (J) showing the ectopic entry points (arrows) in contact with the PLLp (red asterisk); (K) Dorsal view of (J') showing the TGp (yellow asterisk), PLLp (red asterisk) and a lack of ALLp/SAp nerve bundle elongation (see empty space depicted with white asterisk). (L-L'') are different single confocal planes from medial (L) to lateral (L'') showing that ectopic entry points lost somatotopy and now contact with the PLLp (see red asterisk). Anterior is always to the left. Axes are indicated in the figures. TGg/p, trigeminal ganglion/projection; ALLg/p, anterior lateral line ganglion/projection; PLLg/p, posterior lateral line ganglion/projection; SAg/p, statoacoustic ganglion/projection. The contour of the otic vesicle is indicated in white circles.

Figure 5: Expression of *robo2/slit1* genes marks SAg neurons and afferent target fields. Tg[Isl3:GFP] embryos were analyzed for *slit1a* (A-C), *slit1b* (D-F), *robo2* (G-I'), and *robo3* (J-L') expression at 28hpf. Note the diffuse expression of *slit1a/b* along the hindbrain, leaving zones devoid of *slit1* that correspond to the places where the central projection enters (see white arrow heads in A,B,E). *robo2* is expressed in the differentiated Isl3-sensory neurons (white arrows), and *robo3* in the SAg neuroblasts that did not differentiate yet (red arrows). (A-A'', D-D'') are coronal sections corresponding to half-sided embryos. (B-C, E-F) are transverse sections corresponding to (b-c, e-f), respectively. (G-I, J-L) are serial transverse sections along the AP axis.

Figure 6: *robo2* and *robo3* label different SAg neuronal populations according to their differentiation state. (A-A') Tg[Isl3:GFP] embryos were analyzed for *neuroD* (A-A'). Note that the majority of *neuroD*-positive cells are not differentiated yet and do not display Isl3:GFP. (B-D') Tg[neuroD:GFP] embryos hybridized with *neuroD* (B-B'), *robo2* (C-C'), and *robo3* (D-D'). Note that in Tg[neuroD:GFP] embryos *neuroD* and GFP expression do not fully overlap: all *neuroD*-expressing neuroblasts display neuroD:GFP

(see red arrow in B'), but *neuroD* is not expressed in the early differentiated neuronal population which still expressed GFP due to its high stability (see white arrow in B'). Note that *robo2* is expressed only in a subpopulation of the neuroD:GFP cells (see red arrows in C'), which is the same that expresses Isl3:GFP. *robo3* is expressed in a subpopulation of neuro:GFP cells (red arrow in D') but not in the earliest-differentiated ones (white arrow in D'). (E-H') Tg[Isl3:GFP] embryos hybridized with: (E-E') *robo2/neuroD,* demonstrating that within the *robo2*-positive population, some cells express Isl3:GFP (see white arrow in E') and some *neuroD* (red arrow in E'). (F-F') *robo2/robo3,* showing that GFP-positive cells expressing *robo2*, do not express *robo3* (see red arrow in F'), and cells expressing *robo2/robo3* do not display GFP (see white arrow in F); (G-G') *snail*, labeling the delaminating neuroblasts and the non-differentiated neurons; and (H-H') *cadh10*, which label a subpopulation of non-differentiated neuroblasts. All images are transverse sections of embryos at the level of the otic vesicle.

Figure 7: *robo/slit* signaling regulates axonal branching and nerve bundle fasciculation. Tg[Isl3:GFP] embryos were co-injected with MO-p53, mRNA for *H2B-mCherry/lyn-TdTomato* and MO-slit1a, Mo-slit1b, Mo-robo2 or double MO-slit1a/b. (A-F) Examples of phenotypes observed at 48hpf. Control embryos are the ones injected only with MO-p53. Note the variety of effects ranging from ectopic entry points (see white arrows in B,F), ectopic branches (see white arrow heads in C,E,F), defasciculation (see black asterisk in D), and combinations of primary phenotypes (E,F). (G) Statistics of MO injections. (H) Analyses of the percentage of morphant embryos displaying the different phenotypes upon different combinations. Statistical differences were calculated in respect to MO-p53 control embryos, *p<0.1, **p<0.01, ***p<0.001.

Movie S1: SPIM time-lapse analysis of the dynamics of differentiation of the TGg in Tg[neuroD:GFP] embryos injected with TdTomato mRNA to label cell membranes at 1cell-stage. Embryos were imaged from 16hpf onwards and 5min time-lapse images were acquired. Embryos were grown at 25C while recording.

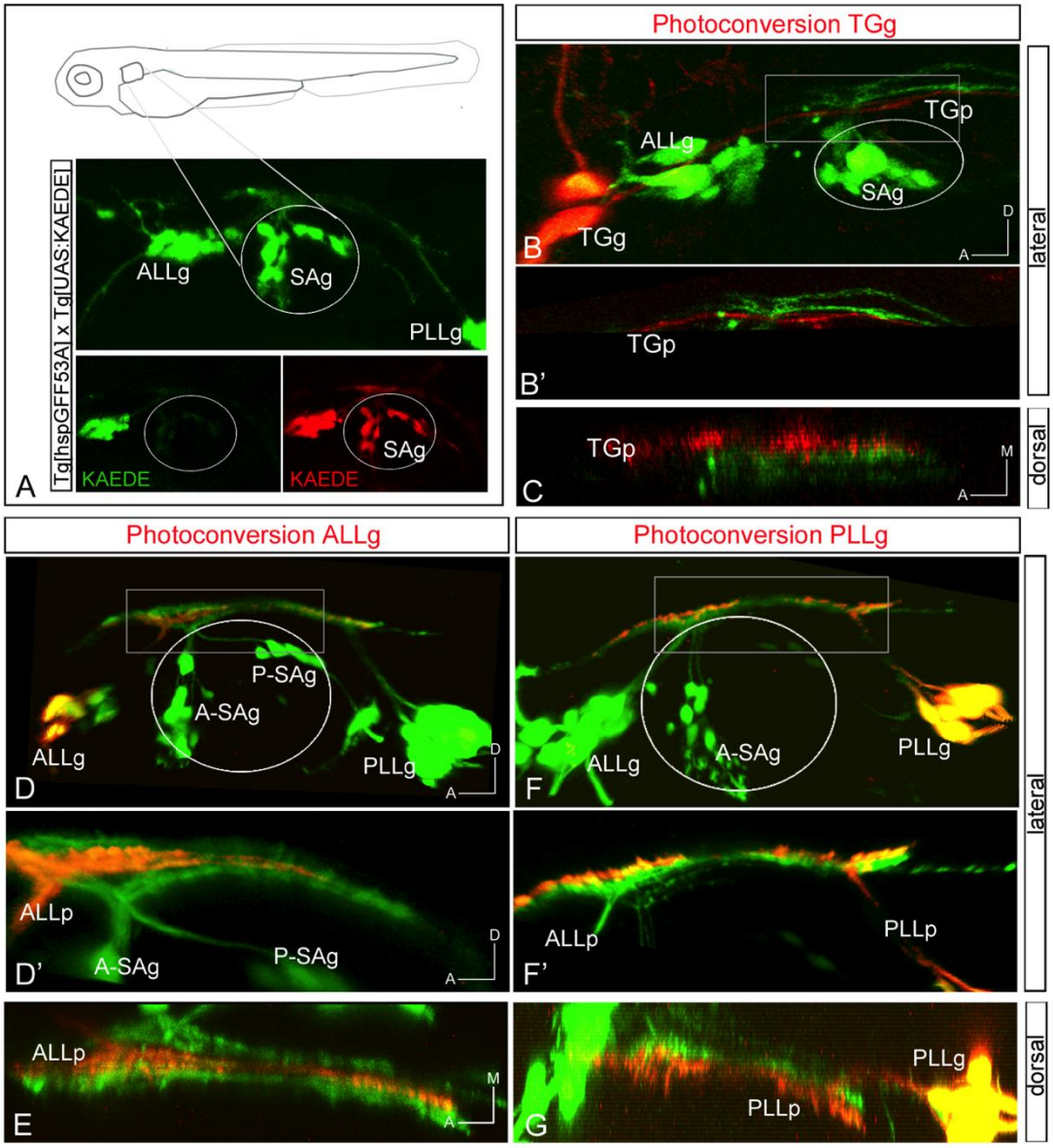
Movie S2: SPIM time-lapse analysis of the dynamics of differentiation of the SAg in Tg[Isl3:GFP] embryos injected with TdTomato mRNA to label cell membranes at 1cell-stage. Embryos were imaged from 18hpf onwards and 23min time-lapse images were acquired. Embryos were grown at 25C while recording.

Movie S3: SPIM time-lapse analysis of the dynamics of differentiation of the PLLg in Tg[Isl3:GFP] embryos injected with TdTomato mRNA to label cell membranes at 1cell-stage. Embryos were imaged from 18hpf onwards and 5min time-lapse images were acquired. Embryos were grown at 25C while recording.

