Dissertation submitted to the Combined Faculties for the Natural Sciences
and for Mathematics of the Ruperto-Carola University of Heidelberg,
Germany for the degree of Doctor of Natural Sciences

Presented by
Antonella Lauri
born in: Naples, Italy

Oral-examination: 15-01-2013

The evolution of the neural crest from an annelid perspective: conserved cell types and signaling pathways in *Platynereis dumerilii*

Referees: Dr. Francesca Peri

Prof. Dr. Jochen Wittbrodt

"Organic life, we are told, has developed gradually from the protozoon to the philosopher[1], and this development, we are assured, is indubitably an advance. Unfortunately it is the philosopher, not the protozoon, who gives us this assurance."

Bertrand Russell, Mysticism and Logic*, 1918*

[1] 'Evolutionary biologists' produce as many abstract thoughts as solid data; and often have to deal with untestable scenarios. Therefore in this context they can be analogized to philosophers.

Acknowledgments

First of all I would like to express my gratitude to my PhD supervisor Detlev Arendt for many reasons: for giving me the possibility to work in his lab and for the possibility to pursue different projects according to my wishes, for his constant encouragement to do better, and for his constructive criticism.

I also would like to thank the members of my thesis advisory committee at EMBL, Prof. Jochen Wittbrodt, Dr.Francesca Peri, Dr. Jan Korbel and Prof. Roberto Di Lauro, for their supervisions and support during my PhD work.

I wish to thank all the Arendt lab, the people with whom I shared my last past four years.

A very special thanks goes to Dr. Mette Handberg-Thorsager, a great scientific colleague, who became a friend, and with whom I loved to share thoughts, insightful discussions, injection sessions at night, pizzas and embryos; but also general thoughts about life !

Without Dr.Tomas Larsson I would have been lost in the big world of the phylogenesis, he has always been very patient with me and gave me many good advices during difficult moments of my PhD..

I also would like to thank Dr. Maria Antonietta Tosches for the scientific discussions, the help , for the fun shared during our collaboration and for critical reading of this manuscript

I am grateful to Dr.Raju Tomer, who developed the PrImR resource and help generating the average expression patterns for this study.
Moreover, thank to Raju and Neetu for sharing science with me and friendship, I felt home when I was with them.

I am grateful to Heidi Snyman and Daniel Bucher for helping me to deal with the german bureaucracy, deadlines and for their help in the lab, thanks to Diana Hofmann for taking care of the animals and be always available.

I must also acknowledge Dr. Heather Marlow, she teached me to work with Nematostella vectensis, she helped me understanding the expression patterns in Nematostella, gave me materials and especially for the proofreading of this manuscript.

A thanks to Thibaut, with whom I share a great project, injection sessions again and thoughts about life.
A special thank to the other people who helped me proofreading this manuscript: Silvia, Tomas and Mette. Thank to Silvia for the german translation and the funny moments!
Thanks to all the other people in the lab : Dr.Pavel Vopalensky for creating a nice atmosfere in the lab, for his collaborative spirit and for scientific discussions.
Thanks to Oleg (together with Tomas) for the great Bioinformatic service and for the transmission electron microscopy.
I will also like to thank the core facilities at EMBL that helped me to develop my project.

I will forever be thankful to my past and current flatmates, and the 'Italian community at EMBL': with you guys I felt always like at home. A special thank to Maria Rosaria, Irene, Giulia ,Yuri and Flavia, they always sustained me during difficult moments. I couldn't have survived the boring life of Heidelberg without them and without the music of Macumba.

And last but no least, I will be always thankful to my family for the 'warm' support they gave me through my entire life, our long skype conversations made my german winters less cold: grazie mamma, papa', Peppe, Valentina, Strega, nonna, zia. A special thank to my Mary and Raffy, they have always been close to me, and they have been always there for me.

In particular I wish to thank my half, Michele. Without him nothing of what I have achieved personally and scientifically would have been possible in these years.He has been always with me, although physically separated, and gave me the stability and the peace required to be concentrated on my work, even when stability was not there: grazie tesoro…..

TABLE OF CONTENTS

LIST OF ADDITIONAL TABLES (not included in the figures)

LIST OF FIGURES

ABBREVIATIONS

c-NCc	Cranial neural crest cells
car-NCc	Cardiac neural crest cells
t-NCc	Trunk neural crest cells
v/s-NCc	Visceral/sacral neural crest cells
NPB	Neural plate boder
NCS	Neural crest specifiers
PNS	Peripheral nervous system
DESN	Dorsal ectodermal sensory neurons
DRG	Dorsal root ganglia
DRGsn	Dorsal root ganglia sensory neurons
DR	Dorsal root
DF	Dorsal fin
VESN	Ventral ectodermal sensory neurons
VR	Ventral root
Psn	Peripheral sensory neurons
Rzc	Retzius cells
RBc	Rohon Beard cells
cg	Central ganglia
pg	Peripheral ganglia
ppn	Pheripheral parapodial nerve
VNC	Ventral nerve cord
ascM	Asciculum muscle
Trk	Tyrosinase receptor kinase
NT	Neurotrophin
ROR	RAR-related orphan receptor
MuSK	Muscle-Specific Kinase
TNF-R1	Tumor necrosis factor-related 1
TRADD	Tumor necrosis factor receptor type 1-associated DEATH domain protein
TNFRSF	Tumor necrosis factor receptor superfamily
Aply	*Aplysia*
Dpulex	*Daphnia pulex*
Pdu	*Platynereis dumerilii*
Nv	*Nematostella vectensis*
Ct	*Capitella teleta*
Bf	*Brachiostoma floridae*
GalG	*Gallus gallus*
h	*human*
Dm	*Drosophila melanogaster*
Sp	*Strongylocentrosus purpuratus*
m	*mouse*
Xl	*Xenopus laevis*
Lg	*Lottia gigantea*
Hr	*Helobdella robusta*
Sk	*Saccoglosus kovalensky*
Lymnea	*Lymnea staginalis*
mitF	Microphthalmia-associated transcription factor
trp	Transient receptor potential cation channel
vGlut	Vesicular glutamate transporter
VAChT	vesicular acetylcholine transporter
5-HT	5-hydroxytryptamine

SUMMARY

The neural crest is indisputably one of the major vertebrate innovations.

Neural crest arises at the neural plate border and is the source of many cell types, such as those of the peripheral nervous system (sensory, autonomic neurons and supporting cells), pigments and cartilage. This region of the neural plate also gives rise to Rohon Beard cells (RBc, primary sensory neurons) that differentiate from the same precursor cells of the neural crest (Rossi, Kaji, & Artinger, 2009), (Jacobson, 1981).

Despite the recent proposal for neural crest-like cells in basal chordates (Jeffery, Strickler, & Yamamoto, 2004), and the postulation of the origin of neural crest from migrating Rohon Beard cells -like cells, the evolution of the neural crest remains obscure. The aim of my PhD was to shed light on the evolution of such a special cell population in bilaterians.

Using classical whole mount in situs, Edu pulse experiments, live imaging and drug treatments I studied the development of the *pax3/7+* lateral neuroectoderm of the marine worm *Platynereis dumerilii.* I used *Platynereis* because it is a protostome that retains ancestral features and it has been successfully used in previous studies to investigate cell type evolution.

I investigated the lateral trunk region because it has been recently proposed that this domain corresponds topologically and molecularly to the dorsal neural tube, where the vertebrate neural crest originates (Denes et al., 2007)

I found that the *pax3/7+* territory is set very early in development and expresses Rohon Beard cells and neural crest specific genes, such as *prdm1-a* , *msx*, *ap-2* and *snail*. Furthermore, I found that canonical Wnt signaling controls the patterning of the annelid lateral neuroectdoderm, as in vertebrates.

Next, I analyzed the fate of the cells emerging from this lateral territory. I found that sensory differentiation genes are turned on in *ngn+* precursor neurons in a temporal sequence, similar to the one occurring in the neural crest derived sensory neurons (Marmigère & Ernfors, 2007), (Lallemend & Ernfors, 2012). The annelid neurons that arise from the lateral *pax3/7+* domain have molecular features of the Rohon Beard-like cells and visceral sensory neurons. I found that also putative supporting cells ensheathing the axons arise peripherally.

Next, I asked whether the other typical cell types that are neural crest-derived in vertebrates are present in *Platynereis*. I found that *MitF* + melanoblasts , putative enteric neurons as well as collagenous skeleton are also present in *Platynereis,* but apparently do not arise from the lateral domain.

The development, survival and axon-pathfinding of the neural crest derived-sensory neurons depends on the neurotrophic signaling (Davies, 1994), (Gershon, 1994), (Tessarollo, 1998), (Sieber-Blum, 1998),(Ernsberger, 2009) Furthermore, the evolution of the neural crest has been associated with the emergence of this pathway, considered for long time a vertebrate innovation (Wittbrodt, 2007). This prompted me to search for the neurotrophic molecules in *Platynereis dumerilii*. I found that all the molecules of the canonical neurotrophic signaling are present in the worm and show vertebrate-like molecular features. They are widely expressed in the nervous system, therefore they likely act during neuronal development. This finding refutes the belief that neurotrophic signaling is a chordate novelty: a hypothesis based on a lack of conservation in other protostomes such as *Drosophila* (Pulido, Campuzano, Koda, Modolell, & Barbacid, 1992)and *Lymnea* (Beck et al., 2003) .

Collectively, these annelid data suggest that the formation of Rohon Beard-like sensory neurons, putative visceral sensory neurons and supporting cells were already a feature of the cells emerging from the lateral neuroectoderm (a neural plate-like territory) at the dawn of bilaterians. A gradual co-option of genetic modules acting in other tissues into the neural plate-like territory might have driven the evolution of *bona fine* neural crest.

Moreover, this study suggests that a vertebrate-like neurotrophic signaling was already in place in the ancestor of bilaterians and put forwards new hypotheses on the evolution of such a signalling from non bilaterian molecules and on the involvementof the neurotrophic signaling in the evolution of the neural crest.

ZUSAMMENFASSUNG

Die Neuralleiste ist unumstritten eine der bedeutenden Wirbeltierinnovationen.

Sie entsteht am Rand der Neuralplatte und ist Ursprung vieler Zelltypen, wie die des peripheren Nervensystems (Sinnesneuronen, autonome Neuronen und Stützzellen), der Pigmente und von Knorpel. Aus dieser Region kommen auch die Rohon-Beard-Zellen (RBZ, primäre Sinneszellen), die sich aus den gleichen Vorläuferzellen wie die Neuralleiste differenzieren.

Trotz der kürzlich gemachten Vorschlage über neuralleistenähnliche Zellen in basalen Chordatieren und über den Ursprung der Neuralleiste aus migrierenden Rohon-Beard-ähnlichen Zellen bleibt die Evolution der Neuralleiste unklar. Ziel meiner Doktorarbeit war es daher, Aufschluss über die Evolution dieses besonderen Zellbestandes in Bilaterien zu geben.

Ich untersuchte die Entwicklung des *pax3/7+* lateralen Neuroektoderms des marinen Wurms *Platynereis dumerilii* mithilfe von Whole-Mount-In-situ-Hybridisierung, Edu-Puls-Experimenten, Live-Imaging und unter Zugabe verschiedener Substanzen. Ich arbeitete mit *Platynereis*, da dies ein Urmünder (Protostomia) ist, welcher Urmerkmale bewahrt und bereits in anderen Studien erfolgreich zur Untersuchung der Evolution von Zelltypen benutzt wurde. Ich erforschte die laterale Rumpfregion, da jüngst vorgeschlagen wurde, dass diese Region topologisch und molekular dem dorsalen Neuralrohr der Wirbeltiere entspricht, in der die Neuralleiste ihren Ursprung hat (Denes et al, 2007).

Ich habe herausgefunden, dass die *pax3/7+* Region bereits sehr zeitig in der Entwicklung festgelegt wird und kennzeichnende Gene für Rohon-Beard-Zellen und Neuralleistenzellen wie *prdm1-a*, *msx*, *ap-2* und *snail* exprimiert. Desweiteren fand ich, dass der kanonische Wnt-Signalweg genau wie in Wirbeltieren die Musterung des lateralen Neuroektoderms des Anneliden kontrolliert.

Darüber hinaus analysierte ich den Werdegang der in dieser lateralen Region aufkommenden Zellen. Ich fand, dass in *ngn+* Vorläuferneuronen Differenzierungsgene in einer zeitlichen Abfolge exprimiert werden. Dies ist ähnlich zu den Vorgängen in Sinnesneuronen, die aus der Population der Neuralleistenzellen in Wirbeltieren entstehen. Die Neuronen des Anneliden, die aus der *pax3/7+* Region entspringen, besitzen molekulare Eigenschaften der Rohon-Beard-Zellen und viszeraler Sinnesneuronen. Ich fand auch, dass mutmaßliche Stützzellen, die die Axonen einhüllen, peripher entstehen.

Überdies untersuchte ich, ob auch die anderen typischen Zelltypen, die in der Neuralleiste der Wirbeltiere ihren Ursprung haben, in *Platynereis* vorhanden sind. Ich entdeckte, dass *MitF+* Melanoblasten, mutmaßliche enterische Neuronen und ein Kollagengerüst zwar in *Platynereis* vorhanden sind, jedoch nicht aus der von mir untersuchten lateralen Region stammen.

Die Entwicklung, das Überleben und das Pathfinding der Axonen der Neuralleistenneuronen hängt von Neurotrophinsignalen ab. Außerdem wurde die Evolution der Neuralleiste mit der Entstehung dieses Signalwegs assoziiert, der lange Zeit als eine Wirbeltierinnovation angesehen wurde. Das brauchte mich darauf, auch in *Platynereis* nach Neurotrophinmolekülen zu suchen. Ich fand, dass alle Moleküle des kanonischen Neurotrophinsignalswegs in *Platynereis* vorhanden sind und wirbeltierartige molekulare Eigenschaften haben. Sie sind weithin im Nervensystem exprimiert und agieren daher wahrscheinlich während der neuronalen Entwicklung. Diese Erkenntnis widerspricht der Annahme, dass der Neurotrophinsignalweg eine Neuheit in der Linie der Chordatiere ist; eine Hypothese, die aufgrund des Fehlens von vergleichbaren Zellen in anderen Urmündern wie *Drosophila* und *Lymnea* aufgestellt wurde.

Zusammenfassend weisen diese Anneliddaten darauf hin, dass die Ausbildung Rohon-Beard-ähnlicher Sinneszellen, mutmaßlicher viszeraler Sinnesneuronen und von Stützzellen bereits eine Eigenschaft der vom lateralen Neuroektoderm stammenden Zellen in der Frühzeit der Bilaterien war. Eine allmähliche Kooption genetischer Module anderer Gewebe in den neuralplattenartigen Bereich könnte die Evolution einer *bona fide* Neuralleiste angetrieben haben.

Zudem legt diese Studie nahe, dass ein wirbeltierartiger Neurotrophinsignalweg bereits im Urahn der Bilaterien vorhanden war und schlägt neue Theorien für die Evolution eines solchen Signalswegs ausgehend von Molekülen von Nicht-Bilaterien und die Beteiligung des Neurotrophinsignalwegs in die Evolution der Neuralleiste.

INTRODUCTION

I. The evolution of the nervous system in bilaterians

The main features defining a vertebrate are: a dorsal nerve cord with a prominent brain, the neural crest cells and their derivatives, the placodes, the notochord, the backbones and also the neurotrophines. Understanding how and when those novelties have originated is one of the major questions in the field of evolutionary developmental biology (EvoDevo) (R. A. Raff 2000).

Although classical morphological comparisons and embryology have provided much insight into conserved traits among animals, the advent of molecular comparisons have revolutionized our view of the evolution of cell types and have revealed deep homologies that were otherwise hidden.
Due to this kind of comparison it is now widely accepted that, despite their different positions in the body plan, both the invertebrate ventral nervous system and the vertebrate dorsal one derive from an ancestral nervous system that was present at the base of Bilateria, in the '*Urbilaterian*' ancestor 500 MYA(De Robertis E M & Sasai 1996) (fig.1).

-The dorso-ventral inversion and the conservation of mediolateral patterning

Molecular evidence suggested that a dorso-ventral inversion (D-V inversion) of the body plan occurred during evolution (as originally proposed by Anton Dohrn in 1875) and relocated the ancestral nervous system (similar to the ventral nerve cord of extant invertebrates) to the dorsal side, to form the vertebrate neural tube (Arendt and Nübler-Jung 1994),(Arendt and Nübler-Jung 1999). For example, BMP is expressed on the ventral non-neural ectoderm in vertebrates, and on the dorsal side in annelids and insects, always in opposition to the neurogenic field.
Moreover, it was later discovered that similar molecular coordinates pattern the neural plate from medial to lateral in vertebrates, annelids and arthropods (Arendt and Nübler-Jung 1999), (Denes et al. 2007).

-Homologous cell types

Nevertheless, despite enthusiasm by evolutionary biologists to uncover features of the *Urbilaterian ancestor*, little is known about the repertoire of cell types that were most likely present. It is also unclear which cell types might have arisen by convergence in different groups of animals. For example, while a clear homology between photoreceptor cells is assumed across phyla (Arendt 2003), (Arendt et al. 2004), (Ullrich-Lüter et al. 2011), the relation between peripheral sensory organs in different animals in not as straight forward. This is particularly apparent when considering that neural crest cells give rise to a subset of the sensory neurons in vertebrates, yet do not have any convincing homologues in invertebrates thus far.

-Choice of species for comparison

Now, assuming that variations in developmental modes have driven the evolution of new cell types (R. A. Raff 2000) , we need to compare the development of animals belonging to groups that are phylogenetically informative. In this way we are able to understand if a certain trait is an innovation in one group, or if it was present in the ancestor and then subsequently lost during the evolution of one specific group.
Animals are considered 'phylogenetically informative' if they are good representatives of the major transitions that have occurred during evolution. For example, representatives

of the transition from non-bilaterians to bilatererians, from protostomes to deuterostomes and from chordates to vertebrates. For instance, comparison of neural development of the Anthozoa (among Cnidaria the less derived non-bilateral Eumetazoa, fig.1) and of the Bilateria is needed to understand how changes in body plan and in the nervous system occurred during evolution. Such changes include the acquisition of bilateral symmetry, the establishment of an orthogonal nervous system with cell bodies clustered in ganglia and the nerves into thick bundles, rather than sparse in a net.

For these types of comparisons, it is also important to choose animals that have accumulated little 'noise' during their divergence from the common ancestor. These animals are 'slow evolving': examples are the annelids, used to understand the appearance of bilaterians, and cephalorcordates, used to understand what the ancestral chordate looked like (fig.1).

Studying only classical invertebrate model systems such as *C.elegans* and *Drosophila* would be misleading. This is because they evolved quickly, thus making it likely that homologous cell types are hidden or have been lost by the additional traits specific to that group of animals and their modified life cycle and niches.

I.1 *Platynereis* as a model system for studying the evolution of the nervous system

I.1.1 Why annelids?

Annelids are representatives of the super-phylum Lophotrochozoa, which makes up a large diversity of the larger protostome clade. Lophotrochozoa, Ecdysozoa (such as *Drosophila*) and deuterostomes form the three major branches of the Bilateria (fig.1). Members of the Lophotrocozoa have not been extensively studied molecularly compared to classical model systems. An extensive comparison between different protostomes, including lophotrochozoans such as *Platynereis*, and deuterostomes is needed to address Bilaterian synapomorphies.

The advantage of studying polychaete annelids lies also in the fact that they are 'slow evolving animals'. This means that their development and cell type repertoire has not diverged dramatically from their ancestral state in comparison to other protostomes.

As a result, they possess few specific morphological features that evolved in the phylum after the bilaterian radiation (Tessmar-Raible and Arendt, 2003). Accordingly, they are among the first fossils identified from the Cambrian records (Morris & Peel, 2008, Shu 2008). Moreover, polychaete annelids possess a prototypical ladder-like nervous system shared with other protostomes.

I.1.2 Why *Platynereis dumerilii?*

Platynereis dumerilii is a marine annelid easy to culture in the lab, producing large numbers of larvae with synchronized and stereotyped development.

It is amenable to many different molecular techniques, as is the case with conventional model systems for developmental biology, such as frogs and fishes.

The genome is now sequenced (from Dr. Tomas Larsson and Oleg Simakov in our lab, and other laboratories, unpublished), and large scale single and double whole mount in situ hybridization (Tessmar-Raible et al. 2005) are routinely used to assess the mRNA expression of the genes of interest. Moreover, reflection microscopy allows the

fluorescence detection of the NBT/BCIP precipitates after in situ staining (Jékely and Arendt 2007).

A tool which relies on this stereotyped embryonic development to perform *in-silico* alignments for the expression of different genes and to thereby assess co-expression at cellular resolution has been recently developed (PrImR: profiling by image registration, Tomer et al. 2010).

Recent developments of injection techniques (Tosches et al., unpublished) and live imaging (this work, contribution of Silvia Rohr and Dr. Mette Handberg-Thorsager) have led to the possibility to follow live development of the larvae.

Techniques to obtain transposon-mediated transgenic animals have also been developed (this study, Arendt lab and other labs) and the first functional studies via morpholino-based gene knock down have been performed (Tosches et al, unpublished).

Furthermore, belonging to the annelids, *Platynereis dumerilii* possesses many ancestral features, compared to other invertebrate models (F. Raible et al. 2005). Its nervous system can then be compared to one of other invertebrate or vertebrate model systems in order to reconstruct the ancestral nervous system that was present at the base of Bilateria.

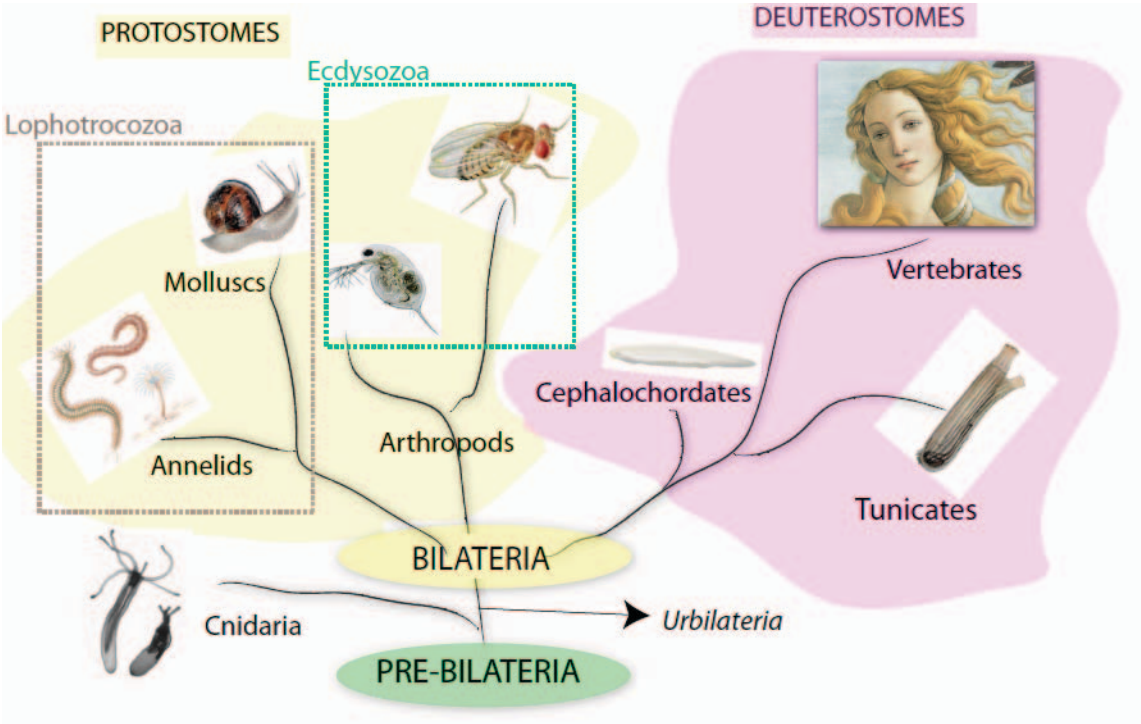


Figure 1. Tree representing the position of the main Eumetazoan groups. Among bilaterians, (Eumetazoa with three germ layers and bilateral symmetry, also called triploblasts) molecular phylogeny has identified three distinct groups: Lophotrocozoa (annelids, molluscs), Ecdysozoa (insects and crustacean) and Deuterostomia. Lophotrochozoa and Ecdysozoa fall within the Protostomia (animals that develops the mouth first from the blastopore). The Deuterostome clade (that develops the anus first from the blastopore) is comprised of chordates such as the cephalocordates, the tunicates and vertebrates. The Cnidaria, a group of diploblastic animals with no bilateral symmetry, are an outgroup to the Bilateria.

I.1.3 The life cycle of *Platynereis dumerilii*

-External fertilization

The adult *Platynereis* worms form *epitokes* (6 in fig.2) that swim in the seawater searching for a partner. The female releases eggs and the male releases sperm in the seawater and external fertilization occurs. A detailed representation of the life cycle is in fig.2(adapted from Fischer and Dorresteijn 2004), a schematic representation of the larval features at 48h and 72h is shown in fig.3.

-The pelagic phase

In the first pelagic phase of their life, the embryos (*trochophore larvae,* 2 in fig.2, fig.3A) swim by means of the ciliary bands (from anterior to posterior they are called: *prototroch, metatroch, paratroch and telotroch ,* (Fischer et al., 2010) toward the light; during this phase they belong to the plankton. A brain region and a trunk region are already distinguishable and separated by the main ciliated band (*prototroch*). The brain region is formed by a cluster of sensory cells called 'the apical organ' (Marlow et al., unpublished a primitive form of the brain), eyes (Jékely et al. 2008) and photoreceptors (Arendt et al., 2004).

The ventral nerve cord represents the trunk nervous system, and most posteriorly, a growth zone (pgz: posterior growth zone) is present.

-The benthic phase

At around three days after fertilization the trunk region elongates and visible lateral appendages appear (*parapodia*). The young worms are now called *nectochaetes* and they start to swim by means of the musculature that continues developing during this time (4 in fig.2, fig.3B).

After this stage the development is no longer stereotypical between animals, as they start to eat, settle to undergo metamorphosis, and become benthic. Morphological changes occur (5 in fig.2). For example, the first trunk parapodium is transformed into an appendage bearing cirri, which is more similar to head structures.

Furthermore the worms develop external sensory organs such as palpae and antaenne and grow more segments in the trunk region. This *atoke* worm (5 in fig.2) builds its own tube and lives there growing and maturing gametes.

When maturation of gametes is complete, the *epitoke* worm (6 in fig.2) starts to search for partners, and the life cycle starts again.

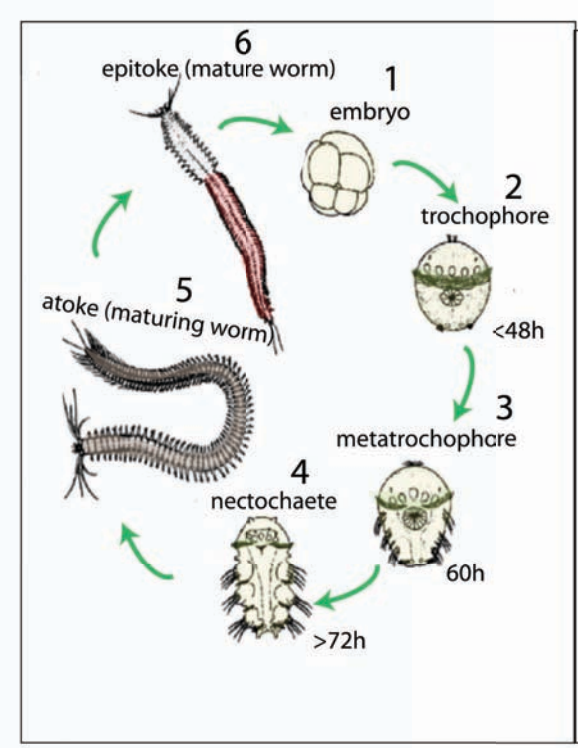


Figure 2. The *Platynereis* life cycle The fertilized eggs develop via spiral cleavage and form the *trochophore* larva that swims by means of the ciliary bands. After 2 days of development, the juvenile worm forms peripheral appendages (parapodia) and starts to swim by means of the muscles that have developed. Metamorphosis will transform the juvnile into a young worm that matures gametes (modified from an original image, courtesy of Guillaume Balavoine).

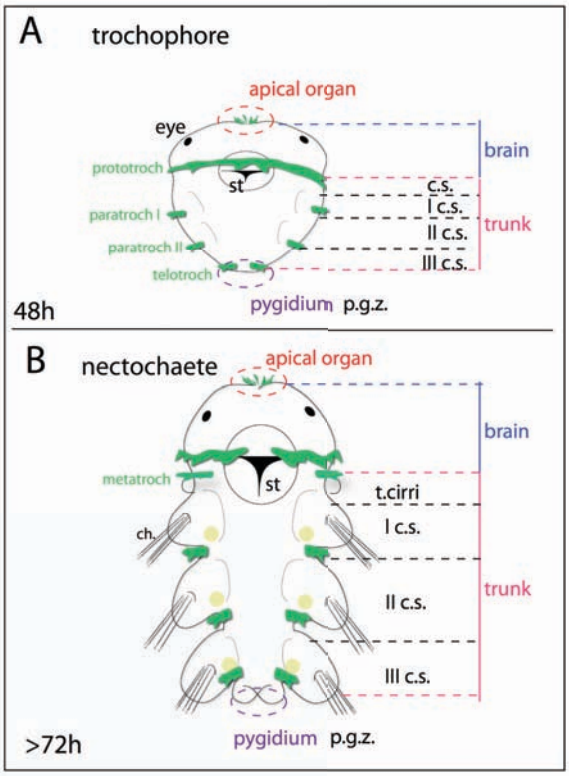


Figure 3. The *Platynereis* larval morphology of the trochophore and nectochaete stages. Main features of the trochophore larva (A) and of the nectochaete larva (B). Apical organ, brain and trunk regions are indicated. C.s.: criptic segment, I-III ch.s. : I- III chaetophorous segment, pgz: posterior growth zone, t.cirri: tentacular cirri, st: stomodeum, the different ciliary bands are indicated in B. The metatroch forms after 48h and it is indicated in C.

I.2 The cell type comparison approach to study the evolution of the nervous system

Comparative anatomy and morphology have revealed true homologies between characters in different animals such as the limbs in humans, bats and dogs. Nevertheless, these types of studies are not always effective in discriminating between homology (characters shared by a group of animals because they are inherited from their common ancestor) or homoplasy, which implies convergence of the characters (evolved independently in the two groups that we are comparing).

Moreover, when comparing groups of animals that are phylogenetically distant such as annelids and vertebrates, morphological comparisons are often not sensitive enough to detect 'latent'' homologies (de Beer 1971).

These homologies can be revealed only if a molecular comparison is added to the classical morphological one. For example, homologous regions of the body plan have been revealed by similar *Hox* gene expression in animals as different as mice and flies .

Because body plans have changed enormously during Bilaterian evolution, acquiring many different new features, it is useful to perform a molecular comparison of the basic units defining an organism: the *cell types* .

Comparison of the molecular fingerprints at the cellular level across animals has been demonstrated to be a powerful approach in assessing homology, as for example, in the case of photoreceptors (Dufour et al. 2006),(Detlev Arendt 2005) ,(Detlev Arendt 2008a).

I.2.1 The cell specific *molecular fingerprint*

A cell type is defined by its function. A sensory neuron of the ear that perceives sounds differs from a secretory cell of the adrenal gland that is instead specialized in producing and secreting catecholamines.

The genes responsible for such specific phenotypes are 'differentiation genes', and effector genes (Hobert 2008), (Arendt 2005),(Arendt 2008), such as sensory channels for the sensory neurons and specific enzymes for the chromaffin cells of the adrenal gland.

A cell type is also defined by a code of 'developmental genes', transcription factors that determine its development. The latter reflects the developmental history of a certain cell type (Arendt 2005),(Arendt 2008).

To define homology between cells in different organisms one should take into account the molecular signature defined by developmental genes and effector genes (Arendt 2008).

It is indeed easy to imagine that differentiation cassettes can be recruited in cell types which did not share a common precursor cell during evolution, such as different kinds of sensory cells across animals. Therefore, it becomes necessary to take into consideration the conservation of the developmental history at the molecular level. Therefore the ontogeny of the cell type, if conserved, is a strong indication of homology. Clear examples are the ciliary photoreceptors expressing ciliary *opsins* and developing from the *rx* + brain region both in annelids and vertebrates (Arendt et al., 2004).

Moreover, one should also take into account the *position* of the cell within the body plan, a test for homology that is also used in classical morphological comparisons (Remane, 1952), (Patterson 1988) (Laubichler 2000) . For this test, the comparison of patterning genes that define the anterior-posterior and dorso-ventral coordinates in different body plans is very helpful. For example, motorneurons always arise in the *pax6+* domain of the nervous system and mark the medial portion of the neuroectoderm (Ericson et al. 1997),

(Denes et al., 2007).

I.2.2 Emergence of new cell types and increase of complexity in the nervous system

New cell types most likely emerge via segregation of functions (*division of labour model)* from an ancestral multifunctional cell type (Arendt 2008), and through the acquisition of new functions.

One could imagine that in the steps from the Eumetazoa to the evolution of bilaterians, the emergence of an integrated circuit, comprising sensory neurons, interneurons and motoneurons that leads to a muscle response, started with a single cell that was sensory and motor (Mackie, 1970, Arendt,2008; Jékely, 2011). From this original cell type, specialized cells emerged: sensory neurons, interneurons, motoneurons and muscle cell. In this context, the evolution of axon tracts might have been necessary to keep cells in communication, forming a circuit. If this is the case, then the molecular fingerprint should be able also to reveal '*sister cell types*' (Arendt, 2008) which derive from segregation of function.

Although comparing developmental histories of the cells can help to decipher homology, we have to consider that even developmental processes, and therefore regulatory modules (kernels) of a gene network can be modified, lost and co-opted during evolution (Davidson and Erwin 2006).

The plasticity of the gene regulatory networks most likely represents the key to the evolution of new cell types. Changes in regulatory modules can indeed confer new properties to old cells, which then acquire new functions.

The neural crest (that differentiates into a plethora of different derivatives) offers one of the best examples of this, and challenges the concept of cell type conservation. Molecular comparisons between different animals possessing or lacking *bona fide* neural crest cells (several works and this work) (Meulemans and Bronner-fraser 2007) (Sauka-spengler et al. 2007) (Yu et al. 2008) have suggested that new 'kernels' (modules of regulatory networks) were recruited in the original cell type over the course of evolutionary time . This probably occurred in a stepwise manner and conferred new features to the cell, which resulted in one of the most important vertebrate innovations.

In summary, the molecular fingerprint approach, combined with classical morphogical studies, should be able to uncover conserved cell types of the nervous system and shed light on how they specialize or diverge during evolution. To implement this approach, we need to compare the cell type inventory in different informative groups of animals (as discussed previously), and then we will be able to reconstruct the features of the nervous system of the basal bilaterians.

II. The organization of the vertebrate nervous system along the A-P and D-V axes

II.1 Formation of boundaries and functional units in the vertebrate neural tube

Very early in development, the nervous system begins to undergo regionalization into two major axes which run orthogonally in relation to one another: the anterior-posterior (A-P) and the dorso-ventral (D-V) axes. This 'regionalization' is achieved via the nested

expression of transcription factors and signaling molecules, long before the different segments are morphologically visible (fig.4 for details) (James L. Roberts, Nicholas C. Spitzer, Michael J. Zigmond , Susan K. McConnell 2008)

The emergence of such a precise 2-D grid is crucial, it determines where certain neurons are born and how they connect to each other and to their targets.
Surprisingly, many patterning molecules, as well as primary and some secondary organizers are conserved across different animals. For example, orthogonal organizer molecules such as Wnt, Bmp and the *hox* genes are conserved, suggesting that a basic subdivision of the nervous system in A-P and D-V is a shared feature of Bilateria.

II.1.1. Subdivions in the vertebrate nervous system along the A-P axis (fig.4)

In vertebrates, the anterior-most part of the nervous system is further subdivided into different vesicles, the prosencephalon (**forebrain**), mesencephalon (**midbrain**) and the rhombencephalon (**hindbrain**) that will comprise the brain.
Caudally, the neuroectoderm is more homogenous and forms the **spinal cord** just posterior to the medulla oblongata (still part of the hindbrain). Explant experiments and mutants have revealed that the anterior region is not responding to Bmp and Wnt (Fekany-Lee et al. 2000) . For example, Cerberus produced by the prechordal plate (part of the head organizer) most likely inhibits Bmp (Belo et al. 1997), while Wnt promotes the formation of the forebrain(S. W. Wilson and Houart 2004).
In general Wnt and RA (retinoic acid) signaling act more posteriorly, in the trunk/tail region, since degrading enzymes for RA and Wnt antagonists are present in the anterior neuroectoderm. Fgf8 is also crucial in posteriorizing the nervous system (James L. Roberts, Nicholas C. Spitzer, Michael J. Zigmond , Susan K. McConnell 2008)
Anteriorly, the *zona limitans intrathalamica* inside the forebrain defines a primary boundary via the mutual expression of *six3* and *irx3*(Puelles and Rubenstein 2003). The expression of *pax6* and *en1* defines the **forebrain-midbrain boundary** and the opposition of otx2 and gbx2 defines the **midbrain-hindbrain boundary**. The expression of *wnt1*, *engrailed*, *pax2/5/8* and *Fgf8,17,18* confer organizer properties to this region (also called the isthmus organizer) which then functions in patterning the cerebellum in rhombomere 1 (Wurst and Bally-Cuif 2001),(Puelles and Rubenstein 2003).The subdivision of the hindbrain into segmental swellings through the expression of *krox20*, *ephrins*, and *Kreisler* as shown in fig.4., is essential for the diversification of different neuronal domains(Voiculescu et al. 2001) (Wurst and Bally-Cuif 2001), (Pourquié 2009).

In addition to this topology, the '*hox* gene code' is superimposed (Pourquié 2009), (James L. Roberts, Nicholas C. Spitzer, Michael J. Zigmond , Susan K. McConnell 2008) . *Hox* genes play a pivotal role in conferring positional information to the neuronal precursors. They are expressed in a collinear manner along the A-P axis and in vertebrates they are responsive to RA signaling, controlling the patterning of the posterior-most neuroectoderm. *Hox2* and *3* are expressed in the rostral rhombomeres and *hox4* starts caudally, from r7. *Hox* genes are regulated by RA and also through the *cdx* genes, as in *Drosophila* (Moreno and Morata 1999), (Häming et al. 2011) . For instance, the co-expression of *hox1-hox4* starts in the caudal hindbrain at the transition between the hindbrain and spinal cord territory due to the expression of *cdx* genes (Skromne et al. 2007) *Cdx* genes ,in turn, induce the expression of the most posterior *hox* genes, such as *hox* 5,6,9, 10(Shimizu, Bae, and Hibi 2006).

Zebrafish mutants for *cdx 1* and *4* lack the spinal cord, which is replaced by duplicated caudal rhomobomers. The spinal cord itself is divided into different regions, the more anterior one produces LMCs (lateral column motoneurons) that project to the limbs. More posteriorly, autonomic motoneuons are generated and innervate the body wall within a territory that is patterned by the collinear expression of *hox5,6, 8, 9,10* (Pourquié 2009). Canonical Wnt signaling, necessary for the formation of posterior tissue, regulates cdx (Faas and Isaacs 2009)

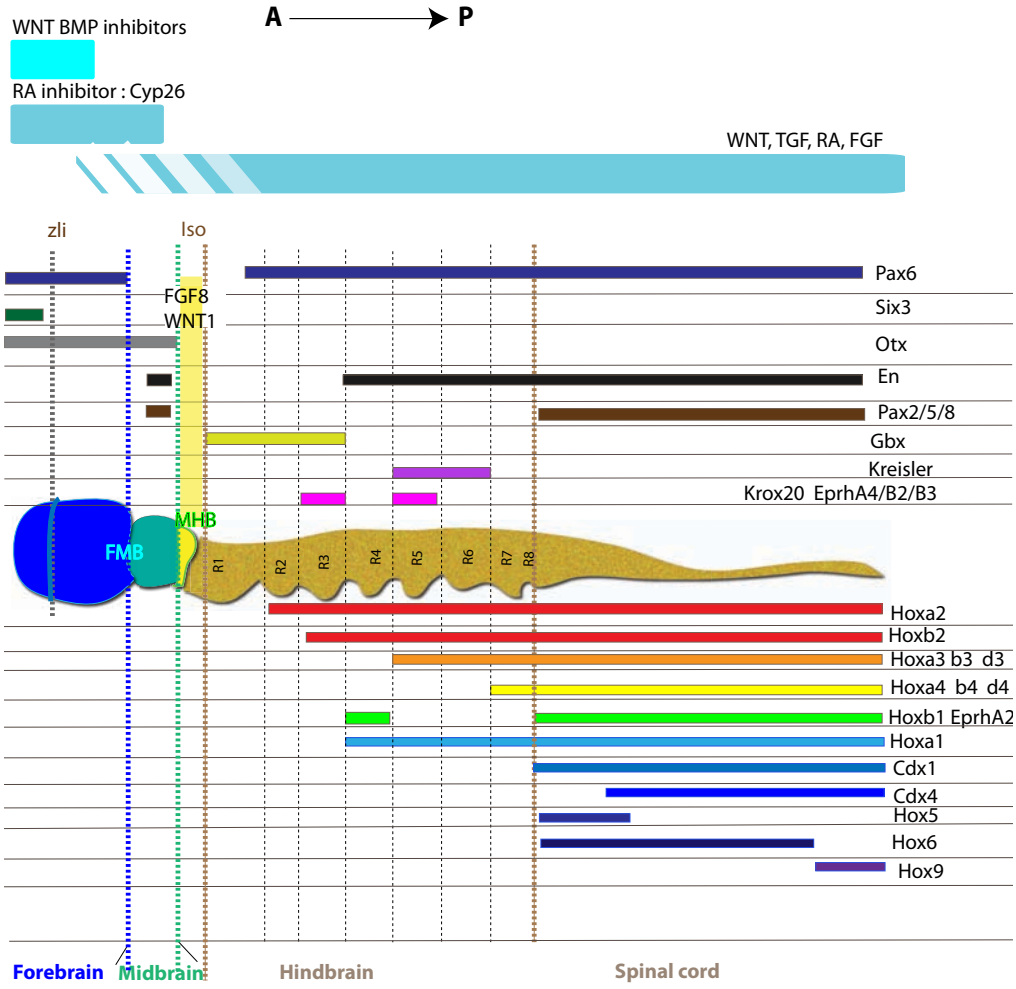


Figure 4. AP patterning along the developing vertebrate neural tube. The anterior neuroectoderm is specified in the region where WNT and BMP antagonists are active. WNT, BMP, RA, FGF signaling act more posteriorly where hindbrain and spinal cord are specified. RA is degraded more anterirorly by Cyp26. Zli: zona limitans intrathalamica. Iso: Isthmus organizer. The drawing is based on the references cited in the main text

II.1.2 The D-V axis

A D-V (dorso-ventral) axis orthogonal to the A-P axis is also present (fig.5), and is essential in determining the precise coordinates where specific neurons will born. For example, in the spinal cord, the ventricular zone is immediately adjacent to the lumen and contains proliferating precursors, while the mantle zone, more dorsally, contains differentiated neurons (Altmann and Brivanlou 2001).
Along the dorso-ventral axis, different classes of sensory neurons are generated in the dorsal-most position, where specific combinations of transcription factors such as *dbx, pax7, msx* are expressed. Motoneurons are formed in the ventral-most part of the *pax6* and *nk6* domains (Briscoe et al. 1999). This will be briefly explained.

-Motoneurons and interneurons in the ventral neural tube : sFRP/Wnt antagonism
Different kinds of motorneuons are formed in the *pax6* and *nk2.2* domain under the control of secreted Shh from the floor plate and notochord (Briscoe et al. 1999),(Briscoe and Ericson 1999). Recent findings have shown that Wnt inhibitors modulate the response of the motoneurons to Shh (Lei et al. 2006).For example, the boundary between *pax6* + PMN domain and the *nk2.2* + p3 domain is kept via the activity of sFRP2, an inhibitor of Wnt expressed in the *pax6* domain (Kim et al. 2001), (Lei et al. 2006). (fig.5). In vertebrates, in the absence of sFRP2, the *pax6*+ precursors are indeed converted into *nk2.2* + precursors. The mechanism is not completely clear, but it leads to the activation of the repressor *Tcf4*. *Tcf4* binding sites are present on the *nk2.2* enhancer and mediate downregulation *nk2.2* in its dorsal-most limit (Lei et al. 2006).

-Sensory interneurons and neural crest in the dorsal neural tube
The same dorsal territory of the neural plate that gives rise to sensory interneurons, also produces neural crest in its dorsal-most limit, in a territory patterned by *pax3/7, dll and msx* (Briscoe et al. 1999)Altmann & Brivanlou, 2001,Liu, Helms, & Johnson, 2004, fig.5, see also chapter III of the Introduction). The patterning of this territory is controlled by canonical Wnt signaling (Muroyama et al. 2002). Indeed, after stabilization of ß-*catenin*, the dorsal territory is expanded. For instance, dorsal transcription factors such as *pax7* and proneural genes such as *mash*1 extend more ventrally, at the expense of ventral genes such as *nk6.2*, that are slightly reduced.

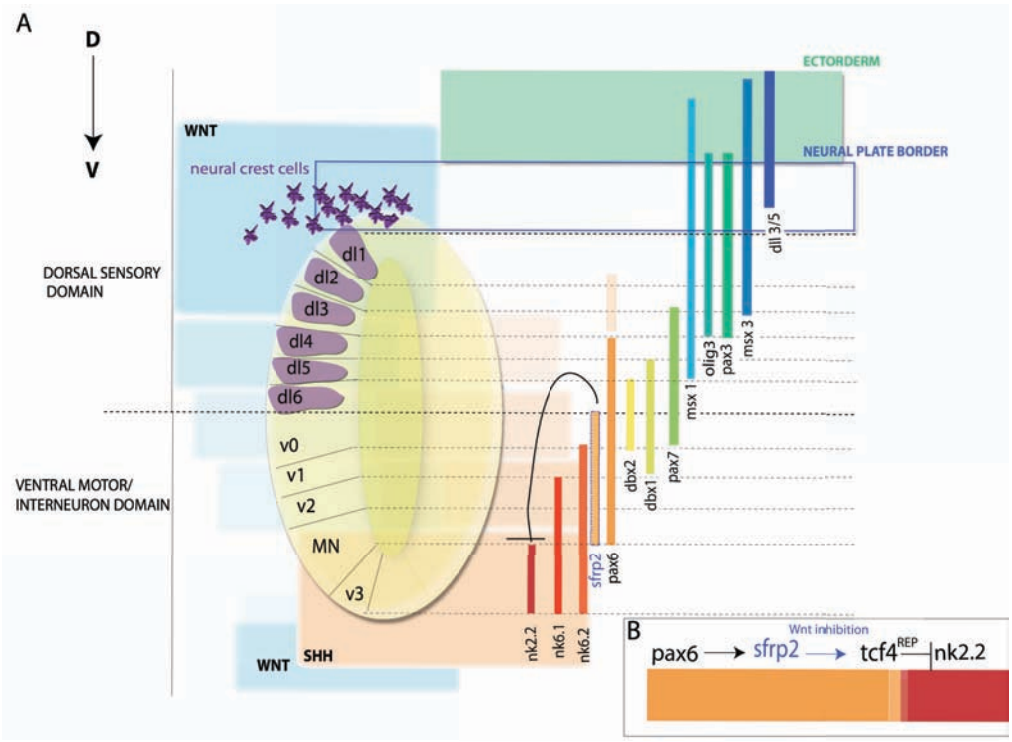


Figure 5. D-V patterning along the developing neural tube of generic vertebrates. The ventral domain is influenced by SHH and produces different types of motoneurons and interneurons. The dorsal-most domain is influenced by different WNT and BMP signaling and produces sensory intereneurons. It also gives rise to the neural crest in the dorsal-most part that borders with the ectoderm, the neural plate border. Nested and specific expression of different transcription factors regionalizes the neural plate along the dorso-ventral axis as shown for the anterior-posterior axis. **B:** At the boundary between PMN and P3 domain, the inhibition of Wnt is mediated via sFRP2 expressed in the *pax6* PMN domain. The repressor TCF4 is activated and downregulates the transcription of nk2.2 in its dorsal most domain. The drawing is based on the references cited in the main text.

II.2 Nervous system patterning and boundary formation in invertebrates

Molecules implicated in AP and DV patterning are conserved across different animals and expressed in comparable territories of the nervous system. For example, primary organizers such as the Wnts seems to act in axis patterning already in Cnidaria (Guder et al. 2006),(Meinhardt 2006); therefore they most likely predated the emergence of Bilateria. It has been proposed that the Eumetazoan ancestor had radial symmetry, similar to the extant Cnidaria, and that its posterior-most side gave rise to the trunk of bilaterian animals via convergent extension movements and the formation of a slit-like blastopore (Dr. Steinmetz PhD thesis 2006, Meinhardt 2006), as has been described in *Platynereis* (Dr. Steinmetz PhD thesis 2006*).* This led to the separation of the *six3-otx* anterior domain and the *hox1-hox4* posterior-most domain.

Beyond this, while a comparison of the anterior-most domains of the nervous system is possible across animals, including Cnidaria (Marlow et al, unpublished), ecdyzozoa, protostomes (Steinmetz et al. 2010), the comparison of the caudal-most nervous system is still controversial. There is no consensus so far, because the expression patterns of only a few genes are conserved across animals.