Figure S4: Entrance points of the sensory axons at the central levels. Tg[Isl3:GFP]xMu4127 embryos at different stages of embryonic development were analyzed in order to study the order of differentiation of the different cranial sensory ganglia and the position of the hindbrain entry point along the AP axis. (A-C) Maximal Intensity Projections; (A'-C') Surface Rendering of embryos in (A-C). (D-F) Serial coronal sections of same embryo from dorsal to ventral. Note that TGg entry point is located in r2, SAg entrance is in r4, and PLLg entry is in r6. In all images anterior is to the left. TGg, trigeminal ganglion; ALLg, anterior lateral line ganglion; PLLg, posterior lateral line ganglion; SAg, statoacoustic ganglion; nt, neural tube; ov, otic vesicle; r, rhombomere.

Figure S5: Quantification of the central nerve bundle width in embryos upon morpholino injection, ***p<0.001.

Figure S6: In situ hybridization of Tg[Isl3:GFP] embryos. Embryos at different stages were assayed for *pax7a* (A,B), *pax6* (C,D) or *pax3* (E,F). Note that the sensory bundle is located within the *pax*-domains of expression. Double in situ hybridization with *pax7a/pax3* (G), or *pax7a/pax6* (H). Note the sensory bundle is located in the *pax7a/pax6/pax3*-positive domain. All images are transverse sections of embryos at the level of the otic vesicle.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lateral
TGp
B'
TGp
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M
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dorsal
Photoconversion ALLg
Photoconversion PLLg
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PLLg
D
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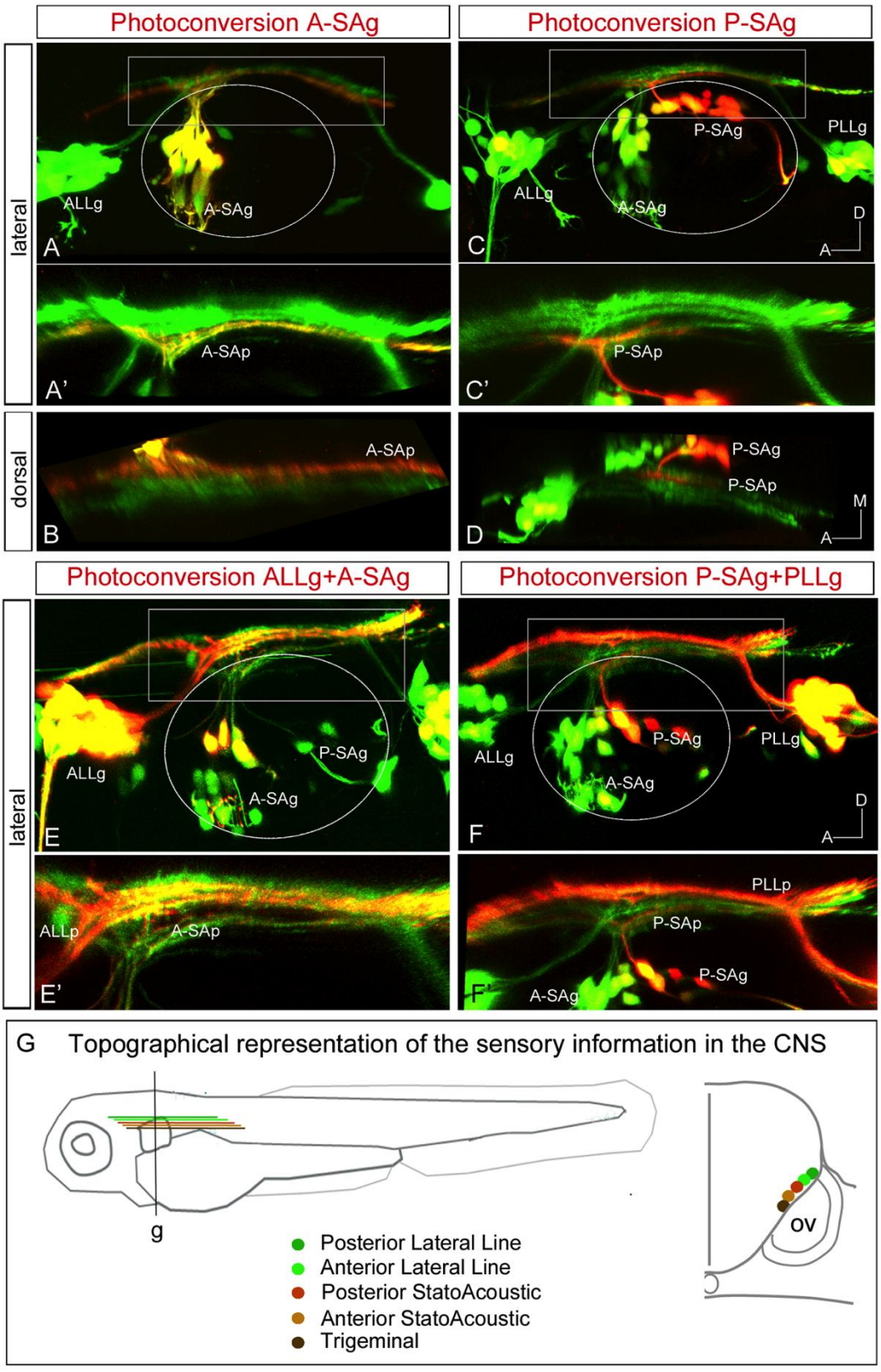
Figure 1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Photoconversion P-SAg+PLLg
ALLg
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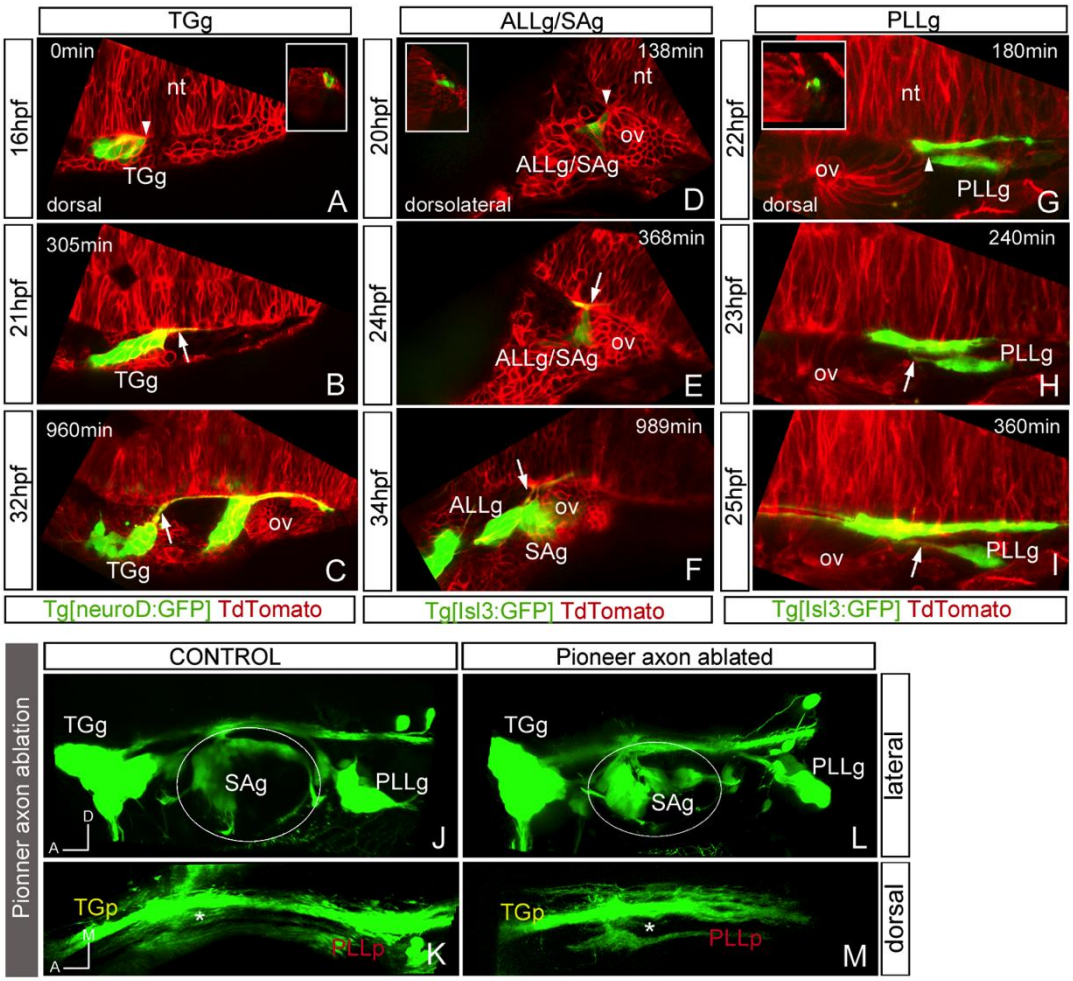
G Topographical representation of the sensory information in the CNS
g
Posterior Lateral Line
Anterior Lateral Line
Posterior StatoAcoustic
Anterior StatoAcoustic
Trigeminal
ov

Figure 2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dorsal
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D
22hpf
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ov
dorsal
PLLg
G
21hpf
305min
TGg
B
24hpf
368min
ALLg/SAg
ov
E
23hpf
240min
ov
PLLg
H
32hpf
960min
ov
TGg
C
34hpf
989min
ALLg
ov
SAg
F
25hpf
360min
ov
PLLg
I
Tg[neuroD:GFP] TdTomato
Tg[Isl3:GFP] TdTomato
Tg[Isl3:GFP] TdTomato
Pionner axon ablation
CONTROL
Pioneer axon ablated
TGg
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PLLg
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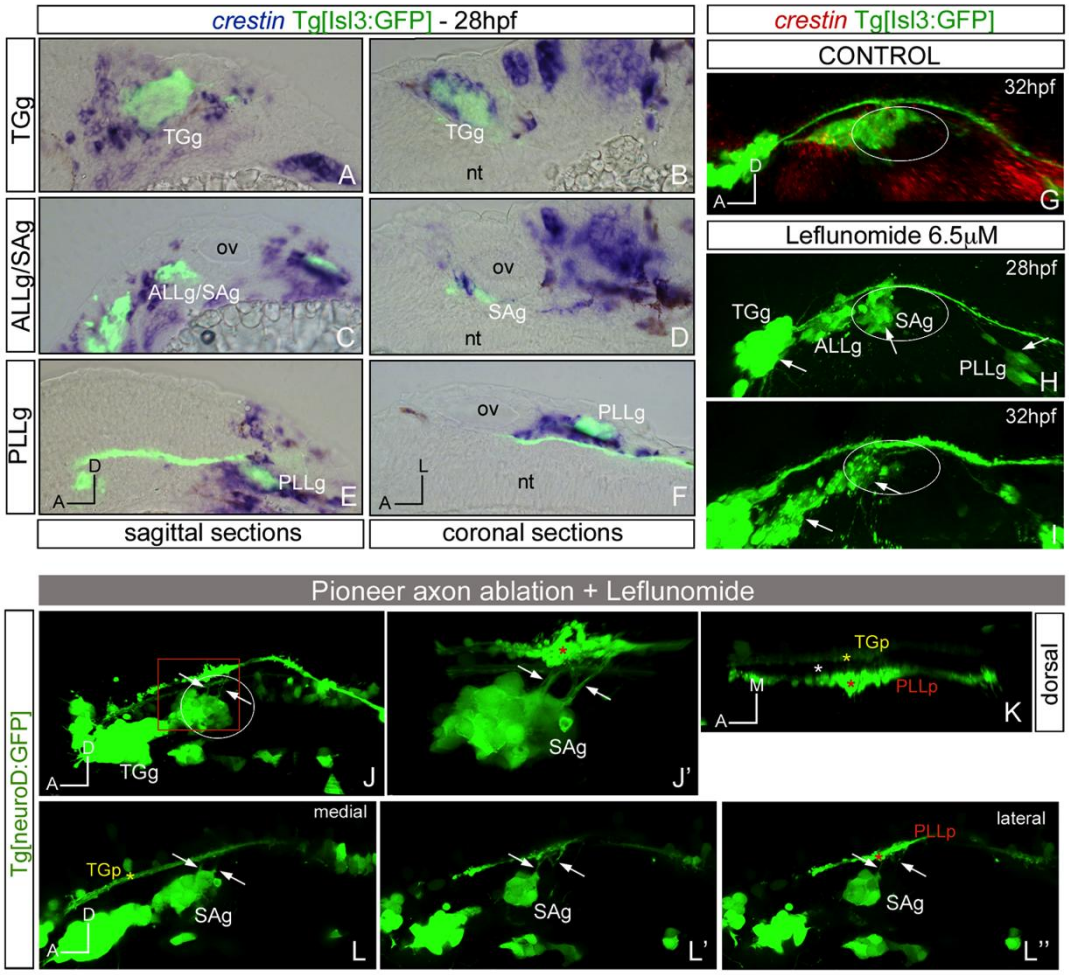
Figure 3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ALLg/SAg
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PLLg
32hpf
Leflunomide 6.5µM
28hpf
ALLg
sagittal sections
coronal sections
Pioneer axon ablation + Leflunomide
Tg[neuroD:GFP]
TGp
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dorsal
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Figure 4

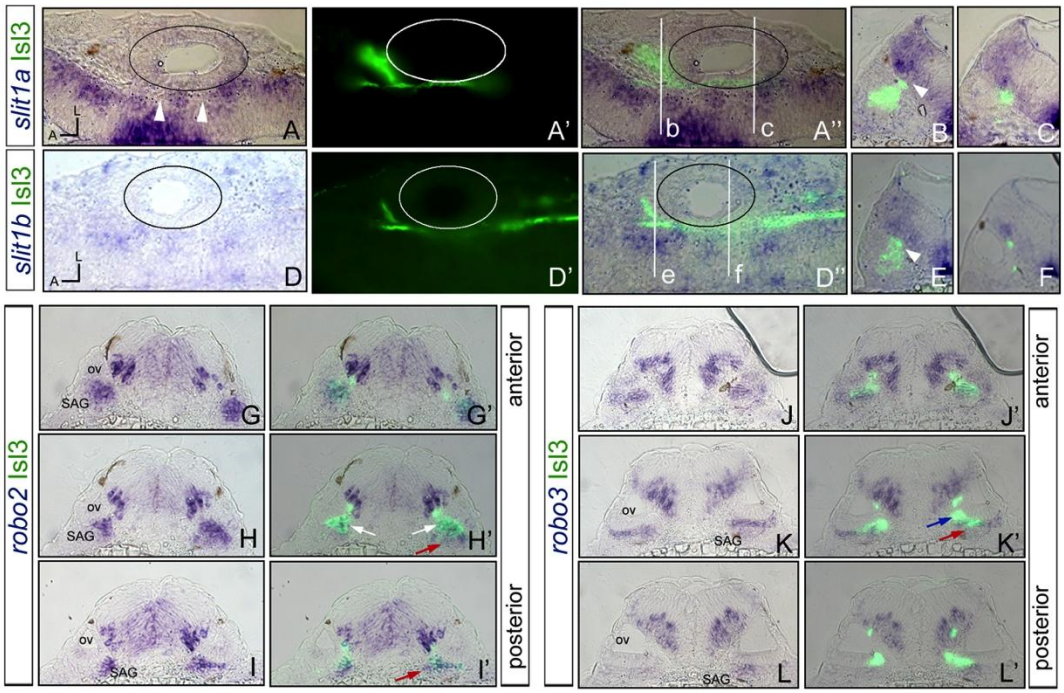


Figure 5

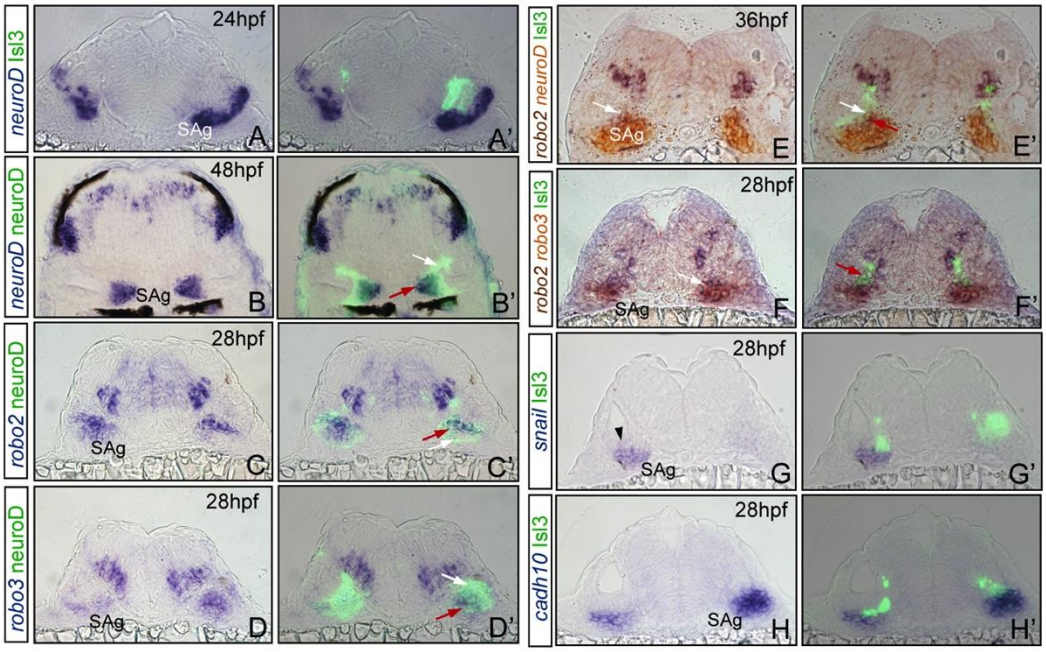
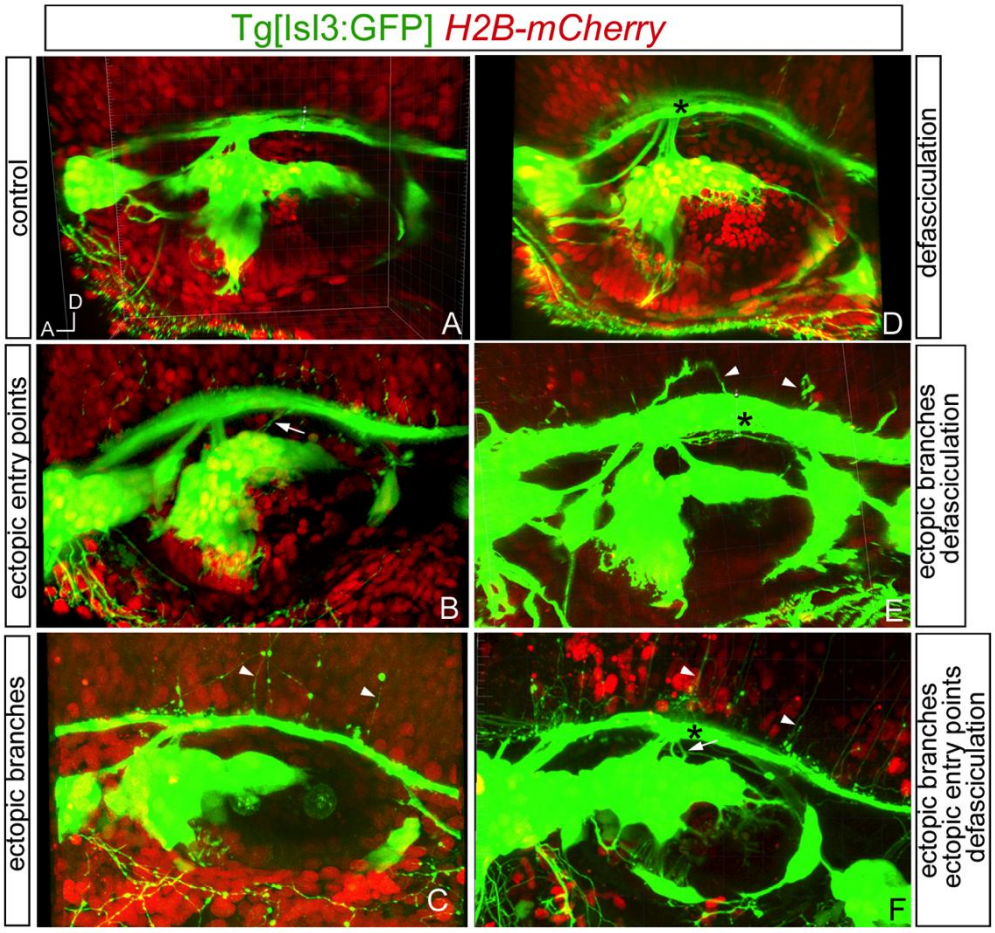