For instance, a MHB is present in tunicates(Lacalli, 2006,Wada, Saiga, Satoh, & Holland, 1998) and hemichordates (Pani et al. 2012) but missing in amphioxus, at least in the stages studied so far. Amphioxus *pax 2/5/8* is expressed broadly in the area that would correspond to the caudal neuroectoderm in amphioxus (Pfeffer et al. 1998), therefore not corresponding to a specific region of the trunk as in vertebrates. Metameric organization of the hindbrain is missing in amphioxus and tunicates: indeed, *amphiKrox20* is expressed in the cerebral vesicle that is the anterior-most part of the amphioxus nervous system(Knight et al. 2000). Therefore, it cannot be compared with the vertebrate expression. Conversely, clear rhombomeres are present in lamprey embryos, with conserved expression of *korx20* (Murakami and Kuratani 2008).
These data indicate that hindbrain segmentation as well as the separation between hindbrain-spinal cord territories arose at the base of vertebrates. Nevertheless, nested expression of *hox* genes is still present in a collinear way in amphioxus, suggesting that this might have predated the compartmentalization to rhombomeres. Furthermore, in amphioxus, it is likely that some of the *hox* genes are regulated by RA (Schubert et al. 2006), constituting a conserved protocordate patterning mechanism of the nervous system.

On the other hand, pioneering work in *Platynereis* from our lab as well as in *Drosophila* have contributed to identify a conserved *gbx+* territory, posterior to the *otx* domain, that might correspond to the vertebrate midbrain-hindbrain boundary (MHB). A caudal domain whit nested hox expression was also observed (fig.6) (Steinmetz et al. 2011).
Furthermore, additional studies have suggested that dorso-ventral patterning is also conserved in protostomes (Denes et al. 2007 and fig.6), indicating that it dates back to the ancestor of Bilaterian animals. This supports the idea that centralization of the nervous system is an ancestral feature as well (fig.6).
This studies revolutionized our view of what the ancestral nervous system looked like. It suggested that a centralized nervous system was already present at the base of Bilateria, with motoneurons arising in the ventral (medial) domain and likely sensory neurons in the dorsal (lateral) domain.

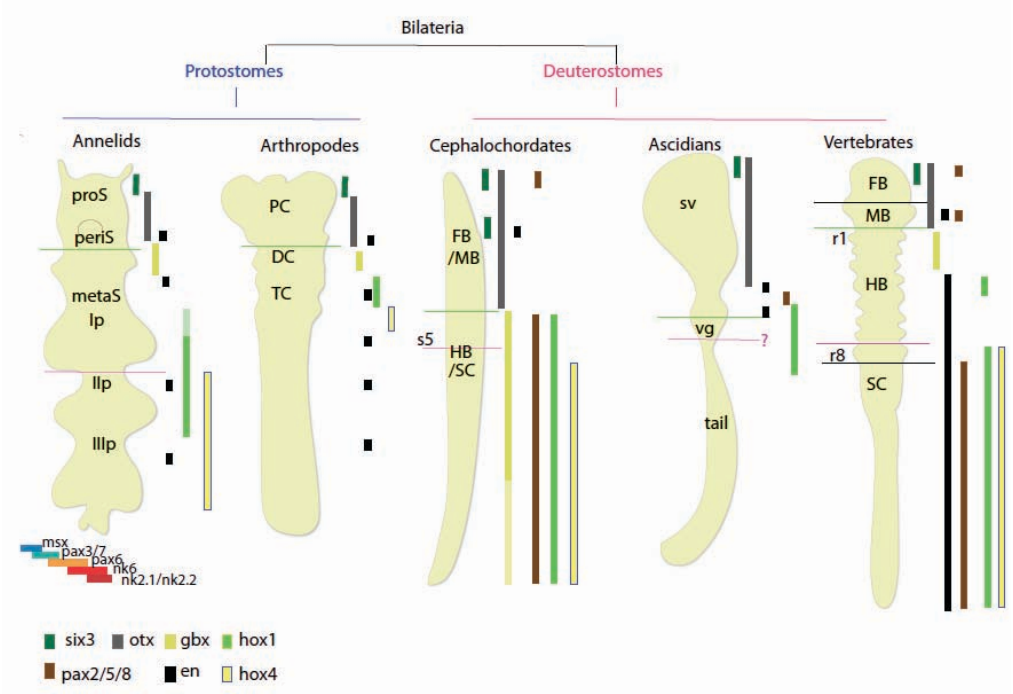


Figure 6. Conservation of patterning genes along the A-P and D-V axis in Bilateria

Green line in vertebrates indicates the MHB. The pink line indicates the start of the caudal hindbrain-spinal cord region. Putative homologus boundaries are indicated with the same colours in the other representatives of Bilateria as well. FB: forebrain, MB: midbrain, HB: hindbrain, SC: spinal cord sv: sensory vesicle, vg: visceral ganglion, PC: protocerebrum, DC: deutocerebrum, TC: tritocerebrum. s5: somite 5, r1, r8: rhombomere 1 and 8, proS: prostomium, periS: peristomium, metaS: metastomium, I-IIIp: I-III parapodial segment. Conservation of mediolateral patterning is also shown to the lower left of the figure.

Intriguingly, as previously noted, the dorsal-most domain of the neuroectoderm is the source of neural crest in vertebrates. Therefore, obvious questions are: how does the dorsal neuroectoderm of annelids (likely giving rise to sensory neurons) compare to the vertebrate dorsal neural tube? Are neural crest-like precursors already produced from the annelid dorsal (lateral) neuroectoderm?

To answer these questions, I analyzed the molecular fingerprint and the cell types emerging from the lateral-most region of the neuroectoderm of the developing larva of *Platynereis*. I focused this analysis of the trunk region, where the mediolateral coordinate were more evident.

III. Neural crest and cell identity at the border of the neural plate in development and evolution

III.1 Neural crest and its discovery

Anterior-posterior and dorso-ventral positional information direct where specific cell types will arise in the nervous system. For instance, the neural crest, a transient population of precursors, originates in the dorsal-most part of the neural plate, at the margin between the neural tissue and the outer ectoderm (fig.4,6).

The emergence of the neural crest represents one of the most important events in the evolution of vertebrates, as it gives rise to components of the vertebrate head and many neuronal cell types, such as the sensory, autonomic neurons and glial cells (Groves and

Bronner-Fraser 1999), (N. Le Douarin and Kalcheim 1999), (Gammill and Bronner-Fraser 2003).

Due to the diversity of derivatives that originate from the neural crest and its importance for the evolution of the vertebrates, in 1989 Thorogood claimed provocatively that '*the only interesting thing about the vertebrates is the neural crest' (*Thorogood, 1989)

The first to observe the neural crest was a professor of anatomy: Whilelm His, who in 1898 named it 'Zwischenstrang' (the intermediate cord). He described it as a band of cells between the neural tube and the ectoderm in developing chicken spinal cord, and he dentified these cells as a source of spinal ganglia (Votano, Parham, and Hall 2004).
The neural crest was later renamed the 'neural ridge' and then the 'neural crest' by Marshall, a professor of zoology in England, who independently identified them (Marshall 1879).
It was difficult to accept that the neural crest can also give rise to non-neuronal tissue, such as cartilage, mesenchyme and blood vessel cell types, as several studies had already demonstrated in the early 30's .

For this amazing developmental plasticity and potential, the neural crest cells have been defined as the 'fourth germ layer', to distinguish their developmental potential from those of the ectoderm, mesoderm and endoderm.

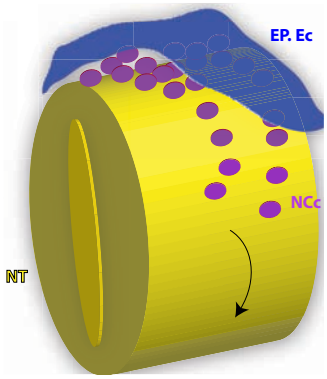


Figure 7. Schematic representation of the neural crest cells (NCc). They originate at the boundary between epidermal ectoderm (in blue, EP.Ec) and the neural tube (NT) and (as indicated by the black arrow) they migrate as a stream of cells from their original location and give rise to a plethora of different cell types discussed in paragraph III.2.

III.2 The neural crest migrates and gives rise to different derivatives Extensive lineage tracing studies in vertebrates and experiments with the quail/chicken system [1] have revealed the migratory and differentiation potential of several sub-popultions of neural crest cells originating at different positions along the A-P axis (Selleck and Bronner-Fraser 1995)(fig.6 and 7).

III.2.1 Different types of neural crest along the A-P axis

-The cranial neural crest cells (C-NCc) are the anterior-most population; they migrate in the head and give rise to the craniofacial mesenchyme, which will differentiate into

[1] Nicole Le Dourain developed the quail/chicken system. It consists on the transplantation of quail neural crest cells on different A-P axis of the chicken neural folds. The quail cells are distinguishable because they have a nucleolar condensation of heterocromatin at the interphase, a feature that is missing in the chicken cells.

cartilage, bone, and connective tissue. They also contribute to the sensory ganglia of the head, such as the trigeminal ganglia (Morris-Kay and Tan 1987). The cardiac neural crest can be considered part of the cranial neural crest, and can form connectives, cartilage, sensory neurons and pigmented cells, as well as the wall musculature of the heart and the outflow tracts.

-The trunk neural crest cells (T-NCc) migrate ventrally through the anterior half of each sclerotome and give rise to sensory neurons and glial cells of the spinal ganglia and the dorsal root ganglia (DRG), which will be examined in detail in the next paragraph. The ones which continue to migrate ventrally give rise to sympathetic ganglia, adrenal medulla and the nerves surrounding the aorta (Bronner-Fraser 1986, N. M. Le Douarin and Teillet 1974, Teillet, Kalcheim, and Le Douarin 1987). Other cells in the trunk migrate dorsolaterally and give rise to pigment cells and melanocytes that spread throughout the skin (Selleck and Bronner-Fraser 1995), (Erickson, Duong, and Tosney 1992).

-The vagal/cervical and lombosacral neural crest cells (V/S-NCc) give rise to the autonomic ganglia, forming the enteric nervous system that allows peristaltic movements(N M Le Douarin and Teillet 1973).

III.2.2 Cranial versus trunk neural crest: different developmental potentials

Cranial and trunk neural crest cells are intrinsically different in terms of developmental potential, and this might obscure distinct evolutionary identities. In contrast to the cranial neural crest, the trunk neural crest cannot give rise to cartilage and bone (Lallier 1991). If transplanted into the head, the trunk neural crest migrates and contributes to the cranial ganglia, giving rise to neurons, glia and melanocytes as well, but failing to differentiate into cartilage.

Accordingly, all the cartilage in the trunk comes from the trunk mesoderm. These distinct fates depend on the *hox* genes that the neural crest cells express at each axial level (Gavalas et al. 2001) (Trainor 2003). For instance, the neural crest migrating into the second brachial arch and contributing to the mesenchyme expresses *hox2,* as does the II brachial arch itself (Creuzet, Couly, and Le Douarin 2005). Accordingly, if the trunk neural crest loses the expression of trunk *hox* genes it acquires the ability to differentiate into cartilage (Abzhanov et al. 2003).

Moreover, cranial neural crest cells differ in their expression of *hox* genes, and this determines their cell fates. For instance, only the anterior neural crest domain is *hox* negative and therefore able to form the facial skeleton (Creuzet, Couly, and Le Douarin 2005). Hence, the *hox* genes confer positional identity to the neural crest along the A-P axis and this is later translated into a different developmental potential.

This concept challenges the idea of the neural crest as a population of stem cells, because, although they are able to differentiate into a plethora of different derivatives, they are restricted toward distinct cell fates. Therefore, they are pluripotent and not totipotent.

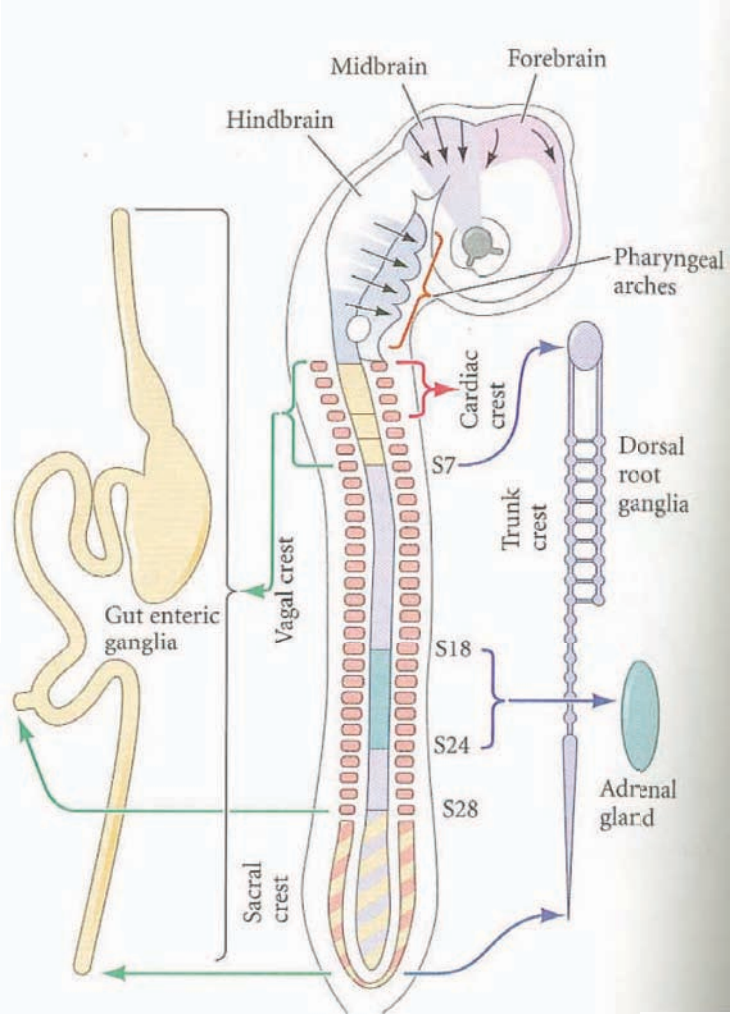
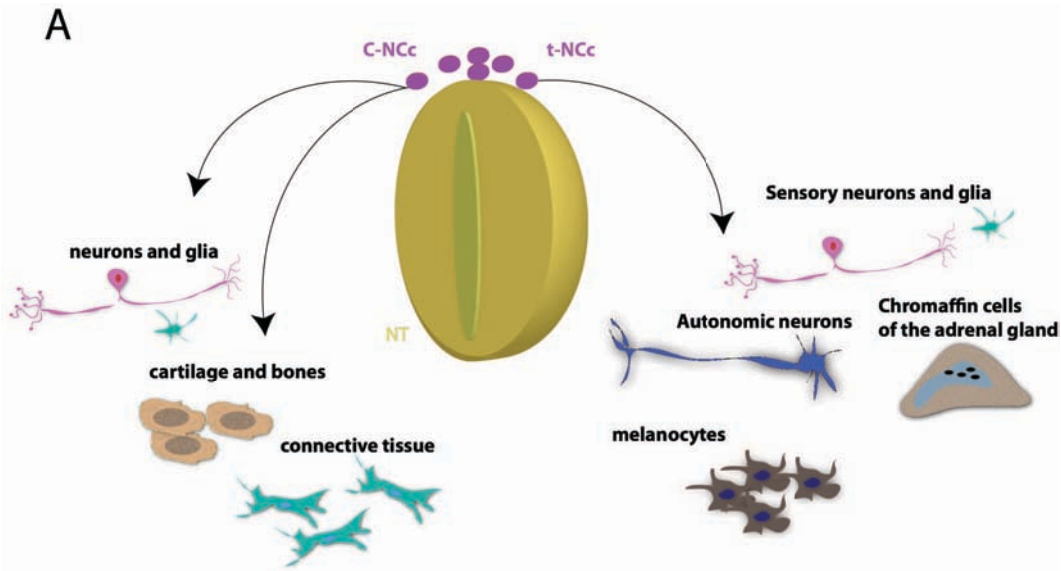


Figure 8. Regionalization of the neural crest in the chicken embryo. Drawing from the book 'Developmental Biology' by Scott Gilbert. Different populations of neural crest cells originate at different axial levels according to their expression of specific Hox genes and give rise to different derivatives.



B

C-NCc Cranial neural crest	T-NCc Trunk neural crest	V/S-NCc Vagal and sacral neural crest	Car-NCc Cardiac neural crest
Mesenchyme	Pigment Merkel cells	Neurons of parasympathetic nervous system of alimentary canal	Connective tissue associated with the great vessels of the heart
Connective tissue (including muscle sheaths)	Dorsal root ganglia	Neurons of parasympathetic nervous system of blood vessels	Aorticopulmonary septum of the heart
Cartilage, Bone, Dentine (odontoblasts)	Neurons and ganglia of the sympathetic nervous system	Enteric ganglia	Smooth muscles of the great arteries
Dentine (odontoblasts),	Chromaffin cells of the adrenal medulla	Ganglia (celiac, superior and inferior mesenteric, and aortical renal)	
Parafollicular cell (ultimobranchial bodies) of the thyroid gland	Epinephrine-producing cells of the adrenal gland		
Cornea and Sclera			
Ciliary muscle and muscles for eye attachment			
Inner ear (with otic placode)			
Sensory ganglia of cranial nerves V, VI, IX, and X			

Figure 9. The neural crest cells and their derivatives. A: schematic drawing showing the different cell types arising from the cranial and the trunk neural crest. **B:** table showing tissue/organs where derivatives of the different populations of neural crest cells are found. C-NCc: cranial neural crest cells, T-NCc: trunk NCc, V-S/Ncc: vagal and sacral NCc, Car-NCc: cardiac NCc. The table as been modified from Votano, Parham, and Hall 2004.

III.2.3 Neural crest in fishes: deviations from the accepted view

Important differences in the onset and migratory behaviour of teleost neural should be noted. In fishes, the migratory behaviour is slightly distinct. First, there are fewer neural crest cells when compared to mammals and birds, and neurulation does not occur by infolding of the neural tube, but rather by thickening of the neural keel. The neural keel forms a slit-like rod that later opens into a lumen (Lowery and Sive 2004) .

Therefore, there are no neural folds in fishes and the neural crest cells originate directly from the dorsal-most part of the neural tissue (D. W. Raible et al. 1992). As this mode is also present in lamprey, it might represent an ancestral state for the vertebrates.

III.3 Neural crest formation on the dorsal most part of the neural plate

Work from the laboratories of Nicole Le Dourain, Marianne Bonner-Fraser and Chaya Kalcheim have uncovered the molecular events inducing the formation of neural crest cells in vertebrates (fig.10). Here, I present a general overview of the gene regulatory network responsible for the formation of the neural crest, reinterpreting some levels of the network. This is based on current knowledge of different vertebrate species (table in fig.11).

The neural crest cells are specified at an early stage in development in many vertebrates. Neural crest forms at the edges of the neural primordium during gastrulation.
Cell labelling and transplantation techniques have demonstrated that the ectoderm can generate neural crest if transplanted to the border between the neural tube and neural crest, as it can respond to several signals coming from the surrounding tissues (Dickinson et al. 1995).

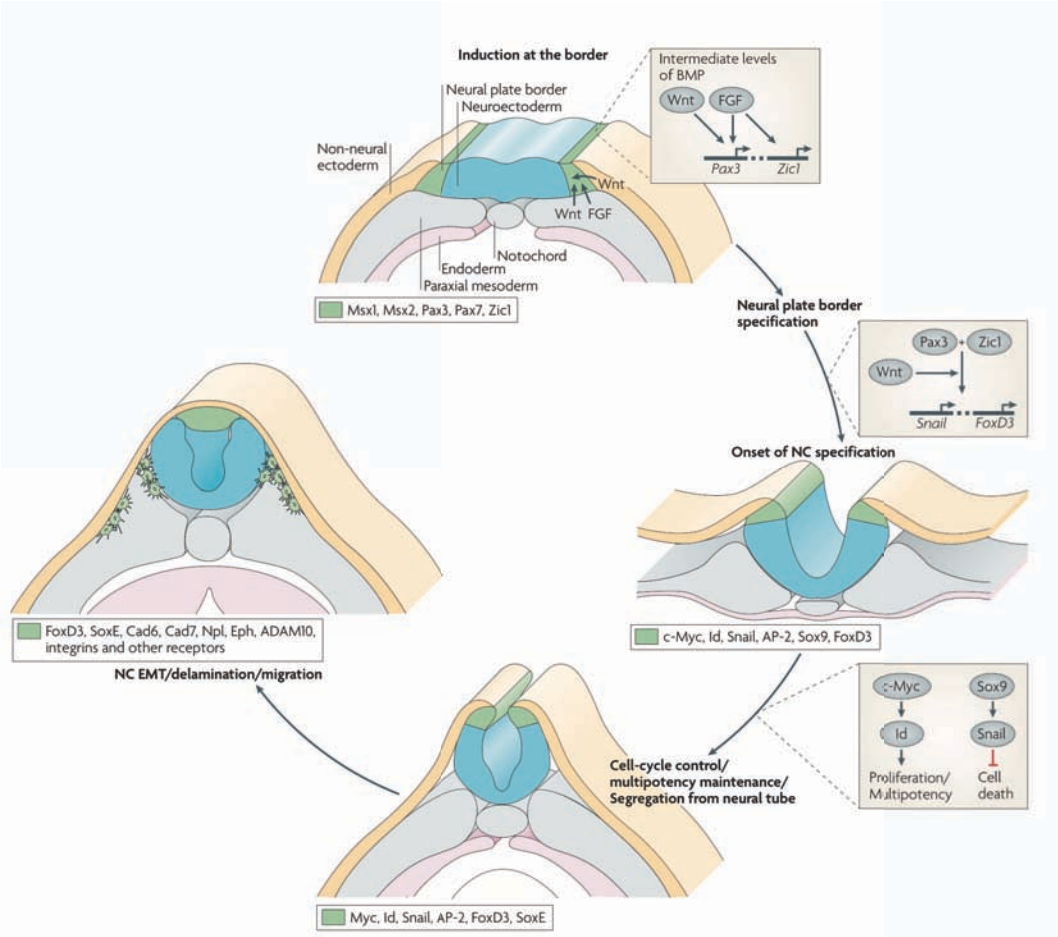


Figure 10. Vertebrate neural plate border formation, induction, and neural crest. The drawing is taken from the review: *'A gene regulatory network orchestrates neural crest formation'*, Tatjana Sauka-Spengler and Marianne Bronner-Fraser, Molecular cell biology, 2008. Green: neural crest and neural plate border, blue: neural tube, yellow: ectoderm, grey: somites and notochord

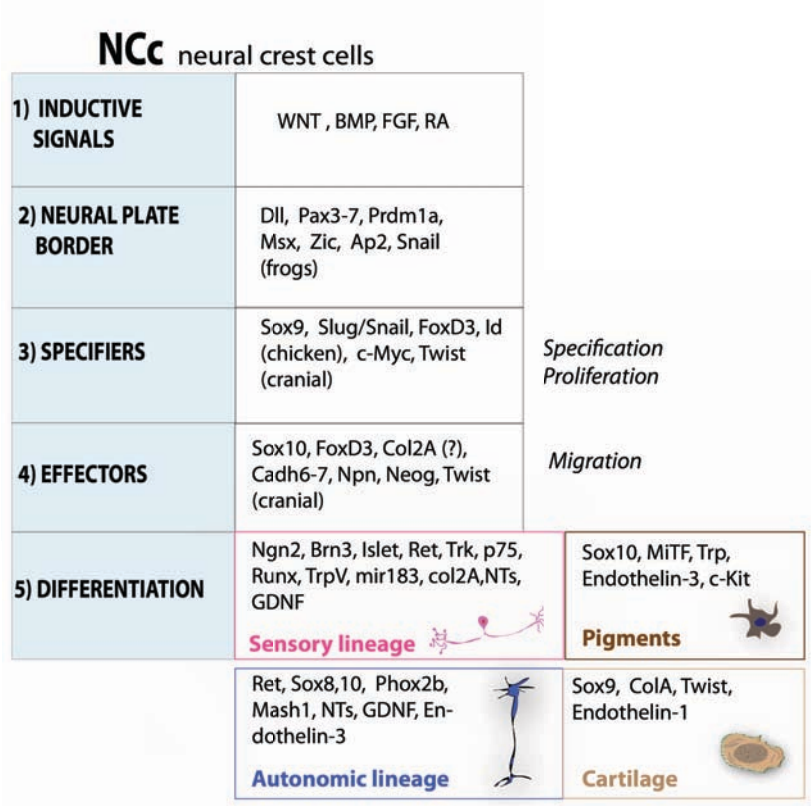


Figure 11. Schematic table representing a putative neural crest gene regulatory network at each step of neural crest specification and differentiationfor several cell types.
The table has been constructed taking into consideration the references indicated in the main text. Col2A, at the level of the effectors has a '?' , as its role in migration has not yet been investigated.

III.3.1 Inductive signals (level 1, table in fig.11)

Inductive **signals in neural crest formation** include the Bmp and Wnts (Dickinson et al. 1995). In mouse and xenopus Wnt1 is expressed in the dorsal neural tube and is involved in the control of proliferation of the neural crest cells, through the activation of the canonical signaling pathway mediated via ß-catenin (Dorsky, Moon, and Raible 1998), (Deardorff et al. 2001)(Hari et al. 2002). It induces the neural crest to differentiate primarily into sensory neurons. Wnt6 (in chicken) and Wnt7 (in frogs) which are secreted at the lateral ectoderm, and Wnt8 (zebrafish) which is secreted from the lateral ectoderm and paraxial mesoderm are also involved in NC induction (Votano, Parham, and Hall 2004). Bmp4 and Bmp7 are first induced in the neural plate from the Shh produced by the notochord and subsequently become restricted to the ectoderm, contributing to establishment of the boundary between neural and non-neural tissue (Selleck and Bronner-Fraser 1995), (Dickinson et al. 1995), (Liem et al. 1995).It has been demonstrated that in the anterior neuroectoderm, Wnt induces Bmp signaling (Patthey, Edlund, and Gunhaga 2009). Later, in the position where placodes originate, Wnt is turned off (Litsiou, Hanson, and Streit 2005). Conversely, neural crest cells will originate in the presence of Wnt. Paraxial mesoderm also plays a significant role in inducing the neural crest cell fate via Fgf8 and RA(Villanueva et al. 2002).