Figure 6



G

PHENOTYPE	MO-p53		MO-Slit1a		MO-Slit1b		MO-Slit1a/b		MO-Robo2	
Ectopic entry point	1/26	4%	8/14	57%	3/18	17%	6/15	44%	18/52	35%
Ectopic branches	4/26	15%	4/14	29%	7/18	39%	11/15	69%	29/52	37%
Defasciculation	3/26	12%	7/14	50%	7/18	39%	12/15	75%	39/52	69%

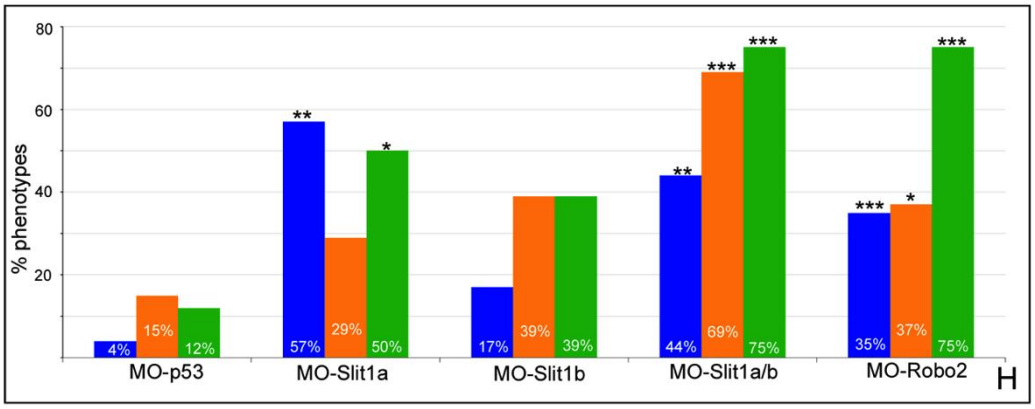


Figure 7

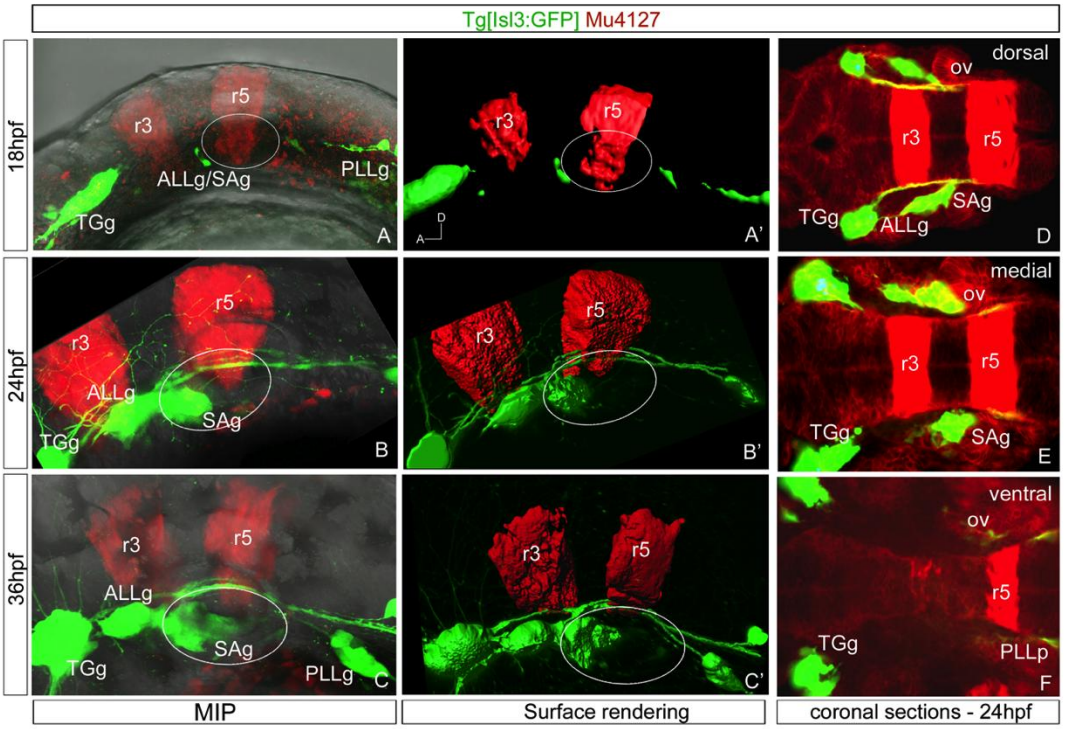


Figure S4

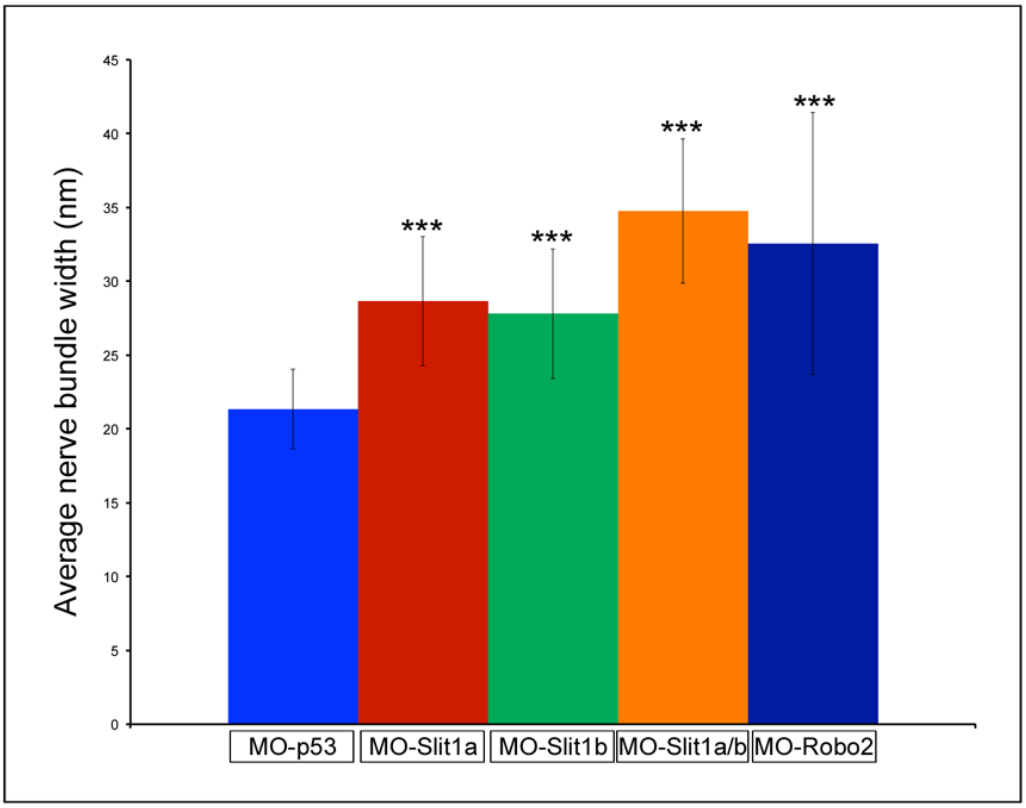
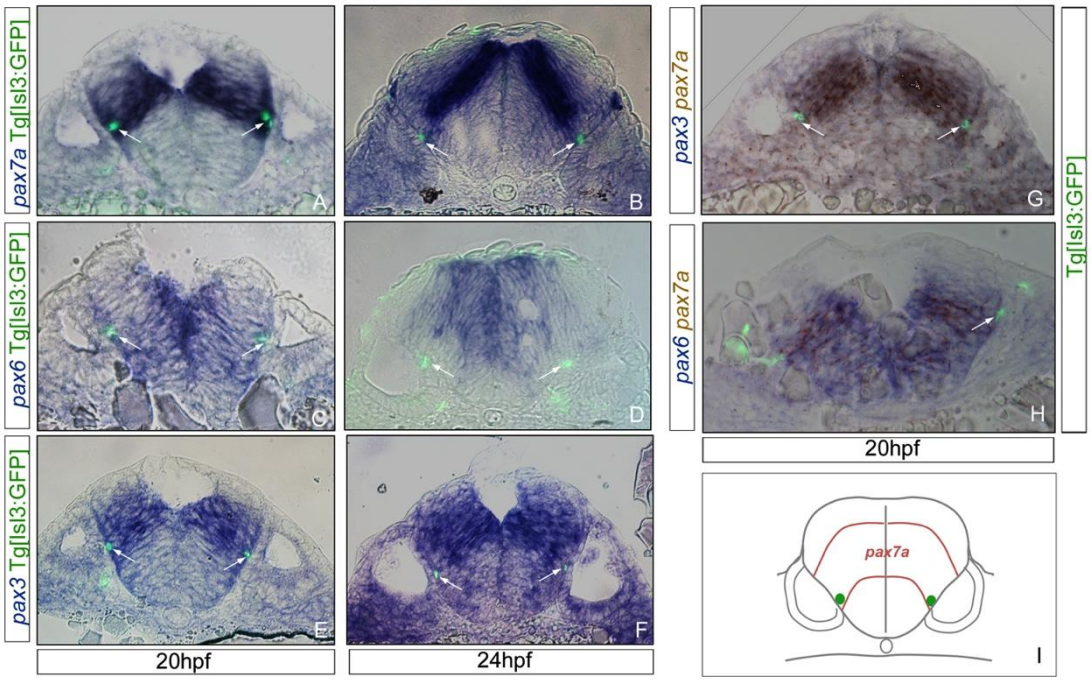


Figure S5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pax3 pax7a
pax6 pax7a
Tg[lsl3:GFP]
A
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H
I
pax7a
20hpf
24hpf
20hpf

Figure S6

DISCUSSION

In order to efficiently process enviromental stimuli, the organism needs to build a correct and precise network of connections between the sensing machinery (the Peripheral Nervous System, PNS) and the sensation processing machinery (the Central Nervous System, CNS).

Recent findings involved Neural Crest Cells (NCC) migration strains in the guidance of sensory afferents. Sensory systems and NCC interact with each other in order to establish correct sensory placodes and ganglia subdivision, coalescence and maintenance (Steventon et al. 2014b; Theveneau et al. 2013b). Loss of sensory neurons gives rise to sparse NCC migration and, the other way around, NCC loss or migration impairment affect ganglia coalescence and placodal subdivision from preplacodal areas (Culbertson et al. 2011; Begbie & Graham 2001). It seems clear that sensory neural cells and NCC contact each other, through a chase-and-run behavior that functions through cadherins and Robo/Slit signalling, in order to correctly form, position and divide developing sensory systems (Theveneau et al. 2013b; Shiau & Bronner-Fraser 2009). These interactions lead to the formation of NCC envelops around sensory neurons, that eventually coalesce into ganglions and corridors used by sensory central afferents to reach the hindbrain entry points (Freter et al. 2013; Sandell et al. 2014; Schwarz et al. 2008). During the recent years a number of axon guidance molecules, such as Robo/Slit, Eph/ ephrin pairs and Semaphorins have also been involved in the connection of the periphery of the organism to its central processing machineries.

However, initial steps of dorsolateral ganglia development such as first sensory neurons differentiation, happen with no NCC contribution (Freter et al. 2013) and we show that central afferent topography, also called somatotopy, is conserved in embryos where NCC migration was inhibited. In accordance with previous studies, we show that in the absence of NCC the coalescence and subdivision of ganglia are impaired and nerve bundles fasciculation display defects, although the basic medio-to-lateral order of sensory projections at the central level is maintained. Therefore, a need of a mechanism by which central connection topography is established arises, which can not be exclusively relying on NCC.

Our results show that:

- There is a correlation between the medioventral to laterodorsal topography of sensory afferents projections in the hindbrain and the time of sensory systems differentiation, being the trigeminal the first to differentiate and "invade" neural tissues, and the posterior lateral line the last.

- Contrary to the paradigm describing sensory neurons "sending" central afferents (Appler & Goodrich 2011, Rubel & Fritzsch 2002), firsts sensory neurons to differentiate do so with their cell bodies attached to the site of entrance, and the contact remains while their projection elongates within the hindbrain. They will eventually leave trailing axons when later they will be pushed toward the periphery upon morphogenetic movements. This is likey due to the growth and expansion of the lumen of brain tissues and a probable chase-and-run reaction to the streams of migrating NCC.

- NCC actually forms corridors and envelope ganglia giving support and maintaining their coalescence. In our view these corridors are involved, together with the trailing axons left from early-differentiating neurons, in guiding later axonal growth from later waves of neuronal differentiation.

- Robo/Slit pairs of repulsive axon guidance cues display expression profiles consistent for a role in the control of the size of the map, and therefore of the network.

4.1 When the time of differentiation is sufficient: the case of posterior sensory systems maps formation

From our studies, NCC corridors seem to be necessary but not sufficient to lay down first differentiating neuronal axons and to guide late-differentiating neuronal projections to their correct position in the brainstem, whereas pioneer contacts between first-differentiating neurons and neural tube border cells seem to be both necessary and sufficient to establish proper first to second order connections. Nevertheless, NCC envelops around sensory ganglions are important in the maintenance of the overall ganglionic structure (Culbertson et al. 2011).

As I have shown in the chapter before, the absence of slit1/robo2 signalling results into an enlargement of the trigeminal, otic and lateral line sensory nerve bundles, to contralateral ectopic projections inside the brainstem and, in a smaller degree, to ectopic entry points, probably due to NCC related problems as shown in chick by Bronner-Fraser (Shiau et al. 2008). The absence of NCC results in lack of coalescence and peripheral migration of sensory ganglia, in line with Theveneau and Mayor chase-and-run model, to some degree of structural problems of the nerve bundles, especially in the case of the PLL, but, by itself, does not lead to a higher number of entry points and still, no spatial medio to lateral alterations in central projections organization. On the other hand, the loss of pioneer neurons leads defects in central projections, and to ectopic entry points, but only when coupled with the absence of the NCC direction-restraining corridors.

The loss of pioneer neurons together with the absence of NCC, leads late-differentiating neuron sensory afferent projections to ectopically enter the brainstem and eventually innervate a different nuclei following the axons of other sensory systems. These late arriving follower projections could have a high affinity for the trailing axons of their own population of neurons (homotypic affinity), but also a lower affinity for other sensory systems axons (heterotypic affinity). When the first projections are absent, their ability to join the latter bundles is unveiled, leading to a loss of somatotopy but not to the new formation of missplaced bundles.

We observed that the statoacoustic ganglion dies when innervating the brainstem along with another sensory bundle (in this case the PLL central bundle) (data not shown). This would be in line with survival effects of a unique Neurotrophin/Trk code expressed by different brainstem layers (Table1 from Barbacid 1995). Thus, in the absence of contact with the right neurotrophin expressing target population, neurons that have been able to innervate another target population fail to survive.