III.3.2 The neural plate border genes and neural crest specifiers

(Level 2-3, table in fig.11) See these references: (Khudyakov and Bronner-Fraser 2009), (Sauka-Spengler and Bronner-Fraser 2008b).

Neural crest forms on the dorsal most part of the neural tube. In response to inductive signals the expression of specific dorsal genes is induced (paragraph II.1.2, fig.5)**.** Among these**, neural plate border genes** are expressed at the edge between the developing neural tissue and ectoderm and confer a specific 'neural plate border identity' to this boundary tissue. These genes are *msx, pax3/7, dll, ap2-a* (Sauka-Spengler and Bronner-Fraser 2008b), (Luo, Lee, and Sargent 2002).

They turn on genes implicated in **neural crest specification** such as members of the *SoxE* family (*sox8, 9,10*), *slug/snail, id, c-myc, foxD3, ets1* and *twist* in the head region (Sasai, Mizuseki, and Sasai 2001),(Haldin and LaBonne 2010), (Stolt and Wegner 2010), (McKeown et al. 2005), (LaBonne and Bronner-Fraser 2000), (Sakai et al. 2006), (Betancur, Bronner-Fraser, and Sauka-Spengler 2010), (Kee and Bronner-Fraser 2005)

These genes are implicated in maintaining a pool of neural crest via the control of proliferation and maintainenance of multipotency. In particular, snail has been considered to be a crucial neural crest specifier. From a careful analysis of the literature, it is clear that snail is not exclusively a neural crest specifier. In amphibians, *snail* is also expressed at the border of the neural plate, earlier than the other neural crest specifiers (Essex, Mayor, and Sargent 1993). Here, *snail* might be needed to induce the morphological changes of the apical portion of the neural tissue that is responsible for the formation of the neural tube. This is also the case for the lamprey ortholog (Sauka-Spengler and Bronner-Fraser 2008a); therefore it might represent an ancestral function of the *snail* transcription factors.

III.3.3 Effector genes (level 4, table in fig.11), (Sauka-Spengler and Bronner-Fraser 2008b). Molecules such as cadherins 6/7, collagen2A, neuropilin and neogenin are responsible for **delamination and migration** and accordingly, are produced by the neural crest cells.

-Fibrillar collagen type II A

It is noteworthy that collagen type II A (fibrillar collagen bolonging to clade A, see Appendix D4) has long been considered as only a cartilage gene. However, the mRNA for collagen type II A, in addition to *sox8, 10* and *sox5,* can also be detected in non-cartilaginous tissues such as the notochordal sheath and in many early migrating neural crest cells (Yan et al. 1995),(Suzuki et al. 2006) (table in fig.11, fig,12). The role of collagen II A in neural crest development has not yet been investigated in vertebrates, but might relate to the migration of neural crest cells, consistent with the role of other extracellular matrix molecules found to be expressed in neural crest.

Moreover, the mRNA of collagen Type II A is also expressed in neurons and supporting cells in the dorsal root ganglia, cells of the peripheral nervous system (PNS) that are neural crest derived (Suzuki et al. 2006) ,(fig.11, 13).

For this reason, collagen type II A has recently been defined as a general '*mesenchymal gene'* (Votano, Parham, and Hall 2004).

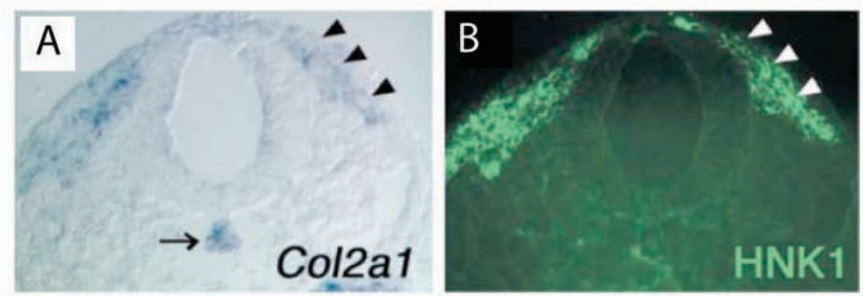


Figure 12. Chicken migrating neural crest cells express *col2a1*. From fig.1 of the paper '*Sox genes regulate type 2 collagen expression in avian neural crest cells*, Suzuki et al.2006'. It depicts the expression of mRNA of *col2A1* (A) in migrating neural crest cells, as identified by the neural crest marker HNK1 (B). Letters indicating the figures have been modified from the original.

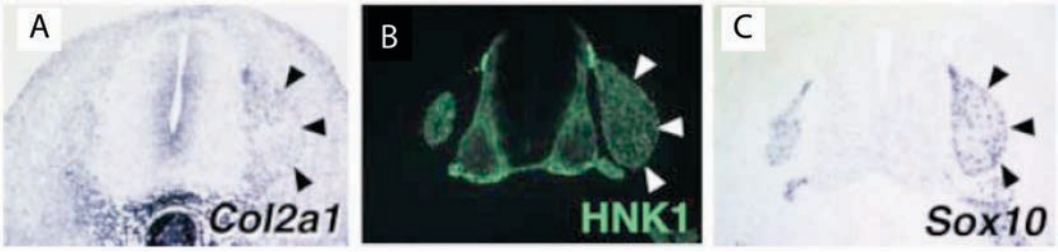


Figure 13. Peripheral neurons in the dorsal root ganglia express *col2a1*. This is fig. 3 of the same paper found in fig.12. It shows the expression of *col2a1* in the cells of the dorsal root ganglia (A), the sensory component of the peripheral nervous system, also labeled here with HNK1 (B) and *sox10* (C). Letters indicating the figures have been modified from the original.

III.3.3.1 Signaling molecules

Signaling molecules are also crucial for the migration and differentiation of different populations of neural crest at this stage. For example, rho and slit, in conjunction with snail, is most likely responsible for the cytoskeletal changes necessary for neural crest migration (Giovannone et al. 2012). Snail also downregulates the *cadherins* (Taneyhill, Coles, and Bronner-Fraser 2007), thereby allowing the neural crest cells to break their contacts with one another, disaggregate, and migrate. These cells then re-establish cell-cell contacts once they reach the target location in the body and differentiate.

As explained previously (paragraph III.2.1) in avian embryos, trunk neural crest cells migrating along the ventro-lateral path move exclusively through the anterior portion of the sclerotomes. This is due to the fact that the posterior region of the sclerotomoes secretes ephrin and semaphorin 3-F (Robinson et al. 1997), (Gammill et al. 2006). These are signaling molecules recognized by the ephrin receptor and the neuropilin receptor, respectively, that inhibit the migration of trunk neural crest. When eprhins and semaphorin molecules are absent, neural crest cells undergo abnormal migration.

-Signaling molecules required for specific cell types neural crest-derived:

Enteric neurons

The neural crest cells migrating ventrally differentiate into the neurons of the enteric nervous system that occur from the foregut to the hindgut. GDNF produced by the gut is detected by neural crest cells which express the Ret receptor and serves to attract the migrating cells to an appropriate position (Young et al. 2001).

Sensory and sympathetic neurons of the peripheral nervous system

The sensory neurons of the dorsal root ganglia, as well as the preganglionic neurons of the sympathetic nervous system require special signaling molecules, the neurotrophins, for their survival, differentiation and axon pathfinding (Liebl et al. 1997; Ernsberger 2009; Davies 1994). This will be discussed in more details in the following paragraphs.

Melanocytes

A distinct molecular toolkit is required for the development of the melanocytes (Sauka-Spengler and Bronner-Fraser 2008b).

When *foxD3* is downregulated, cells that express *mitF* can undergo melanoblast differentiation (Curran et al. 2011; Curran, Raible, and Lister 2010) and can migrate through the use of eprhin signaling. These mitf+ cells detect the ephrin ligand, a chemoattractant, that is expressed along the dorso-lateral pathways (Santiago and Erickson 2002) (table in fig.11).

Cartilage

Endothelin, a secreted molecule, is a crucial signaling for the cranial neural crest that are differentiating in cartilage and bones during craniofacial development (Clouthier and Schilling 2004). Indeed this molecule is expressed in mesoderm and pharyngeal arch and binds to its receptor expressed in the cranial neural crest (Miller et al. 2000; Kempf et al. 1998).

Extracellular matrix

During neural crest migration, components of the extracellular matrix, such as collagen molecules, laminin and fibronectin are expressed throughout the sclerotome (Perris, Krotoski, and Bronner-Fraser 1991; McCarthy and Hay 1991).

III.4 Molecular events responsible for the development of the dorsal root ganglia derived from the trunk neural crest

(this paragraph was written based on Delmas, Hao, and Rodat-Despoix 2011; Marmigère and Ernfors 2007; Lallemend and Ernfors 2012)

Along with the enteric nervous system, sympathetic neurons and melanocytes, the trunk neural crest cells also give rise to the sensory neurons comprising the dorsal root ganglia (DRG of the spinal cord, Lallier 1991; Nicole M Le Douarin and Dupin 2003).The discovery that the spinal ganglia derive from the neural crest is as old as the discovery of the neural crest itself, made by Whilem His in the 1930s.
Early migratory trunk neural crest cells (t-NCc), which already express *ngn2*(Furlong and Graham 2005*),* (Ma et al. 1999a) and are biased toward the sensory cell fate. They migrate as a stream of cells toward the anterior half of each somite, and cluster on the ventral side of the ganglia. Sommer and colleagues have shown that this pre-commitment of the neural crest to the sensory lineage is driven by canonical Wnt signaling (Hari et al. 2002).
It is worth noting the relevant exceptions to the general developmental scenario presented thus far. It has been proposed that, at the time when the neural crest stop migrating, neuroepithelial cells derived from the neural tube also migrate into the dorsal root ganglia and give rise to new sensory neurons, primarily of the nociceptive type.
An elegant study recently published in *Nature Neuroscience* (Maro et al. 2004) revealed that these cells are most likely not neuroephitelial (belonging to the CNS), but are rather boundary cap cells (BCc). BCc are neural crest-derived cells that populate the dorsal and ventral roots, where nerves enter and exit the CNS. Hence, late migrating BCc represent an additional reserve of PNS cell types.

-The first wave of neurogenesis in the DRG
The first migrating neural crest cells are *ngn2+* (between E9.5 and E11.5 in mouse, fig.11A), and they give rise to the large diameter sensory neurons, mechano/propioceptive neurons (type Ia, II, Ib, AßLTMR) that express *trkC* and *trkB* neurotrophic receptor molecules.

-The second wave of neurogenesis in the DRG
The second population of neurons produced (between E10.5 and E13.5 in mouse, fig.14A) are the more numerous small diameter neurons, *ngn1/trkA+*. They can be distinguished in lightly myelinated (Aδ) or unmyelinated (C) fibers and are mostly nociceptive neurons; some of them express also CGRP and PPT peptides. This second wave of neurogenesis also gives rise to supporting cells of the PNS, such as the satellite cells surrounding sensory cell bodies and Schwann cells extending along the nerves.
Functional data in chick and mouse have occasionally demonstrated redundancy of the two Ngn genes in determining the commitment to large versus small diameter sensory neuron fate (Ma et al. 1999b).
It is likely that this segregation of ngn into distinct subsets of sensory neurons has been achieved during vertebrate evolution. This specialization of function would have

followed the duplication of neurogenic transcription factors such as the bHLH Ngns and neurotrophic receptors from single representatives of the protochordate Ngn and Trk molecules (Furlong and Graham 2005), (Benito-Gutiérrez et al. 2005).

In vertebrates, during the early phases of migration, the neural crest cells downregulate *sox10* (a marker of multipotency) and co-express *foxS1* (Montelius et al. 2007)and *ngn* as they become more restricted to the sensory fate.
In the last steps of migration, neural crest cells express the Pou domain *brn3a* and the LIM-homeodomain factor *islet1,* two terminal differentiation markers (Dykes et al. 2011)
Brn3a binding sites have been found in the 5' genomic region flanking genes belonging to the Runt – related family , and multiple lines of evidence have recently demonstrated that Runt genes initiate the subdivision of the distinct lineages of committed sensory neurons and regulate their axon navigation (Kramer et al. 2006; Levanon et al. 2002; Dykes et al. 2010).

Despite the broad role of Runt-related proteins in different developmental processes, in the PNS, *runx1* is selectively expressed in the *trkA*+ nociceptive neurons (Chen et al. 2006). Here, it is involved in the formation of the non-peptidergic *ret*+ cell subtype, which is distinct from the *trkA*+/*runx1*– cell type that later gives rise to peptidergic neurons.
Conversely, when *runx3* is expressed in the large *trkB*/*C* precursors, *trkB* is downregulated and propioceptive neurons arise, thus distinguishing these cells from the *runx3*–/*trkB* +, population that will form mechanoreceptors (Levanon et al. 2002).
Runx3 -/- mice consistently lack this population of proprioceptor sensory neurons, and resemble mice which are mutant for *trkC* and its ligand (Nt-3), (Kramer et al. 2006).
Conversely, *islet1* -/- mice lack large mechanoreceptor *trkA*+ neurons and *trkB* nociceptors, while the *runx3*+/ *trkC* + population is maintained (Sun et al. 2008). This points to a role for *islet* in determining specific sensory modality.

-The third wave of neurogenesis in the DRG
The third wave of neurogenesis (fig.14B) is attributed to the boundary cap cells (BCc), discussed above (Ma et al. 1999b). The molecular fingerprint of these cells is not clear, but they seem to specifically express *krox20 (egr2)* and *sox10* while migrating, to turn on sensory markers such as *ngn1, brn3a* and *foxS1* and give rise primarily to *trkA*+, small diameter sensory neurons (Hjerling-Leffler et al. 2005, Delmas, Hao, and Rodat-Despoix 2011; Marmigère and Ernfors 2007).

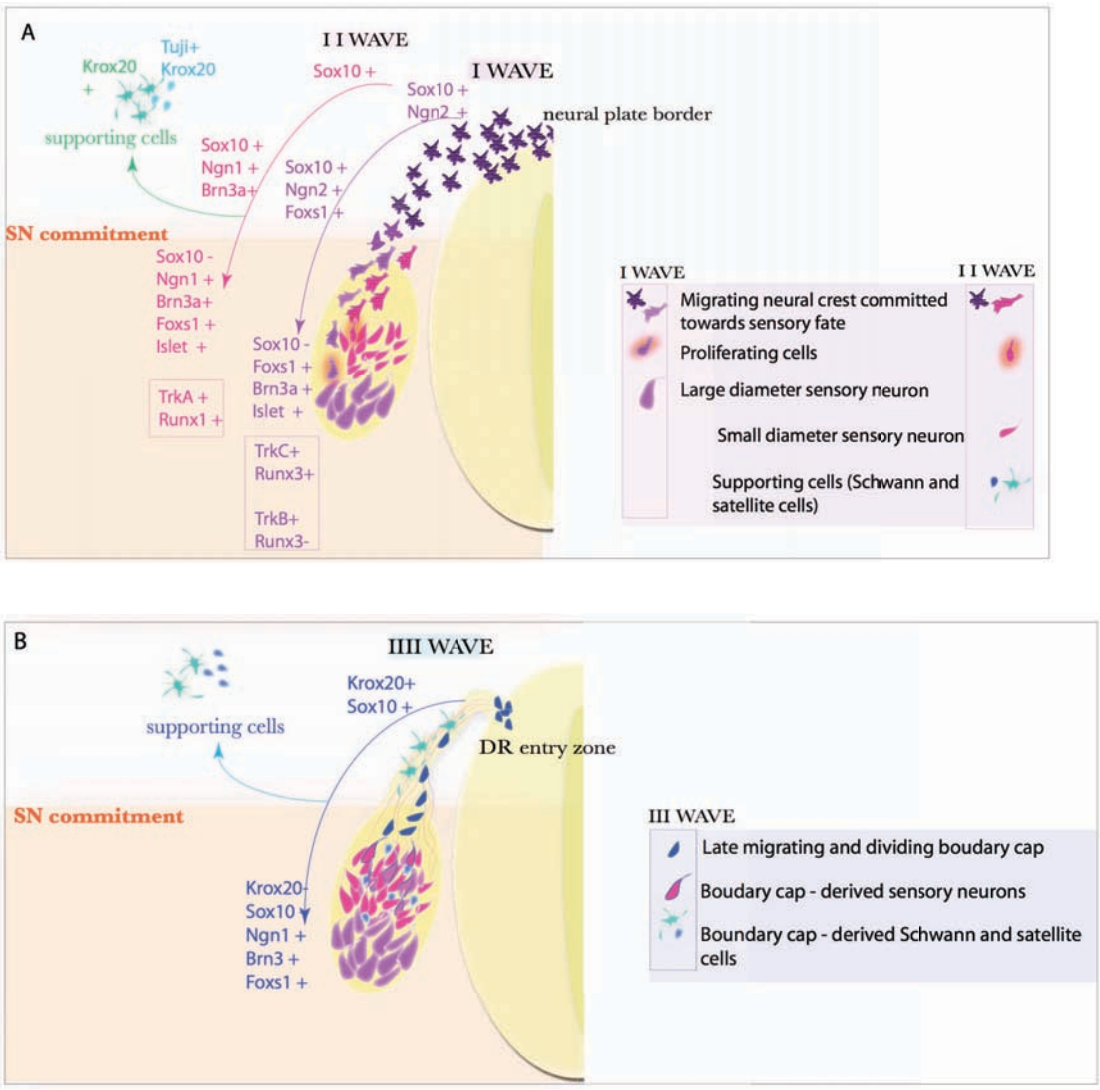


Figure 14. The I, II (A) and III (B) wave of sensory neurogenesis in the trunk of the mouse embryo and the molecular fingerprint of neural crest cells during migration, commitment and differentiation. See text for details
These drawings were done based on Delmas, Hao, and Rodat-Despoix 2011; Marmigère and Ernfors 2007; Lallemend and Ernfors 2012.

III.5 The Rohon Beard cells in anamniotes

In anamniotes vertebrates such as frogs and fishes, the developmental scenario is different. In these animals, the neural plate border region has a greater developmental potential, as it gives rise to both neural crest cells and Rohon Beard cells (RBc) (Rossi et al. 2008), (Jacobson 1981), (Artinger et al. 1999). The latter are the first sensory neurons to arise in the trunk very early, when gastrulation is not complete yet (Lamborghini 1980). Rohon Beard cells (RBc) appear to be absent in vertebrates that have a protected internal development such as mammals.

The RBc are primary mechanosensory neurons with free endings in the skin and the developing muscles. They project centrally and transmit sensory inputs originating from light touch. They are responsible for initiating the first escape response in the early stages of the swimming larva (Kohashi, Nakata, and Oda 2012).

In frogs, RBc are already chemically and electrically active by the time the neural tube closes (Baccaglini and Spitzer 1977).
The RBc arise at the border of the neural plate from the same field that gives rise to trunk neural crest (t-NCc).
RBc are intermingled with pre-migratory neural crest in the dorsal spinal cord early in development and might arise from the same precursor cell (fig.15.A), (Jacobson 1981) (R. a Cornell and Eisen 2000; Moorman 2001). RBc then degenerate slowly through a TrkC and NT-3 signaling-dependent mechanism. During this time, the neural crest cells are specified and produce the sensory neurons of the prospective DRG, that will then functionally replace the RBc (Williams et al. 2000)

III.5.1 Rohon Beard cells and trunk neural crest cells arise at the border of the neural tube and share part of the gene regulatory network

RBc and t-NCcs develop at the lateral most edge of the developing neural plate.

As functional studies (while still few) are beginning to reveal what is most likely a shared genetic machinery acting in the precursors of RBcs and t-NCcs, a tentative gene regulatory network can be proposed (Rossi, Kaji, and Artinger 2009), (details in table in figure 15.B).
prdm1-a, a key regulator of both t-NCc and RBc in anamniotes (Rossi, Kaji, and Artinger 2009), (Olesnicky, Hernandez-Lagunas, and Artinger 2010), is a zinc finger domain transcriptional repressor that acts in fate deci sion in many different tissues (John and Garrett-Sinha 2009)and in this context, at the level of the neural plate border.
In the dorsal neural plate it is likely that prdm1-a acts at different levels of the gene regulatory network. For example, fish mutants for ap-2 (found in the neural plate border) have a reduction in RBc number and reduced *prdm1-a* expression (Li and Cornell 2007), suggesting that ap-2 is upstream of prdm1-a in the specification of the neural plate border. On the other hand, fishes mutant for prdm1-a *(narrowminded)* show a complete absence of Rohon Beard cells (fig.15 B, C), and accordingly, are unable to escape after light touch. They exhibit a reduced number of neural crest derivatives as well, indicating that prdm1-a is required for both the cell types (Hernandez-Lagunas et al. 2005).

III.5.2 Rohon Beard cell or neural crest? Cell fate choice at the neural plate border

-The role of Prdm1-a

Prdm1-a is upregulated in the absence of Notch signaling, which is consistent with a Delta-Notch signaling-dependent decision between the Rohon Beard and neural crest fate by members of the same 'equivalence group'(Cornell and Eisen 2000),(R. A. Cornell and Eisen) .
Hence, blocking Delta signaling leads to an increase in the number of Rohon Beard cells (where delta it is normally expressed) at the expense of trunk neural crest cells and their derivatives, while the cranial neural crest cells seem unaffected (Cornell and Eisen 2000).
Downstream targets of prdm1-a, which is important for the fate decision between RBc and t-NCc, are currently being identified. Nevertheless, recent findings suggest that the split between the two cell types occurs quite early in the genetic program. After local Notch/ Delta mediated sorting, Prdm1-a induces the expression of the multipotency marker *sox10* exclusively in the prospective neural crest precursors (table in fig.15B,C and

table in fig.11) and the terminal differentiation marker *islet* (Olesnicky, Hernandez-Lagunas, and Artinger 2010)(fig. 15.C table in fig.15.B) exclusively in the Rohon Beard cells. This sorting and the Prdm1-a-dependent expression of sox10 act as a crucial node in the genetic cascade that determines the commitment of both populations. Accordingly, the loss of RBc in the absence of *prdm1-a* is partially rescued by the overexpression of *islet* (Olesnicky, Hernandez-Lagunas, and Artinger 2010).

-Differential gene expression in RBc and t-NCc

The sorting between t-NC and RBc fate is determined by differential expression of *sox10* and *islet* respectively, and is then translated into the expression of distinct gene cassettes. Then, only the presumptive neural crest cells express specific molecules such as *foxD3* and *slug/snail*. *foxD3* and *slug/snail* are crucial in the acquisition of migratory behavior, a feature that distinguishes them from other cell types (LaBonne and Bronner-Fraser 2000)(Stewart et al. 2006) .

While no direct interactions have yet been demonstrated, expression profile analysis, functional interference, and zebrafish mutant embryos suggest that Dll and ngns are also downstream prdm1-a in RBc – GRN Ngns (Rossi, Kaji, and Artinger 2009).

Ngns (Rossi, Kaji, and Artinger 2009) and islet(Tanaka et al. 2011) belong to the RBc sensory cascade, but are also among the first factors to be expressed in trunk neural crest cells (as discussed in the previous paragraph) after they have migrated to form the sensory neurons of the DRG (level 4 in the table in fig 15.B).

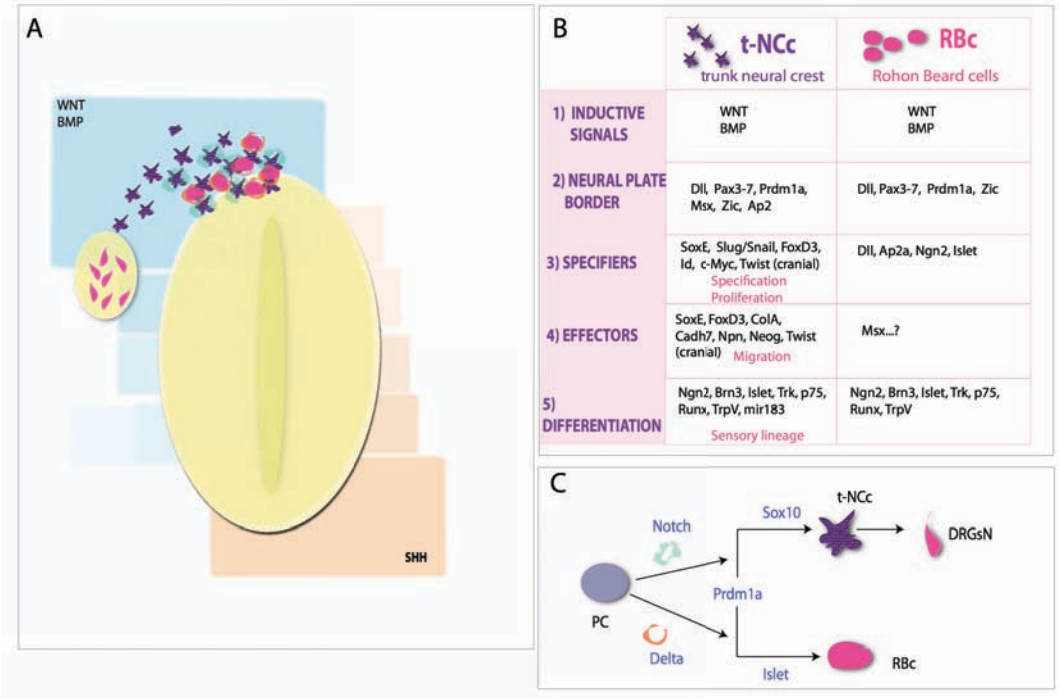


Figure 15. Cell identity and gene regulatory network at the border of the neural plate during early embryogenesis of anamniote vertebrates A: Wnt and Bmp signals coming from the surrounding tissues pattern the neural plate border, where Rohon Beard cells and neural crest originate. Conversely, a gradient of Shh is established on the ventral side, where motoneurons arise. **B:** the table represents the putative gene regulatory network of Rohon Beard cells and trunk neural crest, derived from epistasis experiments as well as direct evidence. **C:** The sorting between the Rohon Beard cells and neural crest is mediated by local Notch and Delta signaling and consequentially determined by the differential expression of *sox10* in prospective neural crest cells and *islet* in prospective Rohon Beard cells. PC: precursor cell, RBs: Rohon Beard cells, t-NCc: trunk neural crest, DRGsN:

dorsal root ganglia sensory neurons. The table and the drawings were done considering the references present in the paragraph.

III.5.3 Did the neural crest evolve from Rohon Beard cells?

Preliminary molecular data presented in the previous paragraph and classical embryological observations have been able to identify a common progenitor precursor for t-NCc and RBc. Based on this, it is appealing to revive the scenario originally proposed by Northcutt in 1993 (Fritzsch and Northcutt 1993).
According to this view, the Rohon Beard cells and the neural crest cells might have indeed shared an ancestral precursor at the border of the neural plate at the base of chordates. Such an 'original' progenitor was likely to have initially given rise to sensory neurons and to later have acquired migratory behaviour and evolved into the neural crest cell during the evolution of vertebrates.
In this view, prdm1-a could be a master gene that was already acting in ancestral cell types. This master-regulator function would have apparently been lost in mammalian early neural crest cells, which accordingly also lack Rohon Beard cells. As mouse mutants suggest, however, prdm1-a still acts in mammals on derivatives of neural crest cells, such as the brachial arches (Vincent et al. 2005).

Next, I will discuss the currently available dataset focusing on molecular and embryological comparisons of cell types originating at the border of the neural plate in the sister group of vertebrates (the tunicates) and in the protochordate lancelet amphioxus.

III.6 What is known about the evolutionary origin of the neural crest ?

To trace the evolution of neural crest, evolutionary biologists began to study the fossil record of basal chordates and vertebrates for evidence of neural crest derivatives. This approach is limited in that it is impossible to trace the embryological origin of fossilized structures. The presence of neural crest derivatives alone is not enough to infer that a neural crest cell population gave rise to these structures

III.6.1 Fossils

- Putative basal vertebrate fossils from the Cambrian (fig.10), such as the jawless craniate *Myllokunmingia fengjiaoa,* possess skeleton in the gill slit and capsules surrounding sense organs. However, as outlined by Brian K. Hall in his book, '*The Neural Crest and Neural Crest Cells in Vertebrate Development and Evolution'*, and as previously noted, the presence of these structures is not definitive evidence that they are neural crest-derived. For instance, collagenous tissues, such as those present in amphioxus gill slits, might have been of mesodermal origin, thereby predating the formation of the NC- skeleton present in extant vertebrates.

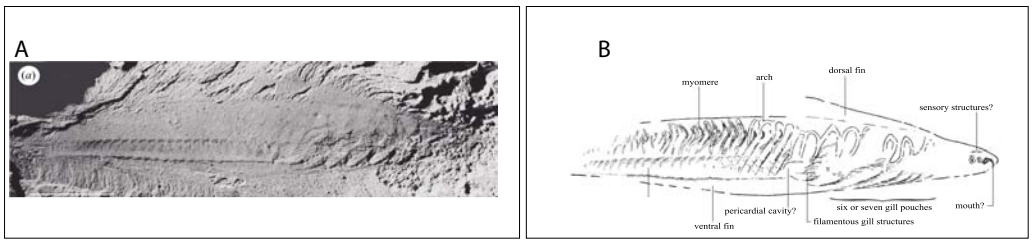


Figure 16. The fossil *Myllokunmingia fengjiaoa* . A: This fossil record is from Yunnan Research Centre for Chengjiang Biota, Yunnan University, China. This is fig.1 of the paper '*New evidence on the anatomy and phylogeny of the earliest vertebrates'*, Hou Xian-guang, Richard J. Aldridge, David J. Siveter, Derek J. Siveter and Feng Xiang-hong, The Royal Society, 2002. **B:** Drawing made with camera lucida that shows the features of t he fossil: such as gill slits, a dorsal fin, V shaped myomers. This is fig.2 of the same paper.

III.6.2 Lamprey

Bona fide neural crest cells are present in the extant basal vertebrate lamprey. Pioneering work from Marianne Bronner has shown that that the overall vertebrate gene regulatory network is conserved, at least for the cranial neural crest cells. Some differences should be noted. For example, lamprey *snail* is expressed at the neural plate border early in development (as mentioned in paragraph III.3.2). A situation similar to that in frogs, but distinct from that in chicken or mouse, where *snail* is expressed in specified and migratory neural crest cells (Sauka-Spengler and Bronner-Fraser 2008a).
Ets1 is never expressed in lamprey migratory neural crest cells. *twist* does not function as a neural crest specifier as it does in other vertebrates, as homologs are expressed only in postmigratory cells of the brachial arches and buccal cartilage (Sauka-Spengler and Bronner-Fraser 2008a). This suggests that a functional role for *twist* as a neural crest specifier could be a gnathostomes innovation.

In addition to their finding of an overall conservation of the NC-GRN, the laboratory of Marianne Bonner has also recently demonstrated a functional link between Prdm1-a and some neural plate border genes (Nikitina, Tong, and Bronner 2011). This indicates that the role of Prdm1-a and these populations might be ancient. The lamprey *prdm1-a* ortholog is strongly expressed in the early neural plate border and pre-migratory neural crest, and is turned off as soon as the cells initiate migration. Lamprey prdm1-a is regulated by ap-2 and msx. This scenario resembles neural crest patterning as has been described thus far only in zebrafish.

III.6.2.2 Rohon Beard cells in lamprey

It has not yet been demonstrated in lamprey that the Rohon Beard cells arise from the same precursors as the neural crest, a finding which would support an ancestral link between RBc and t-NCc in vertebrates. Nevertheless, it is likely that Rohon Beard cells are evolutionarily old cell types and not a specific feature of amniotes. Furthermore, they are present in all 'lower' vertebrates, as described was described in 1914 in the larva of *Ambystoma* by Coghill (Coghill 1914) and in 1948 in lamprey by Whiting (Whiting 1948). In these animals, as is most likely also the case in early zebrafish embryos, Rohon Beard cells are part of a rather simple neural circuit that conveys the dorsal sensory imput into motor imput. This occurs through interneurons that synapse with a chain of primary motoneurons located in the ventral neural tube, those that innervate the developing

myotomes. While not yet fully worked out, such an organization is also present in amphioxus, suggesting that this simple organization was among the first functional units in the trunk nervous system of the last chordate ancestor (drawing in fig.17).

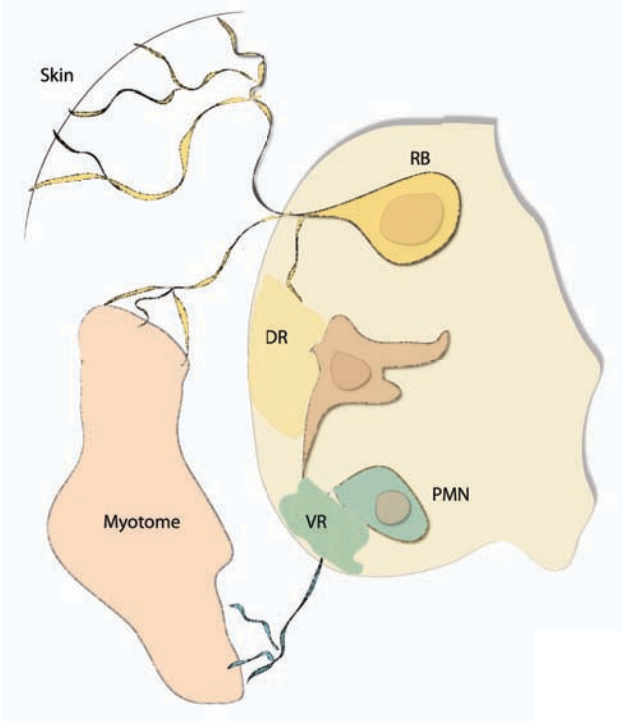


Figure 17. Schematic drawing of the early neural circuit of the spinal cord of anamniotes, adapted from the original description in *Ambystoma* larva made by Coghill in 1984. A similar description of the circuit has been reported in the developing spinal cord of lamprey (Nakao and Ischizawa, 1897), therefore it might represent the ancestral vertebrate mode to integrate somatosensory imputs in the trunk and to coordinate locomotion and escape response. RB: Rohon Beard cell, PMN: primary motoneuron, DR: dorsal roots, VR: ventral roots.

In a series of studies performed between 1984 and 1987 Nakao and Ishizawa followed the development of spinal ganglia in the larva and in the adult of *Lampetra japonica*(Nakao and Ishizawa 1987).

They found that large neurons, most likely Rohon Beard-like cells, arise very early and are situated in the dorsolateral position of the spinal cord, projecting through the dorsolateral tract (DLT). They do not contact synaptic endings on the surface, indicating that they are most likely primary sensory neurons.

By stage 21 mm, dorsal sensory cells, the ' Hinterzellen' (intramedullar cells), are present in the spinal cord as described by Sigmund Freud in his first medical studies (Whiting 1948). They do not show the typical axonal projection pattern of the Rohon Beard cells, and it is not yet clear if these adult 'dorsal cells' correspond to the Rohon Beard cells.

Little is known at the molecular level for the development of trunk sensory neurons in lamprey, and it is not known how many subtypes of sensory neurons are present in the spinal cord. Two Trk receptors are present in lamprey, *Lf*Trk1 and *Lf*Trk2, which are both expressed in the spinal cord, but the nature of these Trk + cell types is unknown (Hallböök, Lundin, and Kullander 1998).

III.6.3 Did the ancestor of chordates have neural crest-like cells?

Chordates (chepalochordates such as the extant amphioxus, the tunicates, such as *Ciona intestinalis* and the vertebrates) possess a dorsal nervous system and a notochord. These common features have inspired biologists to search for neural crest- like cells that might

predate vertebrates. I review here the data that support the presence of a proto-neural crest in chordates and discuss ongoing controversies that have inspired my work in the annelids.

III.6.3.1 Ascidians

I will start with the Ascidians, as recent phylogenetic analysis has revealed that Urochordates are the sister group of vertebrates (Delsuc et al. 2006). Although their development is quite derived, the position of ascidians as sister to vertebrates warrants investigation of their nervous system. This will likely help to elucidate important general features of the proto-vertebrate.

- Migratory-pigmented cells precursors

Recent studies have proposed that migratory pigment cell precursors (neural crest-like), while limited to ascidians, might have arisen in the chordate ancestor after the split of cephalochordates (Jeffery, Strickler, and Yamamoto 2004), (Jeffery 2007). These cells then evolved into a something akin to extant neural crest by co-option of genes and additional cell features. While this migratory cell population has been identified in different species of ascidians (Jeffery 2006), it is not specified at the border of the neural plate, but rather from blastomere A7.6 that gives rise to endoderm and mesoderm. It expresses only a small number of the genes of the NC-GRN, specifically those that are typically also expressed in the mesoderm of vertebrates, such as *zic, ap-2, foxD, twist* (fig.20A)(Jeffery et al. 2008).

Hence, in the absence of functional experiments, it becomes difficult to determine if these genes have a role in a pre-established 'proto-neural crest gene regulatory module'. Conversely, these genes may simply form a part of a mesodermal network, as for other species of invertebrates.

The developmental origin of these mesenchymal cells from endo-mesoderm is inconsistent with the basic definition of neural crest as 'neuroepithelial cells in the periphery'. Furthermore, similar mesenchymal cells, that derive from the endomesendoderm and acquire pigments, are present in other invertebrates, such as the echinoderms that don't have neural crest (Gibson and Burke 1985).

-The dorsal ectodermal sensory neurons

Interestingly, and in accordance with vertebrates, dorsal ectodermal peripheral sensory neurons (DESN) *brn3 +* (fig.18) in tunicates arise from a territory just adjacent to the neural plate, the dorsal epidermal midline (Candiani et al. 2005). This territory is a descendant of the same b-line ectodermal lineage that also gives rise to dorsal neural tube. In particular, blastomere b8.18 gives rise to the dorsal domain where DESN will form, with the left and right precursors juxtaposed when the neural tube is closing (Pasini et al. 2006).

This developing dorsal midline domain could correspond evolutionarily to the neural plate border of vertebrates, by position and gene expression.

In agreement with this hypothesis, genes such as *pax3/7, zic and snail* (fig.20) are expressed in this lineage (Pasini et al. 2006). Furthermore, these DESN cells originate close to the dorsal neuroectoderm, an essential feature that defines the neural crest cells.

Further supporting the original scenario proposed by Northcurtt, Wada (Wada 2001) postulated that the neural crest of the ancestral chordate was a tunicate-like ectodermal

sensory neuron population at the edge of the neural plate (likely homologous to the RBc in vertebrates).

Figure 18. Expression of *Ciona brn3* ortholog in ectodermal sensory neurons in the trunk and in the head. The arrow indicates expression in the motoneurons of the visceral ganglion. This is fig.2 of the paper *'Ci-POU-IV expression identifies PNS neurons in embryos and larvae of the ascidian Ciona intestinalis'*, Candiani et al., Dev Genes Evol, 2005

III.6.3.2 Amphioxus

Amphioxus is a basal chordate (fig.1) and it lacks definitive neural crest cells. Nevertheless, the upstream cascade of the gene regulatory network, which includes genes such as *dll, msx, zic, pax3/7* (fig.20B) is conserved at the border of the infolding neural tube(Yu et al. 2008). No expression of *prdm1* in the lateral neural plate border has been described thus far. Crucial genes of the neural crest network such as *soxE* and *foxD* are only expressed in the mesoderm (Yu et al. 2008), with the current hypothesis asserting that they were co-opted into the original genetic machinery acting at the neural plate border at the base of chordates.

Supporting this scenario, the regulatory region of amphioxus *soxE* does not show convincing ap-2 binding sites, whereas the vertebrate sox10 zebrafish does(Van Otterloo et al. 2012). It has therefore been postulated that the gain of these binding sides, together with neo-functionalization of paralogs, might have driven the expression of *sox10* at the edge of the neural plate under the control of the neural plate border marker *ap-2*.

-The *dll* + cells

Nevertheless, there are a few interesting features that have been reported regarding the dorsal neural tube of cephalochordate. For instance, when the neural tube is closing, *dll* is expressed in the perspective outer ectoderm, as well as in some cells at the edge in the dorsal neural tube (Holland et al. 1996).

The cells in the ectoderm extend filipodia and move over the neural tube, (fig.20A) showing migratory behaviour similar to that observed in the vertebrate neural crest. The fate of the *dll* + cells, is currently unknown. It is currently unknown if some of these cells neural tube territory to give rise to sensory neurons.

-The Retzius cells

Work done by Retzius (Retzius 1891) and Bone (Bone 1960) has shown that amphioxus lacks spinal ganglia, but possesses sensory neurons in the dorsal neural tube. Gene expression studies have revealed that a cluster of *islet* + neurons is visible in the dorsal neural plate by the early neurula stage, when the neural tube is not closed yet, and these cells might correspond to those identified molecularly as the *dll* + population (Jackman, Langeland, and Kimmel 2000). They are located between somite 4 and 5 (fig.19B) along the AP axis, a region compared to the caudal hindbrain of vertebrates. This region

contains *islet* + putative motoneurons, arranged in clusters on the ventral side, similarly to the organization found in the vertebrate hindbrain.

This preliminary evidence suggests that the *islet* + dorsal cells might correspond to the Rohon Beard-like cells described by Bone. In support of this scenario, a recent study of the expression patterns of neurotransmitters in amphioxus shows that the cluster of *islet* + cells in the dorsal neural tube is glutamatergic, as evidenced by their expression of *VGLUT(vesicular glutamate transporter)* (Ramoino and Pestarino 2012). Glutamate is a common excitatory neuroransmitter for sensory neurons of the dorsal root ganglia and Rohon Beard cells. Glutamatergic neurons are also present in the cluster of sensory cells (DESN) in the dorsal neural tube of tunicates, a cell population reviewed in the preceding text. These findings reinforce an evolutionary relationship among these cell types.

A detailed molecular characterization of the dorsal sensory cells of amphioxus is necessary in order to understand their evolutionary origin and to compare their identity with sensory neurons in other phyla. The cluster of sensory cells on the dorsal domain is located at the level of the somite 5-6. This domain expresses *hox1-hox4* (Schubert et al. 2006). Therefore, it is likely that a duplication of this caudal hindbrain *hox1-hox4* + territory (where the original Rohon Beard cells were present, and at the level of somite 5-6 in the extant chephalochordates) gave rise to the spinal cord of vertebrates, where iterated dorsal sensory neurons and peripheral ganglia arising from sensory committed neural crest are located.

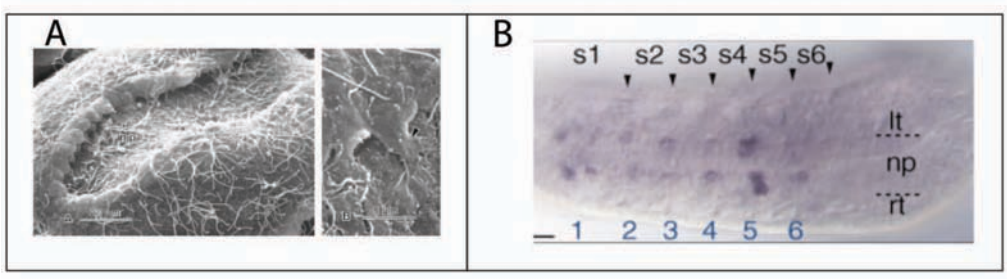


Figure 19. The cells in the dorsal neural tube of amphioxus. A: (scanning electron microscopy) showing migrating epithelial cells during neurulation in amphioxus. This is fig.2 of the paper *'Evolution of neural crest and placodes: amphioxus as a model for the ancestral vertebrate?'*, L.Z. Holland, N.D. Holland, *J. Anat.* 2001. **B:** Islet expression in the 11h amphioxus larva, this is fig.4 *'Islet reveals segmentation in the amphioxus hindbrain homolog'*, William R. Jackman, James A. Langeland and Charles B. Kimmel, Developmental Biology, 2000. S indicates the somites.

III.6.3.3 The neurogenic ectoderm on the ventral side

To further complicate the scenario presented above, a prominent ventral population of sensory neurons is also present, in both amphioxus and tunicates.

These ventral neurons originate along the ectodermal ventral midline in tunicates (Pasini et al. 2006), and in the neurogenic ventral ectoderm in amphioxus (VESNs) . In both cases, these ventral neurons are responsive to BMP/ Delta signaling (fig.20A, B).

The VESNs express markers such as *tlx* (Kaltenbach, Yu, and Holland 2009), *islet* and the only ortholog of the *trk* receptor (Benito-Gutiérrez et al. 2005). Moreover, they appear to migrate from ventral ectoderm toward more lateral positions.

Furthermore, it has recently been shown that at least some proportion of these cells, the more lateral-dorsal ectodermal neurons, send projections to the central nervous system through the dorsal roots, which represent a portion of the peripheral nervous system of

amphioxus (Lu, Luo, and Yu 2012).

While a striking similarity exists between the molecular profiles of the ventral peripheral sensory neurons of amphioxus and *Ciona* with vertebrate cell types, this characterization The VESNs might represent 'evolutionary' a different class of sensory neurons. They originate in a different position in the body plan compared to the neural crest derivatives; they arise in the neurogenic epidermis, and not in the neural tissue.

Based on the position and the expression of some placodal genes such as *six1/2*, *eya* and sensory makers also expressed in the placode-derived sensory neurons such as *tlx* and *brn3* (Kaltenbach, Yu, and Holland 2009),(Meulemans and Bronner-Fraser 2007) in the region, it was proposed that the ectodermal sensory neuronsof amphioxus might be homologous to the vertebrate placode-derive sensory neurons. Actually, due to the lack of WMISH with cellular resolution in amphioxus, it is not clear if these markers are really expressed in the precursors of ventral sensory neurons. Nevertheless, Lu, Luo, and Yu (Lu, Luo, and Yu 2012) speculate that this ventral Bmp + domain might correspond to a protochordate pre-placode, which, in the more anterior region, condensed during evolution on the most dorsal side, just adjacent to the neural plate border.

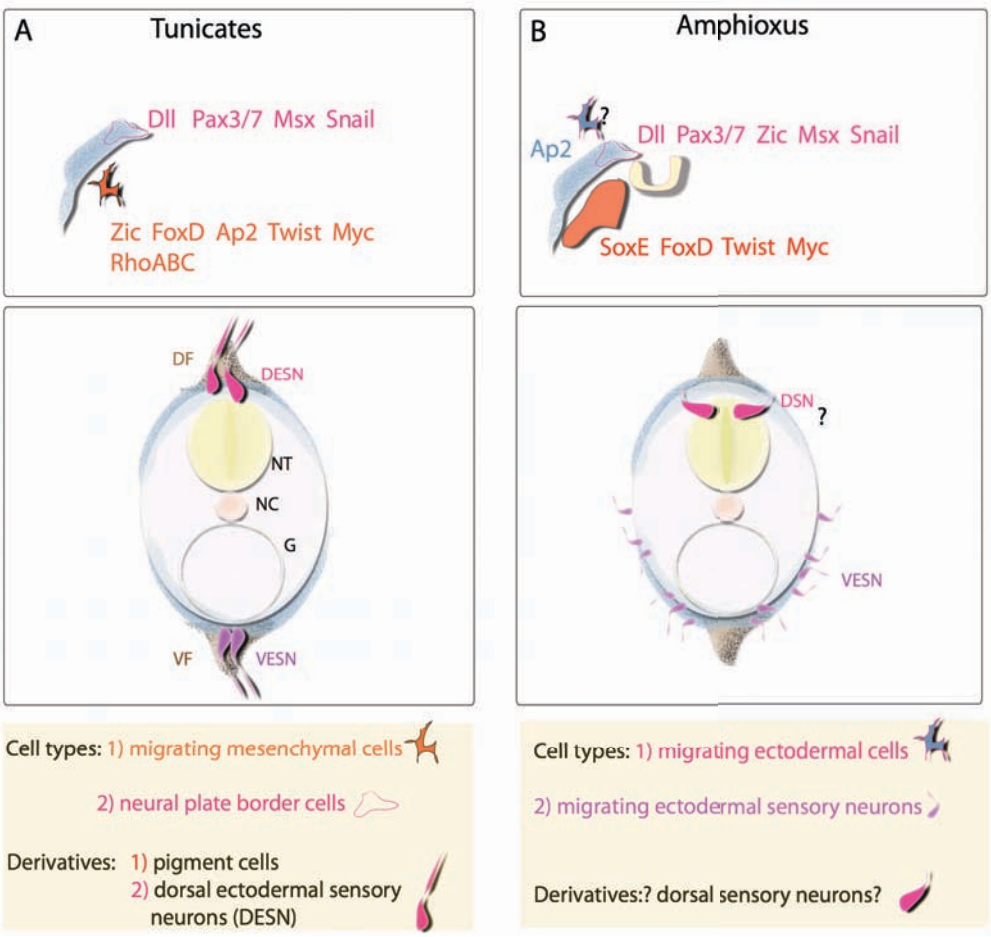


Figure 20. Relevant cell types and derivatives in tunicates and amphioxus. A: In tunicates, migrating mesenchymal cells (indicated in orange) differentiating into pigment cells are derivatives of the A7.6 lineage that give rise to endoderm and mesoderm. They express some of the neural crest specifies, as indicated. A different population of progenitors at the edge of the neural plate expresses the upstream genes of the NC-GRN and give rise to dorsal ectodermal sensory neurons (DESN) localized on the dorsal midline and projecting through the fin. Ventral sensory neurons (VESN) originate far from the neural plate border domain, on the BMP side. **B:** in Amphioxus, cells at the ectodermal edges migrate and fuse over the forming neural tube. They most likely

correspond to the cells expressing some of the genes of the NC-GRN: Dll, Pax3/7, Msx, Snail. Ap2 is expressed in the outer domain in the ectoderm. Crucial neural crest specifies are expressed in somatic mesoderm and chordamesoderm (indicated in red). Dorsal sensory neurons (DSN), Islet and VGLUT +, which have been homologized to Rohon Beard cells, are found in the dorsal neural tube of the animal. It is not clear if they originate from the Dll + precursors and little is known in relation to their molecular signature. Similar to the tunicate situation, ectodermal sensory neurons are present on the ventral side (VESN. They migrate towards more dorsal positions during development. In the drawing, the ectoderm is in blue, the neural tissue is in yellow and fins are in light brow. NT: neural tube, NC: notochord, G: gut, DF, VF are the dorsal and ventral fin. The drawing was based on the references cited in the main text.