	Knockout strain						
Phenotype	p75	NGF	TrkA	BDNF	TrkB	NT-3	TrkC
Sensory activity							
Nociception	Partial	Very low	Very low	Normal	Normal	Normal	Normal
Balance	Normal	Normal	Normal	**Impaired****	**ND**†‡	Normal	Normal
Proprioception	Normal	Normal	Normal	Normal	Normal	Impaired	Impaired
PNS defects‡‡							
Superior cervical ganglion	Normal	5%	5%	Normal	Normal	50%	75%
Trigeminal ganglion	Normal	30%	30%	60%	40%	40%	ND
Nodose–petrosal ganglion	ND	Normal	Normal	**40%**	**10%**[a]	60%	ND
Vestibular ganglion	ND	ND	ND	**15%**	**ND**‡	80%	ND
Dorsal root ganglia	Smaller	30%	30%	70%	70%	**35%**	**80%**
Ia Afferents	ND	Normal	Normal	Normal	Normal	Lost	Lost
CNS defects							
Facial motor neurons	ND	ND	ND	**Normal**	**30%**	Normal	ND
Spinal cord motor neurons	ND	ND	ND	**Normal**	**70%**	Normal	ND
Cholinergic projections	ND	Reduced[b]	Reduced	ND	ND	ND	ND

*Data obtained from [40••–47••,57]. **Characters in bold indicate significant differences found in mice defective for a neurotrophin and its cognate Trk receptor. †ND, not determined. ‡Animals die too early. ‡‡Approximate percentage of remaining neurons. [a]JT Erickson, DT Katz, personal communication. [b]H Phillips, personal communication. PNS, peripheral nervous system; CNS, central nervous system.

Table 1: Summary of the defects observed in mice targeted in genes encoding various neurotrophins and their receptors.

Furthermore, our observation that in the absence of pioneer neurons and NCC axons are not able to innervate their right nuclei runs against the role of molecular signals responsible of the guidance of these axons to their specific brainstem nuclei. This result opens the possibility that sensory axons are involved in the specification of their own nuclei in the brainstem, as experiments from Levi-Montalcini suggested. They showed that removal of the inner ear disrupts Mauthner cell dendrite (Levi-Montalcini 1949), proposing an instructive role of first order projection in their second order target specification. This is the case, among others, for olfactory systems and for lamina neurons formation in Drosophila (Huang & Kunes 1996).

Interestingly experiments from Fritzsch and Elliot showed that implementing a second auditory organ anterior to the native ear in Xenopus led to some degree of overlap between the central afferent nerve bundles of the implemented vestibular ganglion and that of native vestibular ganglion. The fact that only 90 degree rotated ears innervate in a non-overlapping, or partially non-overlapping central bundle, but not the ear

implemented in the same orientation of the native ear that instead projects always in the same central bundle of the native ear ganglion, points to an activity related separation of nerves projections. The observation that the two neuronal populations often project, partially or completely, through the same bundle at the peripheral level, points toward a homotypic affinity relationship between them. However, even if the author speculates about it, no molecular cues were proposed in those studies (K.L.E. Thompson, Thesis dissertation)

Notably, ephrin gradients of expression are present between the statoacoustic ganglion and its peripheral targets, and between the auditory brainstem nuclei magnocellularis and higher nucleus laminaris, but no gradient of ephrins has been reported in the brainstem afferents of the statoacoustic ganglion (Bianchi & Gray 2002; Siddiqui & Cramer 2005; Allen-Sharpley et al. 2013). Hence the ephrin signalling could be used, as in the olfactory coarse map formation, for an axon-axon interaction in neuronal circuit assembly based on time of differentiation, but likely it is not involved in the medio to lateral somatotopic disposition of projections (Allen-Sharpley et al. 2013).

First differentiating sensory pioneer neurons of trigeminal, otic and posterior lateral line systems seem to be both necessary and sufficient in creating an ordered map of central afferents projections based on the time of differentiation. I think that there is a role for activity in bundles separation and unique layer specific, established through innervation, neurotrophic signal involvement in the survival of both first and second order of processing neurons. With this in mind, I took a closer look to the architecture of the different sensory systems map formation and found striking similarities among them, but only when comparing a complexity of processing based order of neuronal cells layers instead of a number of neuronal cell layers order of processing. The difference could not be immediately clear, but I hope to make my point through the following discussion and the scheme in Figure 28.

4.2 Optical illusions: the case of visual maps formation

As I stated in the introduction to sensory map formation, to make sense of the mechanisms that organisms use to build their sensory systems we should consider the level of wiring complexity and the order of the representation of a same stimulus, and compare it during different evolutionary steps of its cytoarchitecture. To represent a stimulus at lower order of complexity, or calculation capabilities and integration, could require less sophisticated network wiring than higher order of processing. As I will try to point out in the following paragraphs, using this classification would allow us to highlight the similarities in the strategies used in the building of the sensory networks in different organisms and sensory systems.

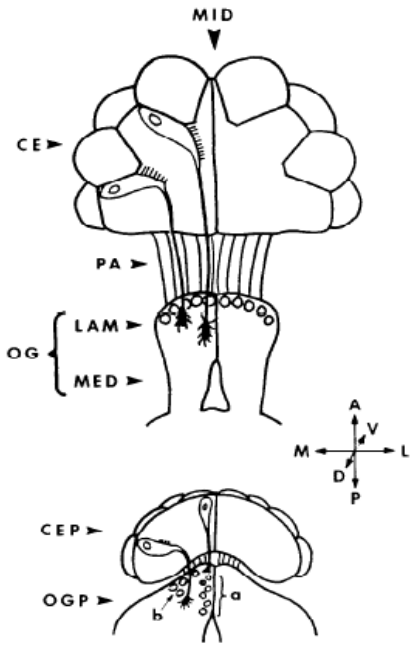

Fig. 23: Drawings of the adult visual system (top) and the developing visual system (bottom) viewed in the horizontal plane. The compound eye (CE) and optic ganglion (OG) are symmetric about the dorsoventral midplane (MID). In the drawing of the adult eye, number of lenses and two photoreceptors are depicted. The photoreceptor axons (PA) project to the optic lamina (LAM). Laminar neuron cell bodies, represented by small circles, are located at the anterior surface of the optic ganglion. The location of the neuropil, composed of optic cartridges, is indicated by the arborizations of the two photoreceptor axon bundles. Posterior to the lamina is the medulla (MED). In the drawing of the developing visual system, two photoreceptors from neighboring ommatidia are depicted in the compound eye primordium (CEP). The photoreceptor axons from the more lateral ommatidium have already reached the optic ganglion primordium (OGP) and recruited presumptive laminar neurons into a nascent cartridge (from Flaster and Macagno 1984).

In a classification based on the level of processing capability, the Eph/ephrin gradient building a retinal representation in the central brain, and the intrinsic OR depending cues in olfactory maps formation, would in both strategies use laws of higher processing areas, the same that the hindbrain nuclei may use to innervate higher brain areas in the diencephalon. On the other hand, the time of neuronal differentiation based anteroposterior retinotopy found in Drosophila would be an ancestral/lower brain strategy, that in my opinion better corresponds to the description of the connections formed between first order neurons of the posterior placodal derived sensory systems and hindbrain second order interneurons. Accordingly, I propose, that vertebrate visual system bipolar

cells (in this view, resembling first order neurons of "ancient" sensory system maps) could wire through a time of differentiation dependent mechanism with retinal ganglion cells, which would be as a hindbrain nuclei -second order neurons using a molecular gradient for their higher connections.

In line with this, receptive neurons (first order) ommatidia of crustacean's eye project in a time of differentiation ordered fashion to neurons in the lamina (second order), where the retinotopy is conserved by the axonal arrival order (Fig. 23, Flaster and Macagno 1984). Thus, the retinotopical projection to second order neuron target seems to depend on the time of differentiation of first order neurons. Then, lamina cells (second order neurons like vertebrate retinal ganglion cells) send their projections to the third order neurons in the medulla (corresponding to the order of processing of mammalian optic tectum).

The same time of differentiation dependent retinotopy map is built in the lamina (ganglion second order neuron nuclei) of the Drosophila by the retinal receptors (ommatidias would be first order neurons like vertebrate bipolar cells) that determine the differentiation of their second order target lamina neurons. Photoreceptors differentiate following their birthdate and induce the differentiation and assembly of target neurons into columnar array. As each incoming bundle of receptor axons induces its own target column, the number of target columns matches precisely to the number of photoreceptors that differentiate. Thus, the map formation along the AP axis is ensured by the precise sequence of differentiation in the retina (Huang & Kunes 1996). Interestingly, the positioning of the projections of R7 and R8 receptors, which are sent directly to the third order of processing in the medullas (equivalent to the mammalian diencephalon), seems to be dependent on axonal interactions and repulsion, reminding the formation of

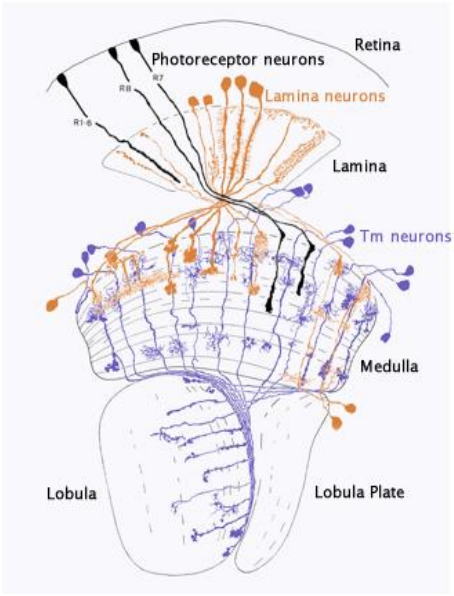

Fig. 24: Schematic representation of some different cell types in the visual system. The photoreceptor neurons (black) fall into three classes based on spectral sensitivity and synaptic specificity: R1-R6, R7 and R8. R1-R6 neurons form synaptic connections with a subset of lamina neurons (brown). R7 and R8 as well as some lamina neurons make specific connections with different classes of Transmedullary (Tm neurons; blue). Tm neurons then make connections in the lobula region (from Sanes & Zipursky 2010; adapted from Fischbach and Dittrich 1989).

the olfactory system map. Again, the authors suggest that the mechanisms responsible to set up this insect map are formally similar to the strategies used by vertebrates (Ashley & Katz 1994). This supports the view that third order maps are not, at least not only, dependent on the time of differentiation of the previous order of neurons, while first order neurons topography representation at the second order neuron level could depend on their time of differentiation.