It is clear that the ontological relationship between trunk neural crest and sensory neurons is most likely an ancestral feature of vertebrates. However, it is not clear from the literature if sensory neurons were generated in the same field as neural crest at the base of chordates, or if they represent ancestors of neural crest cell types.
It is clear that Prdm1-a, the master regulator of both neural crest and Rohon Beard cells in anamniotes, is an important player of at the border of the neural tube in lamprey (Nikitina, Tong, and Bronner 2011), therefore it might represent an ancestral node for the patterning of the neural plate border. Nevertheless, it is not yet known if lamprey Prdm1-a has a function in determining the cell fate of lamprey Rohon Beard cells /neural crest, as has been previously demonstrated for zebrafish. Amphioxus *prdm1* is expressed only in the gill slits (Dr. Elia Benito Gutierrez, personal communication) similarly to the expression of prdm1-a in vertebrate neural crest-derived brachial arches, but no expression is observed in the early neural plate border-like region. Besides, still fewer hints are available on the development of the Rohon Beard-like cells in amphioxus. Such studies would help in reconstructing a scenario for ancestral chordates. Indeed, it is currently unclear whether the amphioxus neural plate border cells and the future dorsal sensory neurons, a population not yet extensively characterized, share precursors cells. This appears to be the case in some species of tunicates.

III.6.4 What does the development of *Drosophila* sensory organs tell us?

I have examined the evidences pointing to a conserved fate of the cells emerging from the neural plate border in chordates. In order to understand if this 'sensory code' was an ancestral feature of the neural plate border-like territory of all bilaterians I need to examine the cell fate of homologous precursors in protostomes.

The development of the sensory neurons in *Drosophila melanogaster* (Ecdysozoa, member of the protostome group) suggests that some genes important in determining cell fate at the border of the neural plate might also be involved in the formation of the sensory organs (SO) of the PNS in insects (Isshiki, Takeichi, and Nose 1997; D'Alessio and Frasch 1996),(Arendt and Nübler-Jung 1999). For example, the ortholog of *prdm1-a* is expressed transiently in the precursors of the sensory organs (SOP) (Ng, Yu, and Roy 2006), a population derived from a conserved *msx* + territory that also expresses *snail* (Ip, Levine, and Bier 1994).

Bearing in mind that the development of *Drosophila* is highly divergent, molecular conservation of some aspects of sensory cell development might be not easily detectable. Sensory organs have also been described in annelids (Purschke 2005),(Hausen 2007), but little was previously known regarding their development and their molecular coordinates.

In order to shed light on neural crest evolution, I characterized the peripheral sensory neurons originating from the lateral neuroectoderm of the trunk in the marine annelid *Platynereis dumerilii*. I then compared the molecular development of the peripheral sensory neurons to that known for other invertebrates.

IV. The emergence of neurotrophic signaling pathway and its possible roles in neural crest evolution

IV.1. The evolution of the neural crest and the expansion of ligands

Signaling pathways such as Bmp and Wnt are essential in conferring cells the capacity to respond to the environment and acquire and maintain a particular identity during development, such as during the onset of neural crest development (paragraph III.3 of introduction).
It has been proposed that a pro-neural crest cell in the dorsal tube of chordates initially diverged and acquired additional features via co-option of transcriptional modules (as explained in the previous paragraphs, Sauka-Spengler and Bronner-Fraser 2008a), as well as via the emergence of new signaling components.

A recent bioinformatic study summarizes the current view on the topic (Wittbrodt 2007). The study has suggested that in particular, the expansion of extracellular ligands might have facilitated the evolution of the vertebrate neural crest and its incredible developmental potential. For instance, the authors of the study calculate a 'gene emergence rate' for specific tissues, (the higher the value, the higher the number of new genes associated with the emergence of a particular tissue).

In this study, the majority of genes appearing at the transition between chordates and vertebrates, correlating with the evolution of neural crest, are ligands. These ligands include neurotrophins, endotelins and GDNF. These "new ligands" most likely allowed for more plasticity of existing signaling systems, thereby allowing the evolving cells to respond to new environmental cues, to migrate, to proliferate and to differentiate into multiple derivatives. For example, it has been demonstrated in chicken that only the neurogenic neural crest (the earliest population of neural crest) expresses TrkC (Henion et al. 1995). These cells are dependent on the NT-3 molecule produced by the appropriate environment in order to survive and differentiate correctly.

IV.1.2 How trophic factors might have driven the evolution of the nervous system

(von Bartheld and Fritzsch 2006)

The emergence of additional trophic factors and new pathways during evolution might have allowed the control of neuronal number, through expansion of the initial cell population (the neural crest prototype cell) by proliferation and cell survival (fig.21).

The evolution of the neurotrophic signalling might have influenced also the expansion of neurons in general in the nervous system. It is easy to envisage that molecules such as the

neurotrophins might have contributed to the establishment of new connections between the old neuronal cell types or between neurons and novel targets, thereby changing the target innervation pattern of existing neurons. Such a change could create a new neuronal type from an initially homogenous cluster (von Bartheld and Fritzsch 2006).

Emergence of new sensory cell subtypes from dorsal root ganglia neurons, with modification of their existing targets and sensory modalities, would follow the model of '*parcellation*' described by Ebbesson in 1980 and 1984 (S. O. Ebbesson 1980) (S. O. E. Ebbesson 1984) (von Bartheld and Fritzsch 2006). If this model is accurate, it would represent one of the best examples of such a 'cell type diversification' in the nervous system (Arendt 2008).

Although the cell type itself (the peripheral sensory neuron derived from the lateral neuroectoderm/neural crest) is most likely ancient (as studies in chordates and this study indicate), it is plausible that different sensory modalities (discussed in paragraph III.4) evolved in combination the expansion of neurotrophin-Trk signaling.
Thus, understanding how new signaling pathways evolved is crucial to understanding how the nervous system itself evolved.
In the next paragraph, I will briefly introduce the main features of neurotrophic signaling. Neurotrophic signaling is known primarily from mammals. Unfortunately, a limited dataset is available for invertebrates, which necessarily renders our understanding of ancestral neurotrophic signaling incomplete.

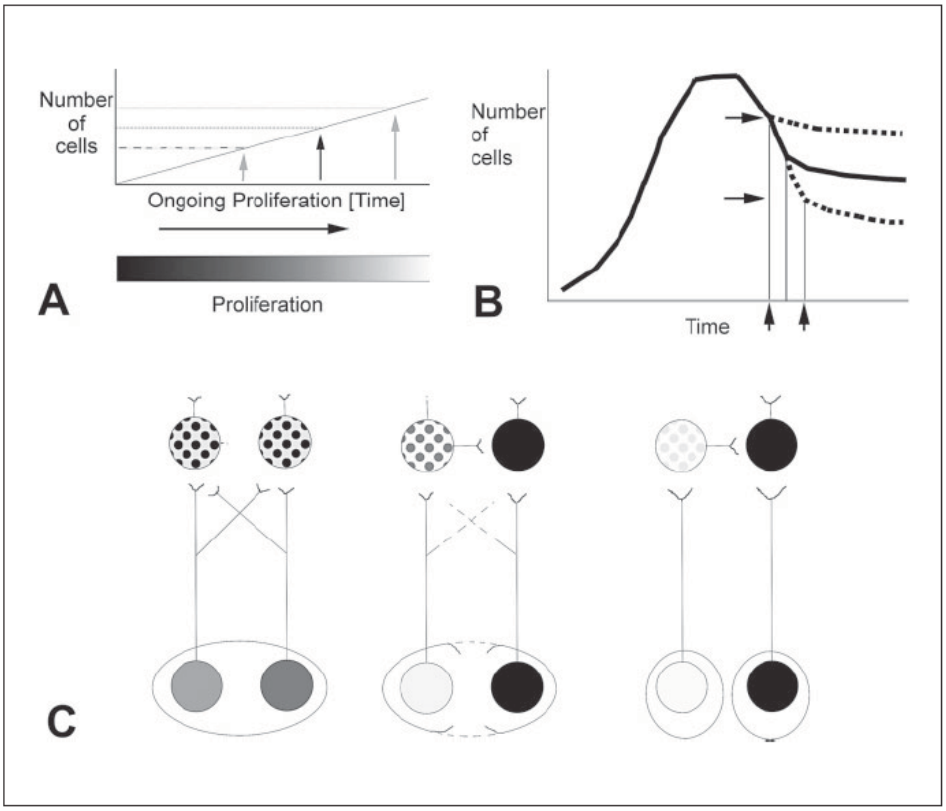


Figure 21. Mechanisms for the emergence of new cell types during the evolution of the nervous system. This is fig.1 of the review: '*Comparative analysis of neurotrophic receptors and ligands in vertebrate neurons: tools for evolutionary stability of changes in neural circuits?* Christopher S.von Bartheld and Bernd Fritzsch, Brain Behav. Evolution, 2006. **A:** expansion of cell number by increasing the proliferation time of a certain precursor. **B:** Expansion of the cell

number by increasing the survival time of a certain cell type. **C:** Cell type expansion and diversification by altering the target connectivity profile in an initial homogenous cell population.

IV.2 General aspects of the neurotrophic signaling in vertebrates

IV.2.1 The molecules involved in the pathway

In vertebrates, the neurotrophic signaling pathway is composed of 4 different ligands : NT3 and NT4/5, NGF and BDNF, which bind to the TrkC, TrkA, and TrkB receptors, respectively. The low affinity binding co-receptor p75 harbours a death domain in its intracellular portion, and is also involved neurotrophic signaling.

-The Trk receptors

The Trk receptors belong to the RTK superfamily of tyrosine receptors. Additional memebers of this family include the Ror (receptor tyrosine kinase- like orphan receptors), the Musk (muscle specific kinases) and the Ddr (discoidin domain recep- tor family) receptors.

The most variable portion of RTK receptor superfamily members is the extracellular portion composed of different domains, as shown in fig.22. The characteristic combination of these domains in each receptor is a consequence of different evolutionary rearrangements that are still yet to be fully understood. They provide binding specificity for a particular ligand. For example, the unique combination of LRR and IgG1 and IgG2 present in neurotrophic receptor extracellular domains confers the ability to bind to neurotrophin ligands (Ibáñez 1998),(fig.23).

-The neurotrophin ligands, the co-receptor p75 and the signaling pathway

Generally, the mature neurotrophin molecule is composed of a core of cysteins (the *Cys knot*) (Vitt, Hsu, and Hsueh 2001) that maintains the functional 3-dimensional structure of the molecule through disulphide bonds. Two molecules dimerize and bind to an extracellular pocked formed by the IgG domains of a Trk receptor homodimer. P75 also binds to the Trk receptor in this complex (fig.23).

After binding neurotrophin, the Trk receptor is autophosphorylated in the intracellular TK domain. The MAPK cascade is activated, leading to the activation of the transcription factor CREB, which in turn promotes cell survival.

When the immature form of neurotrophin (pro-neurotrophin) binds the p75 and Sortilin homodimer, it induces neuronal death and is likely to control the response following neuronal injury (details in fig. 23), (Bibel, Hoppe, and Barde 1999; Eric J Huang and Reichardt 2003; Volosin et al. 2008). In general, the neurotrophins and their receptors are widely expressed throughout the brain, with *trkB* and *trkC* expressed in neurons with more restricted patterns of innervation and *trkA* in neurons with more diffuse contacts that are required for signaling integration (von Bartheld and Fritzsch 2006).

IV.2.2 The function of the neurotrophic signaling

Rita Levi Montalcini received the Nobel Prize in 1986 for the discovery of the first neurotrophin molecule (NGF), (Levi-Montalcini, R. 1975),(Levi-montalcini 1987; Aloe 2004). She observed that a tumoral tissue transplanted close to the spinal cord was able to induce neurite outgrowth from the neuronal ganglia. She hypothesized, based on this

observation, that a diffusible growth factor produced by the tumor might have been responsible (Levi-Montalcini and Hamburger 1951).

Montalcini then isolated and, with her co-awardee Stanley Cohen, biochemically characterized the NGF protein.

This research anticipated the finding that the peripheral innervation of several tissues depends on neurotrophic signaling during development (E J Huang and Reichardt 2001; Buj-Bello, Pinon, and Davies 1994; Liebl et al. 1997; Ockel, Lewin, and Barde 1996; Hory-Lee et al. 1993; Davies 1994; Tessarollo 1998), (Bacher 1973). Skin, viscera and muscles produce the neurotrophin ligand, which then diffuses to bind Trk and p75 on the growing axon of developing neurons. This represents a '*neurotrophic dependency*' mechanism of peripheral neurons (E J Huang and Reichardt 2001). For example, in skin-deprived fishes, the Rohon Beard cells (discussed in the previous paragraphs) die because they lack neurotrophic factors that normally originate from the skin (Bacher 1973). Further research has implicated the neurotrophins in a multitude of other functions in the nervous system, such as in the control of cell survival. As outlined above, the neurotrophins are also involved in the correct genesis of the trunk neural crest.

In contrast, survival defects observed in the absence of neurotrophic signaling in the central nervous system are less pronounced (Minichiello et al. 1999; Minichiello and Klein 1996; Alcántara et al. 1997; Martínez et al. 1998)**.** Furthermore, functions such as memory and learning are also associated with Trk signaling. *BDNF* and *trkB* are expressed in the neocortex and in the hippocampus (Webster et al. 2006; Medina et al. 2004), in addition to *trkA* and *NGF*.

Here, as in the visual cortex, Trk-Neurotrophin signaling persists in adults, and most likely serves a role in the maintainenance and modification of the central circuits by acting to fine tune arborizations and by controlling long-term potentiation (LTP).

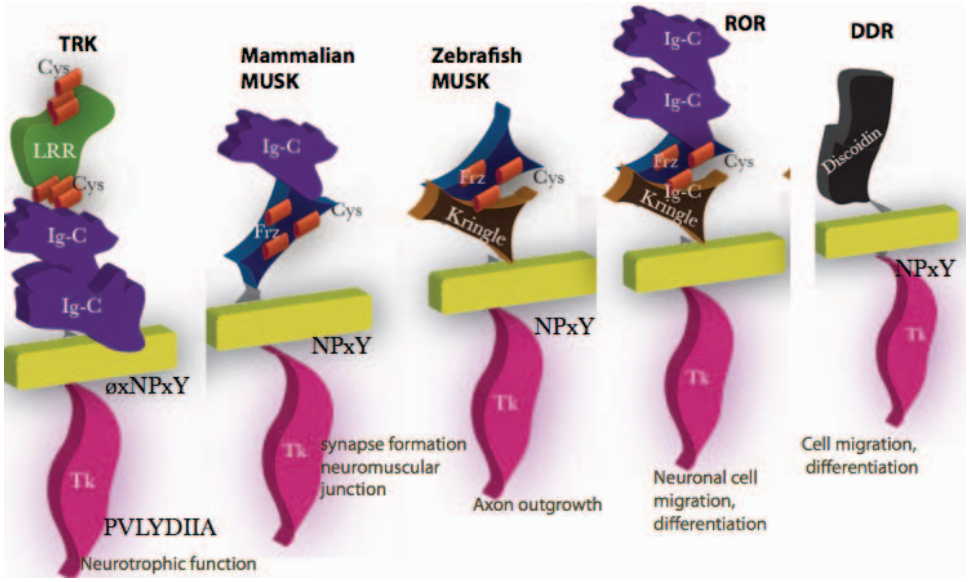


Figure 22. Members of the RTK superfamily

This is a schematic representation of the members of the Receptor tyrosine kinases (RTKs) superfamily. These members include TRK (nerve growth factor receptor), ROR (receptor tyrosine kinase-like orphan receptors), MUSK (muscle specific kinases) and DDR (discoidin domain recep- tor family). The domains are indicated as follows: Tk : tyrosine kinase domain, IgG: immunoglobulin domain, LRR: leucin reach domain,Frz: frizzled domain, Kringle :

Kringle domain, Discoidin: discoidin domain. The NPxY and Yxxø (ø, hydrophobic) sites undergo phosphorylation and bind PTB or SH2 domain-containing proteins.

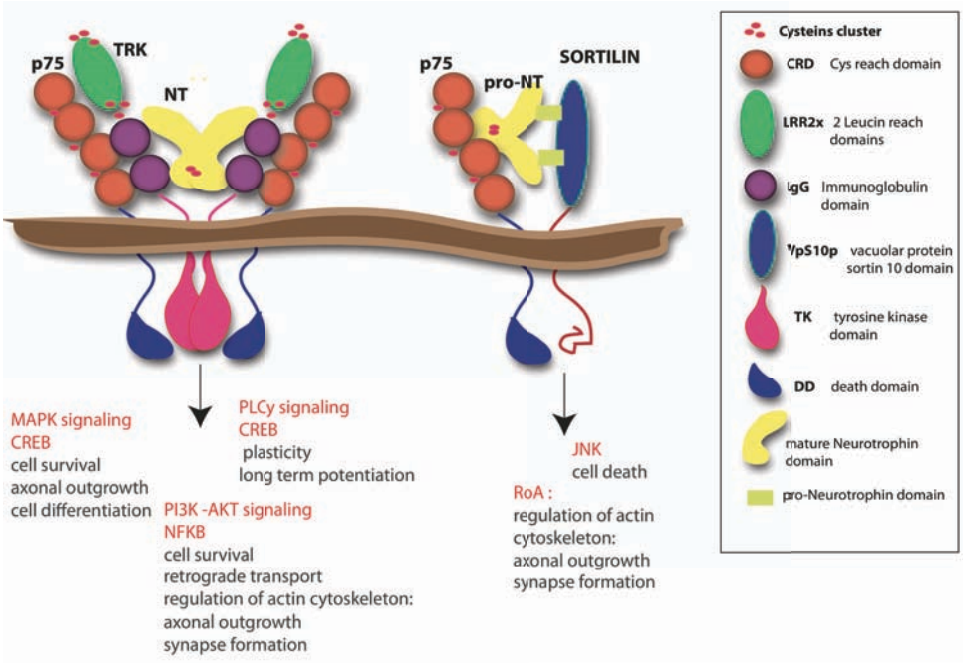


Figure 23. Neurotrophic signaling in vertebrates. When NT dimerizes, it binds to the hetorodimer consisting of Trk and p75 and activates different pathways in the cell. These include the MAPK, PI3K and PLCy pathways for cell survival, axon pathfinding and long term potentiation. When the immature form of neurotrophin dimerizes and binds to p75 and Sortilin, it conveys a cell death signal to the cell. The different domains of Trk and p75 are indicated in the legend on the right. CRD: Cystein rich domain, IgG: immunoglobulin domain, LRR : leucin reach domain. The color coded for the Trk domain is the same as fig.15.

IV.3 Is there a neurotrophic signaling in invertebrates?

Neurotrophic signaling has long been considered a vertebrate novelty.

The ligands and receptors expanded early in vertebrate evolution. This expansion allowed the diversification of new cell types and their accompanying functions, as discussed previously. According to the phylogeny of neurotrophin proposed by Finn Hallböök (Hallböök, Lundin, and Kullander 1998)NGF and NT3 originated after the duplication of an intermediate ancestral gene. BDNF and NT4/5 diversified from yet another distinct ancestral gene in bony fishes.

Similarly, it has been proposed that Trk receptors might have originated from ancestral TrkA/TrkC and Trk B molecules. TrkC and TrkB seem to retain ancestral patterns of expression within the nervous system, as they are expressed in the otic ganglia and in the reticular system of the brain stem, even in basal vertebrates (lamprey) (Hallböök, Lundin, and Kullander 1998).

In contrast, TrkA/NGF associated structures, such as the hippocampus, the sympathetic ganglia and nociceptive neurons, are absent in lamprey and might represent a gnathostome innovation (Butler and Hodos 2005).

In lamprey, two Trk receptors and one neurotrophin have been isolated, but they are distinct from the ones present in fishes (Hallböök, Lundin, and Kullander 1998). The

lamprey signaling system most likely represents pre-duplication descendants of ancestral components.

Nevertheless, little is known about their expression profiles, as discussed in paragraph III.4. While recent datasets have demonstrated the presence of components of the neurotrophic pathway (either one or all of them) in invertebrates (K. H. S. Wilson 2009), it remains unknown whether functional neurotrophic signaling is a vertebrate novelty.

-Amphioxus

Until recently, neurotrophic signaling has been considered as a vertebrate innovation. This idea has been challenged by the discovery of the amphioxus ortholog of Trk, placing the emergence of neurotrophic signaling at the base of chordates (Benito-Gutiérrez et al. 2005). (fig.1).

Amphioxus possesses a single canonical Trk receptor. The extracellular region contains Immunoglobulin domains (IgG) necessary for the binding of neurotrophin. *Amphi*trk is expressed in the cerebral vesicle (a the brain neurosecretory center) and in the peripheral sensory neurons in the ventral ectoderm (Benito-Gutiérrez et al. 2005,VESN, see paragraph III.6.5).

Interestingly, the intracellular portion of the chordate receptor lacks the docking site for PLCγ that has been implicated in long-term potentiation, learning and memory mechanisms. It has been suggested that these functions most likely evolved in vertebrates, but a loss in amphioxus might also have occurred. Nothing is known about *Amphi*NT and p75, which are also present in the genome.

-The protostome neurotrophin signaling

Recently, similar molecules have been discovered in protostomes, such as molluscs (a detailed analysis of the presence and of the domains of the putative Trk receptors present in invertebrates is shown in fig.24, (Beck et al. 2003) K. H. S. Wilson 2009).

These 'Trk-like' receptors are highly divergent from the canonical representatives; the extracellular portion, which is responsible for the binding of neurotrophin and thus for function, is not comparable to the vertebrate-amphioxous extracellular region. Interestingly, while molluscs seem to lack neurotrophin molecules, chimera constructs have demonstrated that the intracellular portion of *Lymnea*Trk (as is also the case for amphioxus Trk) is able to activate the canonical MAPK kinase pathway (Beck et al. 2003). Nevertheless, a ligand for *Lymena*Trk has not yet been found.

The classical model system *Drosophila* has a divergent Trk receptor (Pulido et al. 1992),(Winberg et al. 2001), and possesses very divergent neurotrophin molecules ('NT-like'). These molecules have maintained a function in axon pathfinding, but it is not known through which receptors and pathways they act (Zhu et al. 2008).

Among the arthropods, crustaceans are considered to be less derived than insects. Accordingly, in parallel to my study in *Platynereis*, a genome search revealed the presence of canonical Trk, NT and p75 molecules in the crustacean *Daphnia pulex*, but nothing is known about their signaling or function (K. H. S. Wilson 2009).

Interestingly, *Daphnia* Trk possesses a putative PLCγ docking site that should be experimentally tested. If this proves to be a functional binding site, it could challenge the idea that this is a newly formed domain employed in vertebrates for 'higher' brain functions such as learning and memory.

Due to the importance of the neurotrophic signaling during the evolution of the neural crest, I asked whether a prototype of such a signaling is present in *Platynereis dumerilii*. This led to the discovery of the specificprotostome neurotrophic signaling components at a time when a canonical signaling was unknown outside of deuterostomes.
Furthermore, in order to shed light on the evolutionary appearance of neurotrophic signaling, I also investigated the presence of putative homologous signaling in the cnidarian *Nematostella* (described in the Results section).

A

	TRK	NT	p75
Amphioxus (cephalochordates)	Canonical receptor PLCγ binding site missing	NT wiith C-terminal extension	Wegen (no death domain)
Daphnia (arthropods, crustaceans)	Canonical receptor	Canonical NT and Spz	Canonical p75
Drosophila (arthropods, insects)	no	Spz	Wegen (no death domain)
Aplysia (mollusks)	Only Tk conserved	no	no
Lymnea (mollusks)	Tk conserved + LRR domain +IgC1 domain (immune system)	no	no
Lottia (mollusks)	Tk conserved + LRR domain +IgC2 domain Sequence errors	Canonical NT	N-termus of the 1CRD missing

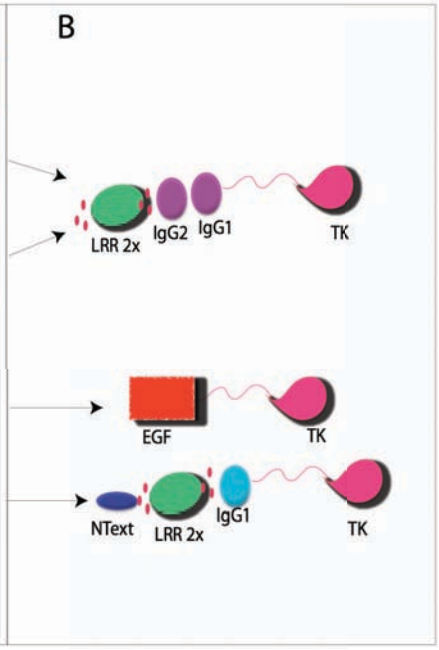


Figure 24. Components of neurotrophic signaling in invertebrates. A: Schematic table representing the presence of TRK, NT and p75. Details of each component are indicated, e.x.: canonical or non canonical. 'No' indicates that no putative homologous molecule has been identified thus far. **B:** Schemaric drawing representing the divergent extracellular domains of the TRK and TRK-like receptors in *Amphioxus, Daphnia, Aplysia and Lymnea. EGF:* epidermal growth factor, *Text:* N-terminal extension, for the other domain abbreviations in this fig. see legend in fig.24

Aim of the thesis:

The neural crest arises from the dorsal neural tube and gives rise to a multitude of derivatives, such as sensory neurons of the peripheral nervous system, cartilage and pigmented cells (N. Le Douarin and Kalcheim 1999). The evolution of the neural crest remains one of the biggest open questions in evolutionary developmental biology. Conflicting datasets have made it impossible to determine whether neural crest evolutionary precursors were present at the base of chordates.

If neural crest precursors predated the evolution of vertebrates, what did they look like?

Molecular evidence has shown that conserved molecular coordinates pattern the annelid trunk from ventral (medial) to dorsal (lateral), as in vertebrates (Denes et al. 2007). Building on this finding, the aim of my thesis was to characterize the cells emerging from the dorsal-most part (lateral column) of the *Platynereis* trunk nervous system, investigate whether neural crest specific genes are expressed, and determine the development, behaviour and molecular fingerprint of the cell types emerging from this territory.
My final aim was to compare the molecular coordinates and the cell fate of the dorsal neurectoderm in invertebrates with the neural crest in vertebrates to find out if 'latent' neural crest precursors were present at the base of bilaterians.

For this purpose, first I aimed to use the molecular fingerprint approach at the cellular level. Cell types are the minimal units conserved over large evolutionary distances (Arendt 2008), and that they diversify over time, molecular features have been shown to be conserved as barcodes for *'ancient cell types'*. To achieve this, a screening of the neural crest genes via WMISH, expression profiling via *in silico* alignment was needed. Furthermore, in order to follow the development and the behaviour of the cells emerging from the lateral domain over the time, it was necessary to optimize transient transgenesis and develop live imaging protocols as well as cell and axonal retro-labelling.

Inductive signals such as the Wnt pathway are crucial to determine where the neural plate border arises in vertebrates (Dorsky, Moon, and Raible 1998), (Deardorff et al. 2001)(Hari et al. 2002). Understanding if such a signaling plays a role also at the lateral neuroectoderm of *Platynereis*, in comparison to vertebrates was also a goal of my study.

Next, I set out to determine the molecular fingerprint of the cell types emerging from the lateral territory and compare with the neural crest derivatives in vertebrates.

Finally, I asked whether the neurotrophic signaling pathway, associated with the evolution of the neural crest (Wittbrodt 2007) and a vertebrate innovation, is conserved in *Platynereis*. When I started this study, no canonical neurotrophic signalling was known in protostomes. Therefore, it was necessary to isolate and investigate the specific annelid neurotrophic molecules and compare them to the vertebrates ones. In order to shed light on the evolution of this signaling I also aimed to compare the protostome molecules with putative orthologs in non-bilaterian animals, the Cnidaria.

RESULTS

1. Cell types in *Platynereis* trunk and the evolution of neural crest

I. The lateral neuroectoderm in *Platynereis*: gene expression and developmental fate

I.1 Early in embryogenesis neural plate border genes and neural crest specifiers are expressed in the *Platynereis* lateral neuroectoderm[2]

Our lab has previously shown that conserved molecular coordinates pattern the neural plate in *Platynereis* and vertebrates from medial (ventral) to lateral (dorsal) (Denes et al. 2007, fig.6). Building on these evidences of molecular conservation, I set out to shed light on the evolutionary origin of the neural crest, a population that originates from the dorsal-most domain in vertebrates (fig.5). Hence, a prerequisite for this work was to expand the analysis of the lateral neuroectoderm and to test how it compares to the vertebrate neural plate border (NPB), where neural crest originates.

In anamniotes the development of the early migratory neural crest takes place at the dorsal territory of the neural tube during gastrulation (the neural plate border, NPB), during the time when primary sensory neurons are already differentiated (Lamborghini 1980), (Baccaglini and Spitzer 1977) , (Rohon Beard cells), (Jacobson 1981) , (Artinger et al. 1999). These are primary sensory neurons originating from the same precursor field of the neural crest, thereby they express many genes that are known for the neural crest cells (Rossi, Kaji, and Artinger 2009) (table in fig.15). These observations led to the speculation that neural crest cells derive from Rohon Beard-like cells evolutionary precursors.

Hence, I started the analysis at early stages of development (between 20-24h), stages that have not been previously investigated (Denes et al., 2007). These early stages should correspond to the open neural plate stage in vertebrates, when the first neural crest and the Rohon Beard cells are specified. Similar to the approach taken in protochordates (amphioxus) and basal vertebrates (lamprey), I investigated the expression pattern of the crucial genes for the formation of neural crest cells and Rohon Beard cells.

Molecular fingerprint at later stages (48h) has been assessed *in silico* using PrImR (Tomer et al. 2010). This tool is not yet available yet for early stages, and co-expression analysis at early stages (24h) via double WMISH is extremely difficult to achieve, and has led to misleading results. Therefore, at these early stages, I could only determine single expression patterns for the genes of interest. I used the ventral and dorsal limit of the prototroch cells between the second and the end of the third row of the ciliated cells, and first row of the telotroch as morphological landmarks for defining the lateral domain at 24h (fig.26 J).I subsequently validated the co-expression of these genes using PrImR at 48h of development (see paragraph I.3).

[2] Dr.Mette Handberg-Thorsager and Franziska Gruhl (at the time a student in our lab, under our supervision) have contributed to the insitu work on the early stages and on the Edu treated embryos. Dr. Mette Handberg-Thorsager has contributed fixing some of the embryos used, supervising Franziska and commenting the results. Franziska has contributed performing Edu treatments, WMISH at early stages , some of the confocal scans and commenting the results.

-Neural plate border genes

Between 20-24h the epiboly movements in *Platynereis* are nearly complete and few neurons are present in the trunk (Appendix, paragraph A1, fig. a1, a2). At these stages, the lateral neuroectoderm is highly proliferative (Appendix, paragraph A2, fig.a3,b2).

I observed that at 20h the lateral neuroectoderm expresses markers of the neural plate border such as *pax3/7, msx* and *dll (*NPB, fig.10 and table in fig.11,fig. 25). This is consistent with the data shown in Denes at al. 2007 for later stages. Furtheremore, I also found that orthologs of *zic, prdm1* and a*p-2* (also considered a neural crest specifier) are expressed in the lateral territory at this stage. I will discuss these genes below, as they have not been described before.

At 24h, the expression of the neural plate border genes in the lateral neuroectoderm occurs segmentally (visible for example in the *msx* and *prdm1* expression pattern in fig.26C and E). Similarly to vertebrates (Khudyakov and Bronner-Fraser 2009), while *Platynereis pax3/7* and *zic* are expressed in the medial-most neuroectoderm and in the neural plate border-like territory, genes such as *msx*, *dll* and *ap-2* are expressed more broadly and extend further into the more dorsal ectodermal domain.

Prdm1:

Recent observations (introduction, III. 5.2) show that Prdm1-a (Blimp-1) is a crucial player in the formation of neural crest and Rohon Beard cells (Rossi, Kaji, and Artinger 2009), (Olesnicky, Hernandez-Lagunas, and Artinger 2010).

Strong expression of a *prdm1* ortholog in the neural plate border of the basal vertebrate lamprey (Nikitina, Tong, and Bronner 2011) also points to a an ancestral function in defining this domain.

Therefore, I cloned the *Platynereis* ortholog of *prdm1-a* and analyzed the expression profile at different stages of development.

The annelid *prdm1* ortholog (phylogenetic tree in Appendix, D.3) is expressed in a small number of cells per segment, in the lateral-most domain (fig.25F, 26E). More posteriorly, *prdm-1a* is also expressed medially. Later in development (fig.27), it is evident that the *prdm1+* cells correspond to precursors of the ciliary bands. They are easy to recognize based on their elongated morphology (cells indicated with a white arrow in the insets in fig.27A, C) compared to the other cells of the neuroectoderm and also because they are multiciliated.

Interestingly, as is the case for the zebrafish Rohon Beard cells precursors (Roy and Ng 2004), *Platynereis prdm1* expression is downregulated in the ciliary band cells as soon as the cells begin to form cilia (differentiate).

Indeed, at 48h (fig.27C) *prdm1* was not detectable in the lateral domain. Accordingly, the cells of the paratroch expose cilia at this stage (cilia are indicated with a pink arrow in the pink inset in fig.27C).

A stripe of *prdm1* +cells remains in the neuronal midline (yellow arrow in fig.27C), a population occasionally labeled with DiI at these stages (fig.29L). This might be

reminiscent of the medial ciliary band present in other polychaetes (Bartolomaeus and Purschke 2005) ,(N. P. Meyer and Seaver 2010). At this stageI observed *prdm1* expression in new precursors of on the anterior most ciliated band that is now developing : the metatroch. These cells do not expose cilia yet, and express *prdm1* (white inset in fig. C, cells indicated with a white arrow).

-Neural crest specifiers

As outlined in the introduction (paragraph ,III.3.2), members of the soxE family (sox8,9, 10) are specific for neural crest cells and in anamniotes it is downstream *prdm1-a* only in the neural crest and not in the Rohon Beard cells (Olesnicky, Hernandez-Lagunas, and Artinger 2010). SoxE family genes are responsible for the mantainance of the neural crest pool (sox10)(Haldin and LaBonne 2010), (Stolt and Wegner 2010). They also activate the migration cascade genes (sox9) (McKeown et al. 2005) and they are sub sequentially implicated in the skeletogenic program activated in the cranial neural crest that give rise to cartilage (sox8,9) (Mori-Akiyama et al. 2003). I cloned the ortholog of *Platynereis* soxE[3] (phylogenetic tree in Appendix, D6) and analyzed the expression profile.

Surprisingly, I found that *Platynereis* s*oxE* is already expressed at 24h in the *Platynereis* NPB-like region *(*fig.26H*)* in dividing cells (H',H'').

Slug/snail are zinc finger repressors and are among the earliest markers for neural crest induction (LaBonne and Bronner-Fraser 2000)(Sakai et al. 2006), (Betancur, Bronner-Fraser, and Sauka-Spengler 2010).

In anamniotes they are also expressed very early in the neural plate border, where they are likely to inhibit epidermal fate and to induce morphogenetic changes required during neurularion (Essex, Mayor, and Sargent 1993). Therefore, they are also considered NPB genes.

Ap-2 is a neural crest specifier (Luo, Lee, and Sargent 2002). It turns on *sox10* expression specifically in the neural crest, accordingly, fish mutants lacking ap-2 show reduction of all neural crest derivatives.

Nevertheless, ap-2 is also considered a neural plate border gene upstream of the specification of Rohon Beard cells. Indeed, it is known that ap-2 regulates the expression of *prdm1-a* in lampreys and fish (Li and Cornell 2007),(Nikitina, Tong, and Bronner 2011), (de Crozé, Maczkowiak, and Monsoro-Burq 2011). Therefore, I investigated the expression of these genes in *Platynereis* and found that both orthologs of *slug/snail (Platynereis snail2* probe courtesy of Dr. Antje Fischer) and of the ortholog of *ap-2* (phylogenetic tree in Appendix, D7) are expressed in the neural plate border-like region (fig.26F,G).

While *Platynereis ap-2* is expressed broadly in the lateral neuroectoderm, comprising also the dorsal ectodermal side, as in vertebrates and in amphioxus, *snail2* and s*oxE* show a specific more restricted expression pattern. S*oxE* is clearly in dividing cells (fig. 26 H, H'), likely comprising neuronal precursors. Co-expression and functional data are needed to understand this patterning in more details.

[3] The primers to amplify partial DNA sequence of this gene were designed by Dr. Alexandru Denes (see Materials and Methods)

Collectively, these data expand the previously published comparison between the neural plate of *Platynereis* and the neural tube of vertebrates (Denes et al, 2007), and support the hypothesis that this lateral region is homologous to the neural plate border in vertebrates, where specification of the neural crest occurs.

-Lateral neuronal precursors

I found that, by 24h, a few precursors emerging from the lateral neuroectoderm, a region spanning from the neural plate border to the dorsal most domain, express *ngn* (fig.26I). Ngns are proneural genes (Furlong and Graham 2005) expressed early in differentiating Rohon Beard cells (Rossi, Kaji, and Artinger 2009) and in cells committed to the sensory fate that are derived from trunk neural crest (Introduction, III.4, III.5.2, fig.11,14), (Ma et al. 1999). Hence, it is likely that *Platynereis ngn+* cells in the lateral neuroectoderm are neuronal precursors.

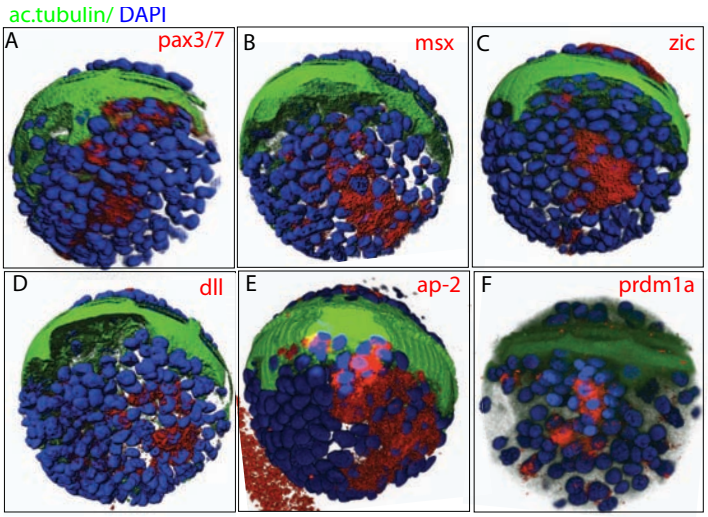


Figure 25. Expression of NPB genes in the lateral neuroectoderm at 20h. At 20h, the neural plate border genes (*pax3/7* in A, *msx i*n B, *zic* in C, *dll* in D, *ap*-2 in E and *prdm1*-a in F) already pattern the lateral neuroectoderm.
At this stage, no segmental pattern is visible in relation to *msx*. The volume rendering has been obtained with Imaris 7.3.1. At 20h it is possible to orient the larva by taking morphological landmarks such as the ciliary bands and the proctodeum into account. Boundaries are hard to distinguish, and for this reason, I do not indicate them here

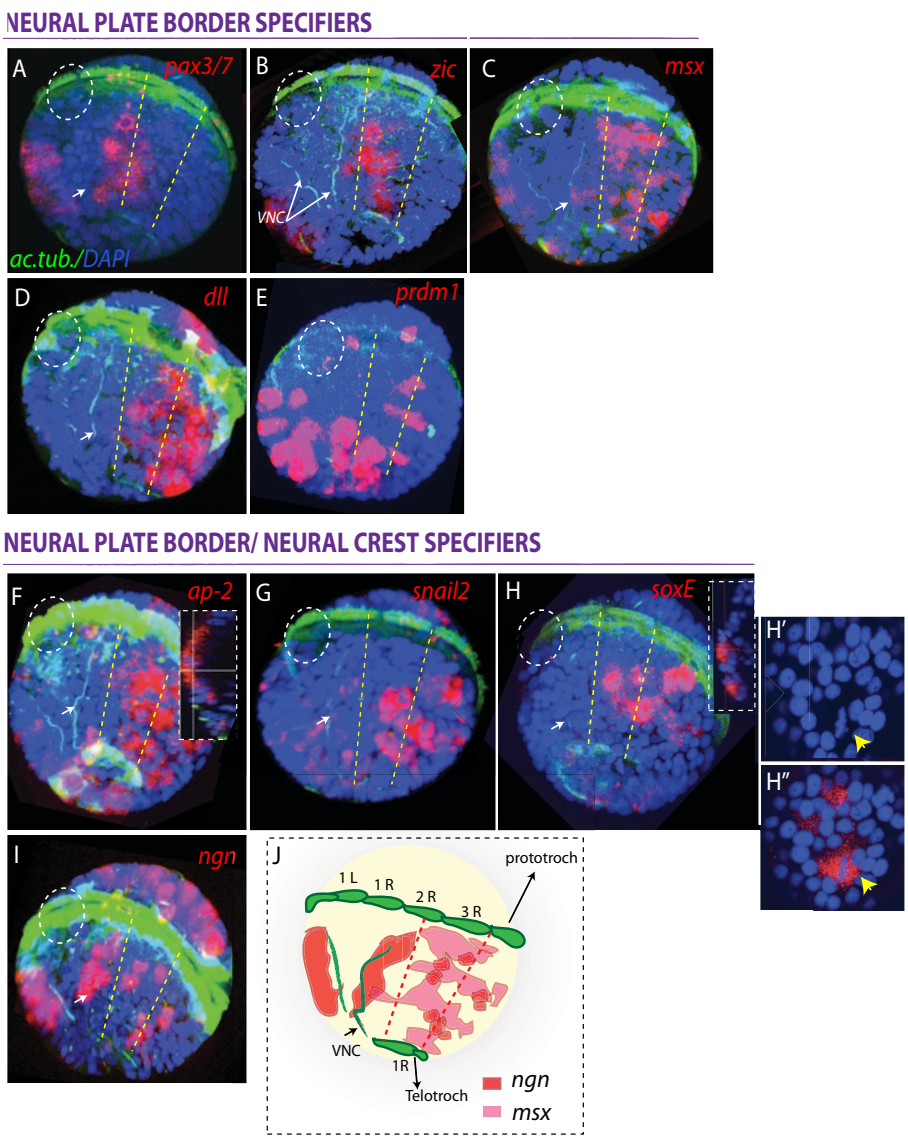


Figure 26. Expression of NPB and NCS genes in the lateral neuroectoderm at 24h. All pictures are lateral views. Yellow dashed lines outline the lateral neuroectodermal portion where all the markers of the neural plate border are expressed at this stage. This domain starts between the 2nd and 3rd row of cilia of the prototroch (2, 3R, 2nd and 3rd right in K) and the end of the 1st row of the telotroch (1R, 1st right in K). In the panel, the distance between the start of this domain and the right axon tract of the ventral nerve cord (white arrow) is constant. **A-E:** WMISH for *pax3/7* (A), *zic* (B), *msx* (C), *dll* (D) *prdm1 (E),* which are segmentally expressed in the lateral neuroectoderm at 24h. **F- H:** WMISH for *ap-2* (F), *snail2* (G) and *soxE* (H). The insets in F and H are virtual cross-sections that show the superficial expression in the neuroectoderm. **H', H''**: some of the *soxE* + cells are actively dividing at 24h (compare DAPI in H' with the staining for SoxE in H'', yellow arrowheads). **I:** lateral view of *ngn* showing few positive neuronal precursors already originating from the area of the neural plate border and extending into the lateral domain. Expression in the medial-most domain is also visible along the tracts of the ventral nerve cord. The neural plate is not yet closed at this stage (ref) **J:** schematic drawing of neuronal patterning in the lateral neuroectoderm represented by *msx* (in light pink) and *ngn* (in light red). The position of the forming ventral nerve cord (VNC) tracts are shown (green), in addition to the stomodeum. In all panels, dashed white circles indicates the stomodeal field. Some of the pictures were rotated using *Imaris*

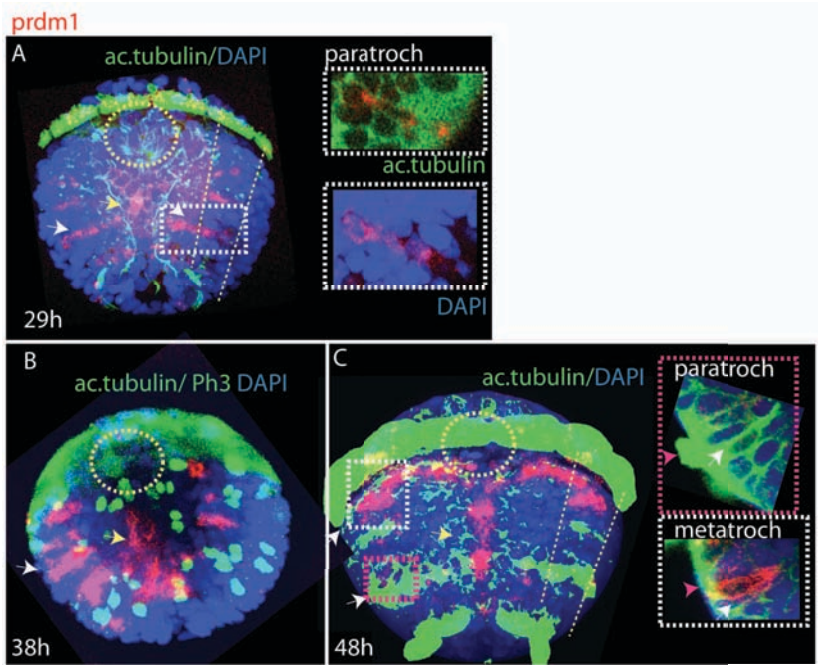


Figure 27. Expression of *prdm1* between 29h and 48h. **A, B:** Later in development, at 29h (A) and 38h (B), *prdm1* is clearly expressed in the precursors of the cells of the ciliary bands, white arrows.In the inset in B a close up of this precursors, recognized for the elongated morphology. Expression in the midline is indicated with yellow arrow. **C**: At 48h, *prdm1* is downregulated in the lateral neuroectoderm (white arrow). Indeed note that the elongated cells of the ciliary band of the paratroch which protrude cilia (pink insets, pink arrowhead) don't express *prdm1* any more at this stage. Strong expression in the midline (yellow arrow) is continuity with a the metatroch precursors (more anterior ciliary band). Note that the metatroch precursors don't protrude cilia yet at this stage and express *prdm1* (white inset in C).

I.2 The proliferative state of the progenitors in the lateral neuroectoderm from 22h to 48h, 30'

Commitment to the sensory lineage is a defining feature of cells found in the dorsal-most part of the neural tube in vertebrates (Delmas, Hao, and Rodat-Despoix 2011; Marmigère and Ernfors 2007; Lallemend and Ernfors 2012, Lallier 1991; Nicole M Le Douarin and Dupin 2003). Primary sensory neurons (the Rohon Beard cells) arise in this territory in anamniotes (Lamborghini 1980). Neural crest cells arise and differentiate into sensory neurons from the same precursor population that forms the Rohon Beard cells (Cornell and Eisen 2000; Moorman 2001).

The neural crest derived sensory precursors undergo extensive proliferation and migration, before forming the dorsal root ganglia along the spinal cord (Delmas, Hao, and Rodat-Despoix 2011; Marmigère and Ernfors 2007; Lallemend and Ernfors 2012, Nicole M Le Douarin and Dupin 2003) (fig.14).

At least three waves of proliferation and differentiation of the *ngn+* precursors occur to form the peripheral dorsal root ganglia in vertebrates, during which the precursors express a set of specific transcription factors in a temporal sequence. First the *ngn+* cells proliferate, they express *brn3* and then *islet* and *runx* genes. Different neurotrophic *trk* receptors are subsequentially expressed in different subsets of sensory neurons (see Introduction, paragraph III.4, fig.14) (Marmigère and Ernfors 2007).

Hence, once I identified the neuronal precursors *(ngn+)* at 24h in *Platynereis*, I asked how do they compare to neuronal precursors of the vertebrate trunk sensory neurons that derive from neural crest. Therefore, I set out to investigate their proliferative behaviour, as well as the developmental fate.

To assess the proliferative behaviour, I performed these experiments using Edu. Edu is a modified nucleoside that is incorporated in newly synthesized DNA, thereby labeling proliferative cells.
Taking advantage of the fact that the Edu incorporation can be coupled to *in-situ* hybridization techniques, Edu-treated animals at various developmental stages were assayed for the expression of indicative markers via WMISH.
Classical pulse-chase experiments with nucleoside analogs were not effective to examine the proliferation state of cells in the trunk in *Platynereis*. For instance, after a pulse of 2h (for example between 22-24h), followed by a chase of the animal until the desired stage (for example until 38h), highly proliferative cells dilute the Edu signal over long time periods, resulting in a false negative result. This experiments led to misleading outcomes (Appendix, fig. b1). For this reason, I decided to perform Edu pulses of only 2 hours at different stages of development and to then fix immediately the larave and analyze via WMISH(from 22h to 48h, 30'). This approach has been widely used in Platynereis to assess the proliferative status of the neuronal precursors (Denes et al., 2007).

To find out how the proliferation relates to the prospective neuronal precursors arising from the lateral territory, I chose to analyze *msx,* a marker of the lateral neuroectoderm and *ngn*, a proneural gene that labels these lateral neuronal precursors. As explained previously and in the introduction, ngn1 and ngn2 mark the early neural crest derived sensory precursors.