Furthermore, the chiasm of optic nerve fibers, a structure that suggests in my opinion a need for navigation through molecular cues, resides between the lamina and the medulla. In the mammalian retina, first order resembling neurons, the bipolar cells, contact second order neurons, the ganglion retinal cells, directly into the retina, creating a first image representation, or map, already at the level of the retina. Only then, the retinal ganglion cells relay this representation to a third level of processing in the optic tectum (mesencephalon) eventually reversing it at the level of the optic chiasm.

Interestingly in Xenopus, retinal cells differentiation progresses in a spatiotemporal ordered fashion, from the centre to the periphery of the neural retina region (resembling the latero to medial disposition in the side-positioned Drosophila eye whose cell population is inverted in respect to a mammalian eye: receptor layer is toward the interior in the mammalian and reverted, toward exterior, in flies and octopus) (Fig. 25).

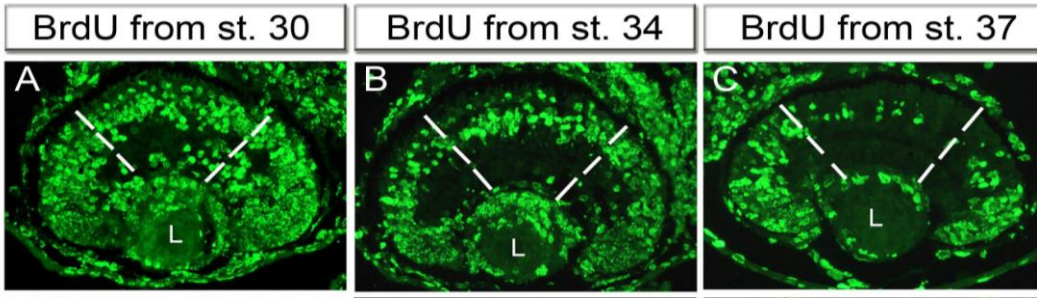

Fig. 25: Cell birth date (that is the time of exit from the cell cycle) of different retinal cells in xenopus. Dividing retinal progenitors have been labeled by BrdU intrabdominal injections from st. 30, st. 34, and st. 37, and analyzed their differentiation fates at st. 42 (mature embryonic retina) (modified from Decembrini et al. 2006)

Hence one may expect a time correlation between the differentiation of bipolar (first order of processing neuronal cells) and the order of projection send to the ganglion cells (second order of processing neurons).

An intermediate cytoarchitecture seems to be present in the octopus anatomical description: second order cells in the inner granule cell layer, this time having the aspect of bipolar cells, are contacted by axons from the retina receptive first order neurons, suggesting that the same time dependent mechanism could maybe work here, while Ganglion-like cells (in this case third order of processing) reside in the deep optic lobe (Fig. 26, Young 1961). Nevertheless, the author also reports that bipolar cells from the inner granule cell layer send projections to the retina at the same time that the optic nerve chiasm, suggesting the need for a molecular gradient located at the border between the retina and the lamina/inner granule cell layer of the optic lobe (between first and second order of processing).

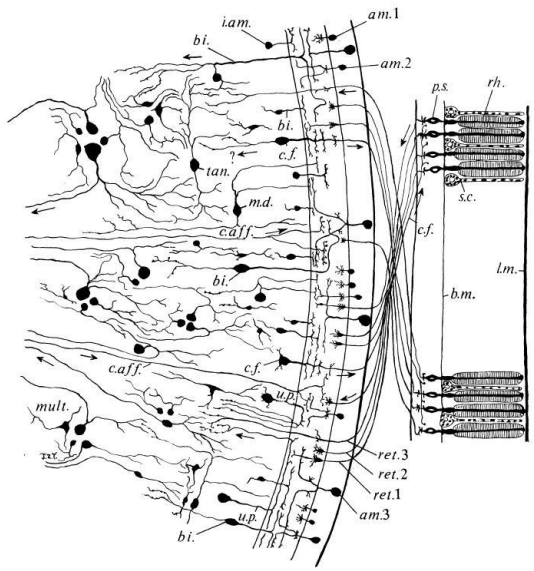

Fig. 26: Diagram of the connections of the outer portion of the optic lobe in octopus (from Young, 1961).

In Figure 27 I summarized the available information of visual maps circuits to depict how visual circuits' architecture is shared from invertebrates to higher vertebrates and built up through similar mechanisms. Nevertheless, cephalopods visual circuit from Young's drawings seems to be quite different. It could be interesting to re-analyze the cytoarchitecture of this specie with nowadays techniques to understand its position in the evolution of visual systems cytoarchitecture.

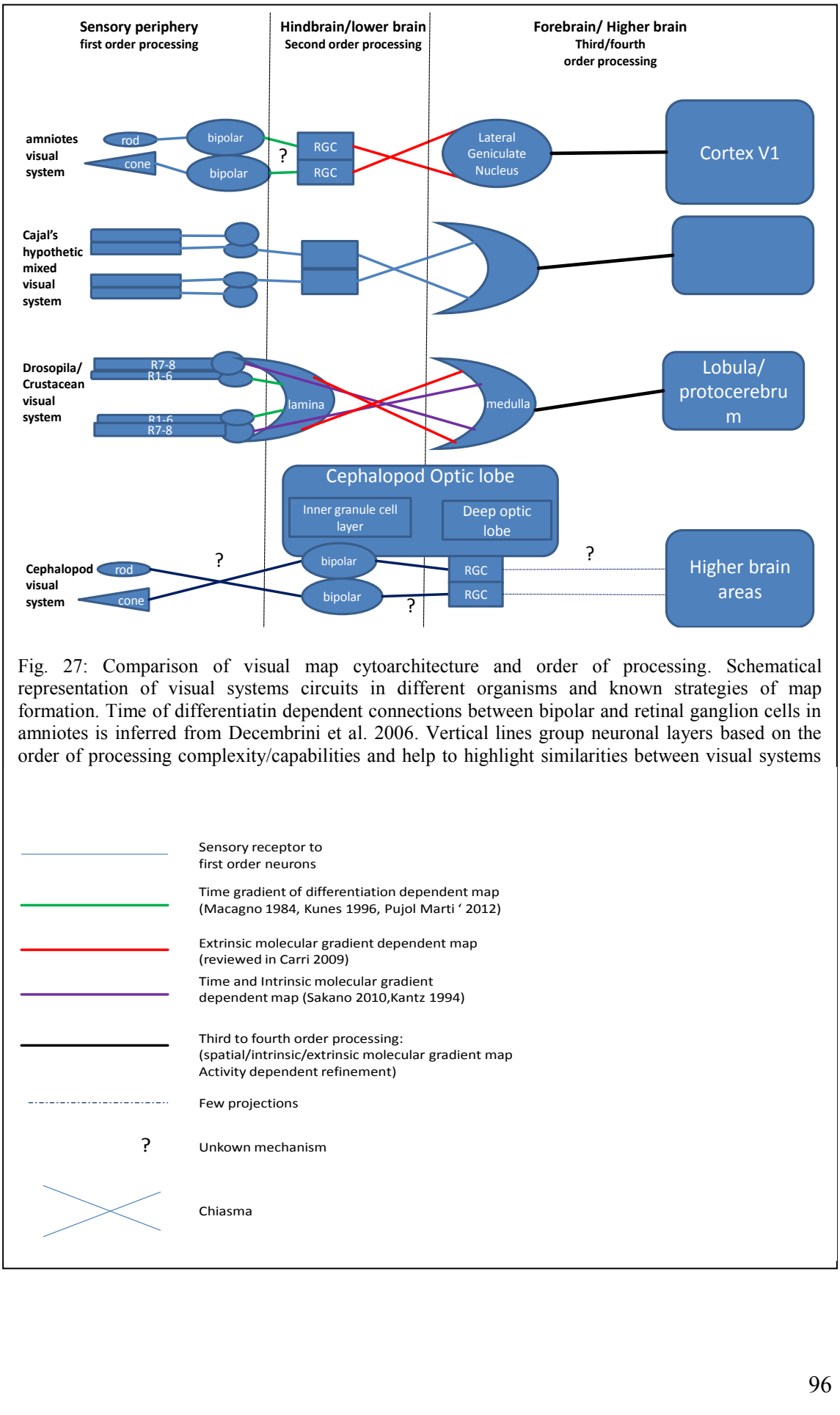

Fig. 27: Comparison of visual map cytoarchitecture and order of processing. Schematical representation of visual systems circuits in different organisms and known strategies of map formation. Time of differentiatin dependent connections between bipolar and retinal ganglion cells in amniotes is inferred from Decembrini et al. 2006. Vertical lines group neuronal layers based on the order of processing complexity/capabilities and help to highlight similarities between visual systems

4.3 Connecting first order to third order of processing: the case of olfactory maps formation

Another interesting observation, this time in olfactory maps, is the formation of an initial continuous anteroposterior order of projections dependent on the time of differentiation and that subsequent differential glomeruli innervation depends on the cell intrinsic Odorant Receptor (OR) expression (Takeuchi & Sakano 2014). Furthermore, cells from the olfactory placode expressing *ngn1* display cell to cell contacts with the telencephalic olfactory bulb during early stages of development (Maier & Gunhaga 2009). In this case Olfactive Sensory Neurons (first order) project directly to the glomeruli of olfactory bulb in the forebrain (second layer of neurons in the olfactory circuit but third general order of processing capability). Thus, we could speculate that a hypothetic first order neuron without sensory appendages would not innervate correctly its targets. Being a first order neuron, the correct innervation of third order targets would rely only on its own ability of navigation that, without sensory OR, would be none. The problem is solved by the coupling the hypothetic sensory appendages free first order neuron with an OR that confers the intrinsic guidance mechanism ability. One may wonder if the spatial correlation of an olfactory map of OSN not expressing, or expressing only one OR, would be only dependent on the time of differentiation.

4.4 Discussing about dimensionality: does the number of mechanisms used to build a map parallels the number of needed dimensions to represent a stimulus?

It is worth to point out that visual inputs at the level of the retina are bidimensional, meaning two dimension input is needed to reconstruct a visual stimulus. Then, third dimensionality is given by the arrival to the same higher brain area of contralateral and ipsilateral inputs. On the other hand, stimuli processed by the other sensory systems are monodimensional. This may explain why in the retina there is an evolutionary step to implement a dorsoventral molecular gradient (as described in Drosophila) on the top of the anteroposterior time of differentiation gradient to represent the two axes needed to reconstruct visual stimuli.

In the audition for example, the only input received by the sensory organ is the frequency of a sound that is monodimensional. Thus, the map depending on the time of differentiation is enough to process sound-evoked stimuli. The idea of the distance that a sound stimulus is coming from is instead intrinsically contained in the intensity of this stimulus, which results in stronger or weaker activation of the neuron innervating a hair cell stimulated by the particular frequency.

Olfactory stimuli are monodimensional: the two-dimension map is intrinsic to the system and is needed for neurons carrying the same odorant receptor to be able to innervate the same glomeruli. Thus, the spatial relationship of innervations inside one glomerulus is not important until one odour stimulus activates the right glomerulus from any receptor placed anywhere in the olfactory epithelium. Again, the distance of an odour source is given by the intensity of the stimulus and not by its actual position in the space.
In other words, for both odours and sounds one can distinguish farther or nearer according to one's position from the source of the stimulus, but an exact position cannot be ascribed.

4.5 Resume

Thus, first posterior sensory organs axons do not actually navigate in search of molecular gradients to reach their targets because their projections enter the brainstem through cell bodies membrane contacts with the tissues they need to innervate.
In this case the topographical order of the pioneers' central projections is dependent on the time of differentiation.
Nevertheless the overall architecture and positioning of bundles is controlled physically and molecularly to avoid the innervation of other layers or nuclei of the brainstem and to avoid, for example, contralateral projections when it is not the moment.
When the late axons need to navigate from the periphery they use first differentiating trailing axons to reach their targets and molecular and physical NCC constrains to not cross to other region of the animal periphery and/or enter the brainstem in ectopic positions. In the sensory regenerative medicine field it could be worth to take into account the idea of NCC regeneration together with the localized delivering of neurogenic stem cells.
In other systems, where the axonal target field is not in direct contact with the neuron that innervates it, like in visual second to third order of processing or in the olfactory system, those axons actually need to navigate through other cells layers and therefore there is a need for molecular instructions, both intrinsic (like in the olfactory systems) or extrinsic (like in the visual system), to reach for the appropriate targets without losing the ability to represent the innervations of the periphery of the organism.
Based on the anatomical description of the different sensory system above I would also propose topography map based on time of differentiation as the groundstate in first order sensory projection maps formation. First order neurons project topographically to their second order targets in a time of differentiation dependent manner, whereas molecular gradients navigation is needed to maintain topographical order of projections to higher order of information processing.
This seems to be a conserved process between all sensory systems. (Fig. 28)

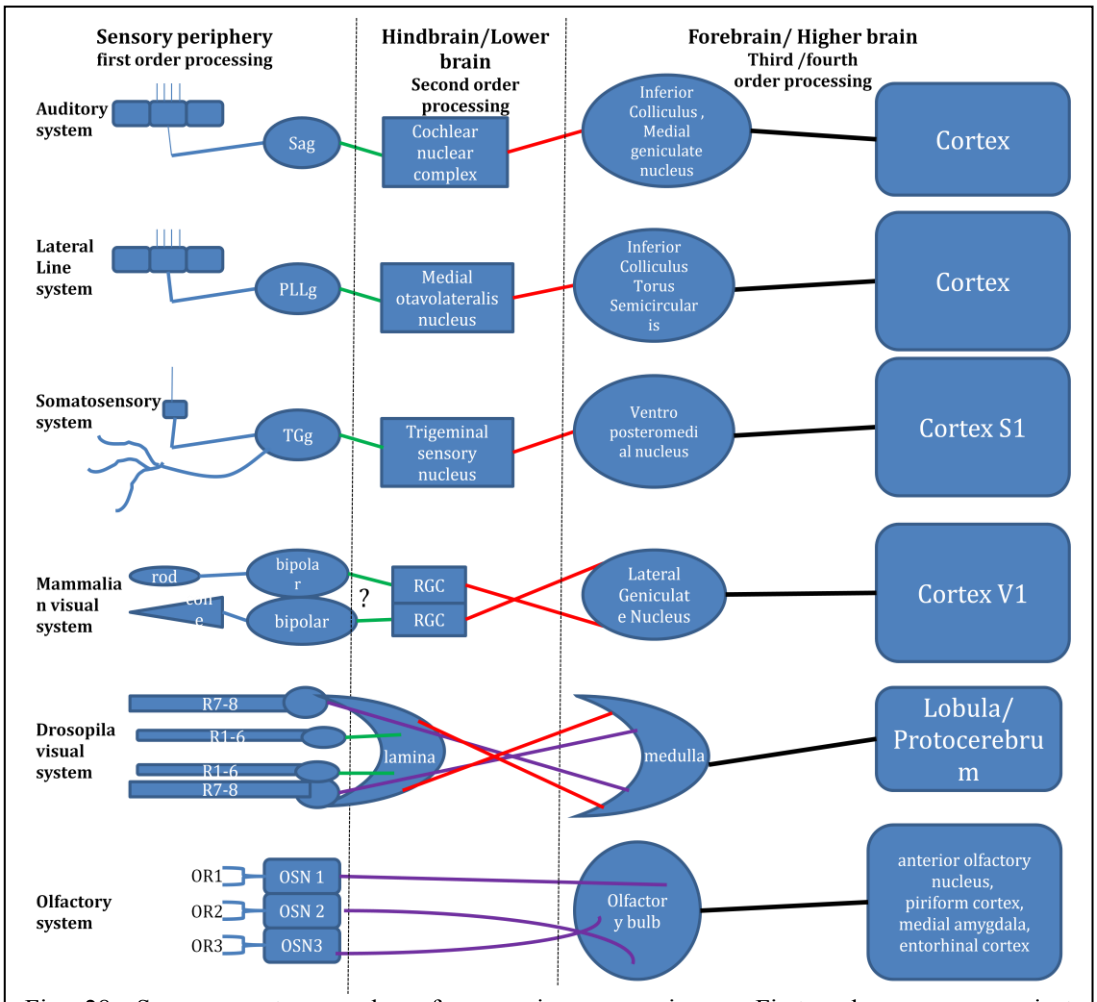

Fig. 28: Sensory systems order of processing comparison: First order neurons project topographically to their second order targets in a time of differentiation dependent manner (green lines), whereas molecular gradients navigation is needed to maintain topographical order of projections to higher order of information processing (red, violet and black lines, see below

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CONCLUSIONS

1. Time dependent somatotopic organization is a shared process, at least, of cranial posterior sensory nerve bundles. This is based on the time of neuronal differentiation,

2. The initial coarse map generated in a time of neuronal differentiation manner, relies on close contacts between first differentiated neuronal cell bodies and the neural tube border cells. It starts with the invasion by sensory afferent projections of the brainstem, with no need for navigation cues,

3. NCC mechanical constriction is involved in the coalescence of peripheral ganglia and guidance of axonal central projections of late-differentiating neurons,

4. Robo2/Slit1 signaling is involved in:
    i. maintaining fasciculation of nerve bundles to avoid bundle expansion

    ii. restraining sensory central afferents at the border of the neural tube

    iii. controlling axonal branches development toward the different brainstem nuclei,

    iv. guiding of late differentiating neurons at the proper entry point previously established by the pioneer axons.

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ANNEX

Neuronal Birth Order Identifies a Dimorphic Sensorineural Map

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