-Edu incorporation between 28-39h and 38-40h

The first *ngn+* precursors are visibile at 24h in the lateral neuroectoderm. At this stage they are highly proliferative (Appendix, fig.b2).
Between 28-30h the cells in lateral domain are still proliferating. Conversely, the *mxs-ngn+* cells become Edu negative between 37-39h (fig.28 C,D), coincident with the onset of expression of *elav* (Appendix, paragraph A.2.2, fig.a4).
I also confirmed that in the more medial neuroectoderm progenitors, likely to give rise to motoneurons and interneurons, proliferation continues on the surface (white arrow in fig.3IF, as previously shown in Denes et al, 2007).
It was only at 40h that I could detect some *brn3* +/Edu negative (-) neurons in the lateral *msx* + domain (yellow arrow in fig.28E). These *brn3* +cells likely represent sensory neurons emerging from the most lateral *msx+/ngn+/Edu-*. This is similar to the developmental progression observed in vertebrates, where *brn3* is expressed in the second step of differentiation of the sensory neurons from early migrating neural crest (fig.14).

-Edu incorporation between 46h, 30'- 48h, 30'

After Edu incubation between 46h, 30' and 48h, 30', the majority of the *ngn* + cells in the *msx* territory were Edu - (fig.28 F,G).

I observed that at this stage the cells in the Edu -/*elav* + lateral domain express *islet* (yellow arrow in fig.28 H,I). These cells are likely differentiated sensory neurons.

Consistent with this idea, *islet* + cells are not mitotically active at 48h, as confirmed by the staining for the phosphorylated form of Histone 3 fig.28 J).

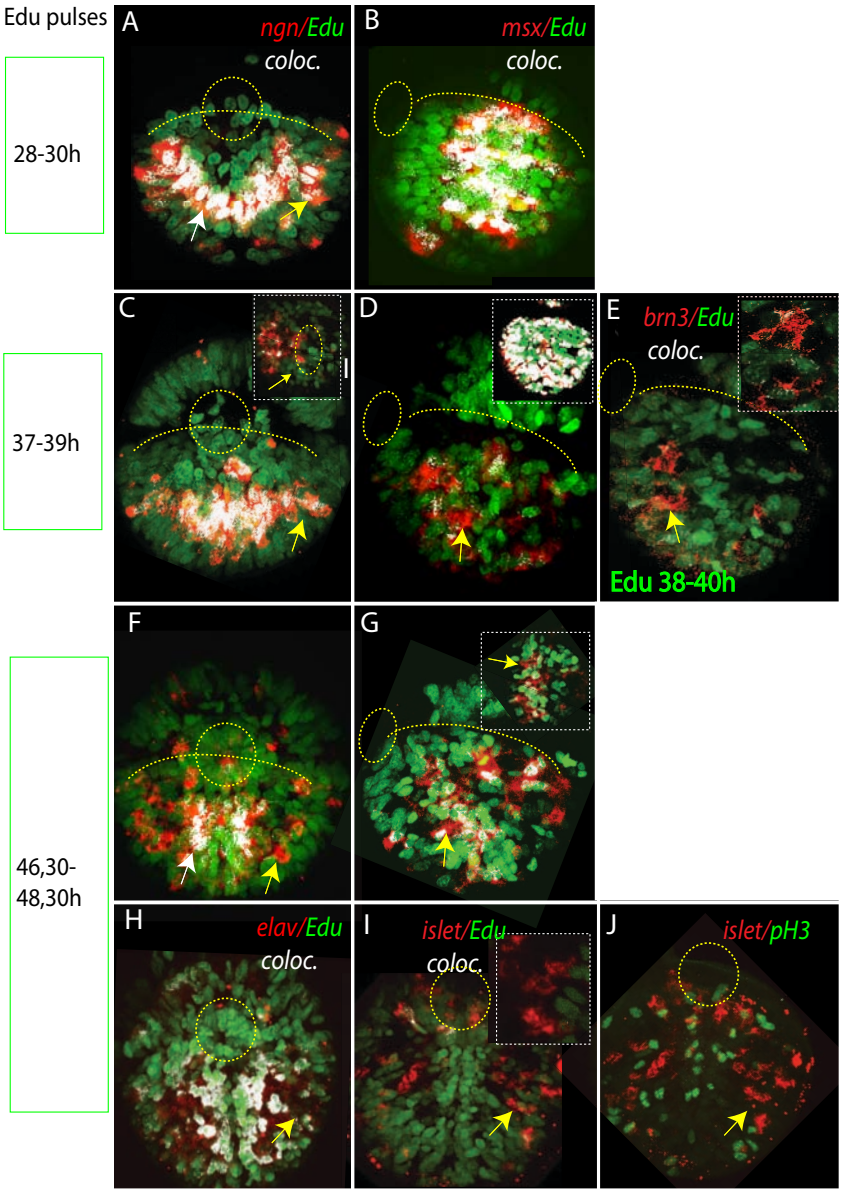


Figure 28. The proliferative state of the *msx/ngn* + lateral cells between 28h-48h,30'

WMISH on embryos incubated with Edu for 2h between 28h-30h , 37-39h, 38h-40h and 46h,30'-48h-30', and then immediately fixed. Co-localization is shown in white. **A,C,F**: ventral views. Co-localization of *ngn* and Edu+ are shown for each time point**.** Yellow arrows in C, F indicate the lateral *ngn*+/ Edu negative cells **B,D,G**: lateral views. Co-localization of *msx* and Edu+ cells are shown for each time point. In D and G yellow arrows indicate the lateral *msx*+/Edu negative cells. Lateral views of the co-localization of Dapi and Edu+ cells (between 37-39h) in the lateral neuroepithelium are shown in the inset in D .The Edu negative segmental stripes most likely correspond to the *msx* + ones. **E:** *brn3* + neurons after Edu incubation between at 40h.The yellow arrow indicates the neurons in the lateral

neuroecoderm.**H,I:** co-localization of *elav* (H) and *islet (M)* with Edu + cells following an Edu pulse between 46, 30′ and 48h,30′. The yellow arrow indicates *elav*+ (I) and *islet*+ *(M) /Edu* negative lateral cells. The inset in I is a close up of the *islet*+ cells in the lateral domain. **J:** the *islet* + cells (red) are not actively dividing during this time, they are not positive for Ph3 (staining dividing cells in green). Dashed yellow circles indicate the stomodeum and dashed yellow lines the ciliary band

These data show that *ngn*+ neuronal precursors in the lateral neuroectoderm undergo extensive proliferation and differentiate as sensory neurons in a step-wise manner. Sensory differentiation markers, such as *brn3* and *islet* are expressed in a temporal sequence similar to that observed in vertebrate early neural crest cells (paragraph III.4 of the Introduction, fig.14).

I.3 Molecular fingerprint of the lateral neuroectoderm at 48h

I found that between 46-48h *islet* + neurons arise from the lateral territory (paragraph I.2), fig.28). WMISH and time lapse movies (Appendix, fig.a3) suggest that they originate from the territory that later expresses neural plate border genes. Next, to perform an exhaustive comparison of the annelid lateral cells with the early *ngn*+ neural crest cells, I set out to obtain a cell-specific molecular fingerprint of the lateral sensory precursors, asking whether genes specific for the NCc/RBc specification are expressed in the lateral cells (Rossi, Kaji, and Artinger 2009), (Introduction, paragraph III.5.2, fig. 15).

To do this, I utilized 'PrImR', an *in-silico* alignment tool recently developed in this lab (Tomer et al., 2010). Based on an average axonal reference scaffold, this tool allows us to generate average expression profiles for each gene and to produce *in silico* co-expression of different genes at cellular resolution. I performed WMISH for the genes of interest and obtained confocal scans in the same ventral orientation. An average for each gene was generated using at least 3 different embryos. Some of the scans used in this study were generated by other members of the lab (see Materials and methods).

I.3.1 The *msx-olig* lateral column

To assay for the presence of neural crest specific markers in the lateral territory I used *msx* expression as a molecular landmark for the lateral domain, as described in Denes et al, 2007. Consistent with the data generated at earlier stages, *msx* also demarcates the lateral column at 48h (fig.29B). A subset of the *msx* + cells, those bordering the medial nervous system, also express *olig*, another NPB gene (Rossi, Kaji, and Artinger 2009; Filippi et al. 2005).

After defining the *msx-olig* synexpression group, I proceeded to assay the *msx-olig* domain for the expression of the other candidate genes I had previously found to be expressed in the lateral column such as *zic* (fig.29C) and *pax37* (fig.29D).

This region is now recognized to give rise to part of the peripheral nervous system. Consistent with a peripheral sensory cell fate, I found that this domain lays outside the medial most *pax6-nk6* +domain (fig. 29D), where motoneurons arise (Denes et al. 2007).

I.3.2 The neural crest specifiers and effector molecules

In addition to corroborate what was previously shown in Denes et al 2007, using PrImR, I was also able to confirm that neural crest specifiers and effector genes are expressed in the lateral territory.

Expression of *soxE* is still detectable in the lateral neuroectoderm at 48h (fig.29E)[4]. *SoxE+* neuroectodermal cells are embedded in the *msx-olig* lateral column.
Although an average expression pattern of Snail2 needs to be generated and analyzed in PrImR, it is clear that *snail2* is also still expressed in the lateral neuroectoderm, (fig.30B, similarly to the *Drosophila* orholog that is expressed in the sensory organs, Ip, Levine, and Bier 1994). This domain most likely corresponds to the *soxE+* domain.

Having confirmed that this lateral domain as a homolog to the neural plate border (because it expresses neural plate border specific markers such as *pax3/7*, *prdm1* and *msx*), I set out to analyze the expression of neural crest specific genes and effector genes (table in fig.11).
Among these genes I cloned and analyzed the expression the a putative *Platynereis neogenin (neog)* receptor, the transcription factor *foxD* and fibrillar collagen (*colA*). Neogenin is a receptor belonging to the Immunoglobulin superfamily and very similar to the receptor Dcc (deleted in colorectal cancer), and similarly it mediates netrin signaling (Lai et al. 2011; Matsunaga and Chédotal 2004) .

In addition Neogenin receptor binds to Rgma (repulsive guidance molecule) and regulates neural crest migration in Xenopus (Gessert, Maurus, and Kühl 2008). Because the sequence that I cloned is quite small, the phylogeny of the *Platynereis* putative neogenin is not clear (Appendix, D9); nevertheless it clusters with other invertebrates putative neogenin receptors. PrImR revealed that *neog* is present in the *msx-olig* column (fig.29F). Vertebrate *foxD3* is a 'Neural crest specifier and effector gene' (table in fig.11), crucial for the migratory behaviour of the forming neural crest (Sasai, Mizuseki, and Sasai 2001; Dottori et al. 2001) .

In *Platynereis,* I found only one ortholog of the *foxD* family. *Platynereis foxD* is primarily expressed in muscle precursor cells in the trunk (yellow arrow in fig.30, this is likely comparable to the amphioxus mesodermal expression of *foxD*, Yu et al., 2008). It is also expressed in neurons of the medial neuroectoderm (not shown, as the vertebrate ortholog FoxD1 and D2). Laterally, *Platynereis foxD* is expressed in a small number of cells in the neuroectoderm that are *pax3/7+* (white arrow in fig. 30B). These cells form a portion of the ciliary band of the II segment that originate from the *prdm1+* cells described in paragraph I of the Results, fig.27.

I.3.2.1 *Platynereis* neuroectodermal expression of *colA* at 48h

Vertebrate fibrillar collagen *colA* is a neural crest effector gene (Sauka-spengler et al. 2007) . It is involved in ectodermal to mesenchymal transition (EMT) and the early migration of the neural crest cells in chicken (Suzuki et al. 2006). Later, *colA* is a

[4] subset of muscle precursors express soxE. This is similar to the mesodermal expression reported in amphioxus (J. Yu et al. 2008).

determinant for the formation of the neural crest-derived cartilage, supporting cells of the peripheral nervous system, odontoblasts and notochord, (Suzuki et al. 2006). *Platynereis colA* is expressed in the lateral *msx-olig* territory (fig.29G). Expression was also detected in the axial mesoderm (this will be discussed in chapter IV of the Results).

Twist is one of the cranial neural crest specifier. (Soo et al. 2002)Interestingly, the cells of the *Platynereis* lateral neuroectoderm do not express *twist*, which is found exclusively in mesodermal cells in *Platynereis* (PhD thesis, Steinmetz 2006). This is similar to the condition found in lamprey, where only cranial neural crest derivatives, and not the neural crest itself, express *twist* (Sauka-Spengler and Bronner-Fraser 2008a). Hence, it is possible that the co-option of twist into the neural crest gene regulatory network occurred only in the gnathostome lineage (vertebrates with jaws).

I.3.4. The peripheral sensory lineage

Similarly to vertebrates (Marmigère and Ernfors 2007; Lallemend and Ernfors 2012), *Platynereis* lateral trunk cells express *ngn* and terminal differentiation markers for the sensory lineage in a temporal sequence (first *brn3* between 38-40h, and then *islet* between 47-48h).

Using PrImR at 48h, I determined that the *brn3, islet+* neurons are embedded in the *msx+/olig+/zic +* domain in the lateral neuroectoderm that I described (paragraph I.3.1). Accordingly, this is most likely the *barHI +* domain (fig. 29K), that is involved in defining the in the sensory subtypes in *Drosophila* PNS (Higashijima et al. 1992), the vertebrate homeobox protein BarH-like 2 is expressed in the dorsal root ganglia(Jones et al. 1997).

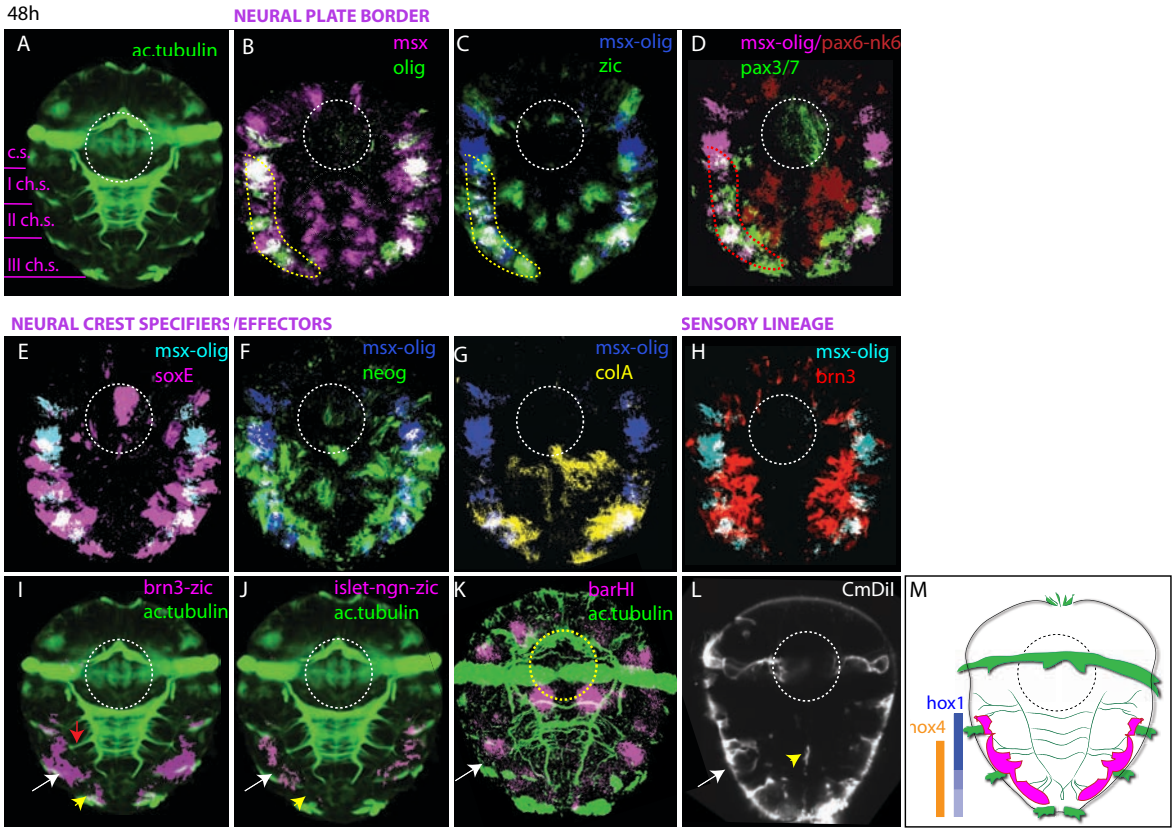


Figure 29. Co-expression of the genes of the neural crest gene regulatory network in the trunk of *Platynereis* at 48h. A-J: Z-projections from 42z onwards of the co-expression profiles made using the colocalization plugin implemented in PrImR. **A:** Z-projection of the average reference of the trunk axonal scaffold of 48h. C.s.: cryptic segment, *ch.s.:* chaetiferous segment. **B-D:** neural plate border genes, dashed lines outline the lateral domain, in C mesodermal *zic* expression is visible (asterisk). **E-G:** neural crest specifiers. **H-J:** sensory lineage markers**.** Yellow arrow in I indicates the undifferentiated posterior segment (*islet-* in J) **K:** *barHI* (white arrow : lateral precursors). **L:** CmDiI passive labelling between 48-52h. White arrow : *DiI* + lateral cells, yellow arrowhead: putative neurotroch rudiment. This DiI pattern was obtained in approximately 80% of the larvae in two different experiments **M:** drawing of the lateral cells in *Platynereis* at 48h in relation to the co-expression of *hox1-hox4*.

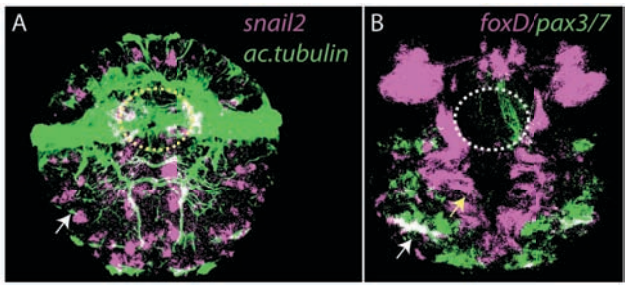


Fig.30 Expression of *snail2 and foxD* at 48h. A: the *snail2* + lateral cells are indicated with a white arrow. **B:** Z-projection from 42z onward of the co-localization of *foxD* and *pax3/7*, generated with PrImR. The ciliary band of the II chaetiferous segment in which *foxD/pax3/7+* cells are indicated with a white arrow. The yellow arrow indicates the mesodermal expression.

At 48h a subset of these cells, that correspond to the multicilited cells of the ciliary bands originating from *prdm1+* precursors (fig.27), *foxD* and *pax3/7+* (fig.30) express the ortholog of *atonal* (fig.31A,B, the inset in B shows a close up of the *atonal* +cells of the left paratroch, recognized by the elongated morphology and the presence of cilia, stained with acetylated tubulin, pink arrow). This domain of expression might be comparable to the expression of *atonal* in the chordonotal organs, a subset of *Drosophila* peripheral sensory neurons (Jarman et al. 1995; Simionato et al. 2008). Members of the transient receptor potential cation channel subfamily V are expressed in sensory neurons in many bilaterians (from Drosophila to mouse (Gunthorpe et al. 2002; J. Kim et al. 2003; Koltzenburg 2004; Kahn-Kirby and Bargmann 2006; Hjerling-Leffler et al. 2007)). Accordingly to their sensory specification, *Platynereis trpV1* (is also expressed in this population of cilated cells, fig.31C, phylogenetic tree in Appendix, D2). These cells correspond to the ones described in Denes et al., 2007 as sensory neurons, and make up only a subset of the sensory neurons of the lateral neuroectoderm.

Runx genes belong to a family of transcription factors implicated in the terminal phases of differentiation of the Rohon Beard cells and of the neural crest-derived subset of the peripheral sensory neurons which form the dorsal root ganglia (Inoue et al., 2007, Park et al., 2012, Inoue, Shiga, & Ito, 2008, (Chen et al. 2006) and paragraph III.4 of the Introduction, fig.14).

Hence, I cloned and analyzed the expression of *Platynereis runx* (phylogenetic tree in Appendix,D.5). The expression at 48h is observed only in medial motoneurons (fig.31D), comparable to the expression observed in vertebrate motoneurons. These neurons likely

correspond to the *hb9*+motoneurons described in Denes et al., 2007). But, around 56h I also observed the expression of *runx* in the lateral neuroectoderm in the position where sensory neurons are localized (fig.31E).

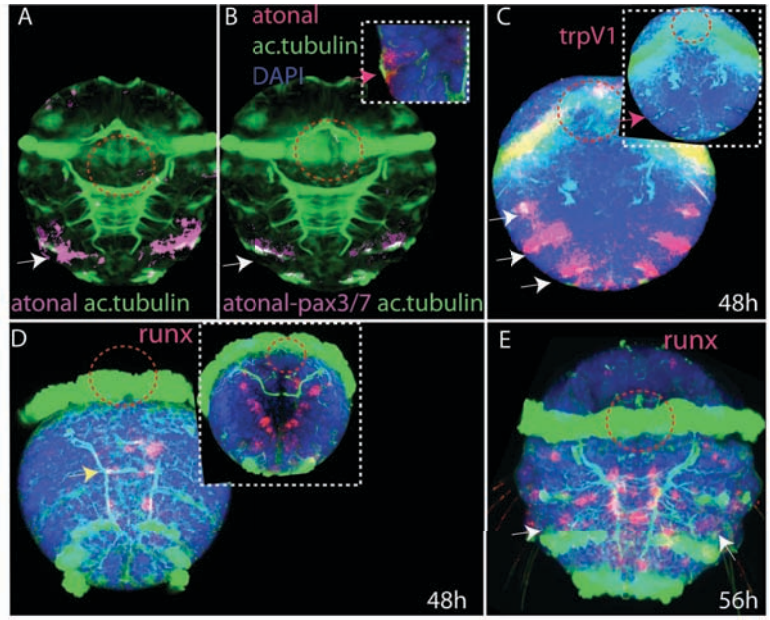


Figure 31. Expression of *atonal*, *trpv1* and *runx* at 48h and 56h. A: Z projection of the average reference of the trunk axonal scaffold at 48h (green) and the average for atonal (magenta), obtained with PrImR.. **B:** Z projection of the average reference of the trunk axonal scaffold of 48h and the co-localization of *atonal* and *pax3/7*. The inset shows the ciliated cells of the left paratroch of the III segments, expressing atonal. The cilia are visible because stained with the antibody against acetylated tubulin (pink arrow) **C:** expression of *trpv1* in the cells of the ciliary bands (pink arrows). The inset shows the ac.tubulin staining of the cilia of the ciliary bands in green (pink arrow). **D:** neuroectodermal expression of *runx* at 48h. Yellow arrows show the putative motorneurons. The inset shows expression in deep mesodermal bands. E: neuroectodermal expression of *runx* at 56h. Expression in the lateral neuroectoderm can be observed (yellow arrowhead).

An additional experiment supported the presence of sensory cells in the lateral neuroectoderm at this stage. After light treatment with the proteinase K (to digest the cuticle), I incubated the live animals in sea water containing DiI for 2h and then imaged the live animals using confocal microscopy (details of the protocol in Materials and methods). In this experiment, In these types of experiments, sensory cells take up DiI from the sourrounding medium. DiI is a fluorescent lipophilic dye that diffuses along the cell membrane and thereby allowing the visualization of nerve cells and their axons. In this experiment the cells in the lateral neuroectoderm could readily be labeled with DiI (fig.29L), a common property of sensory cells.

Interestingly, I could not detect the co-expression of sensory markers in the I chaetiferous segment (fig.2and fig.29I-M) . The expression of sensory markers begins only in the II chaetiferous segment and extends posteriorly (white arrows in fig. 29 I-K). This is the domain along the A-P axis where *hox1* and *hox4* are co-expressed (Kulakova et al., 2007 , fig.29M). In vertebrates, the co-expression of *hox1-hox4* defines the caudal

neuroectdoderm (fig.4) where trunk sensory neurons are also known to develop (Pourquié 2009). This evidence corroborates the idea that the annelid peripheral sensory neurons described in this study are homologous to the trunk sensory neurons of vertebrates.
Hence, it is likely that *hox* genes directed the formation of trunk neurons along the A-P axis at the base of Bilateria.

Prior to the undertaking of this study, the lateral neuroectoderm of *Platynereis* had been compared to the dorsal most part of the neural tube of vertebrates. The molecular data presented in this study so far strengthen this idea and further expand this comparison, providing good arguments (such as the expression of *prdm1, snail* and other neural crest markers) to hypothesize that a neural plate border –like region is present in this territory.

Surprisingly, in this territory I identified *msx*/*olig*+ cells co-expressing specific neural crest genes: *soxE*, *neog*, *colA* and most likely *snail*. Sensory cells are likely to develop from this domain along specific A-P coordinates. This occurs in a step wise manner of terminal differentiation programs similar to the ones occurring in the neural crest derived sensory neurons in vertebrates. For instance, *ngn* is turned on first in the sensory precursors (fig.26I and fig.32), then *brn3* (fig.28E and fig.32 step2), then *islet* (fig.28I,J, fig.29J and fig.32 step3) and finally *runx* (fig.31E and fig.32 step3). Motile cells of the ciliary bands also arise from an *msx*+ lateral region and express sensory markers such as *atonal* and *trpV* by 48h (fig.31 A, B,C and fig.32 step3).

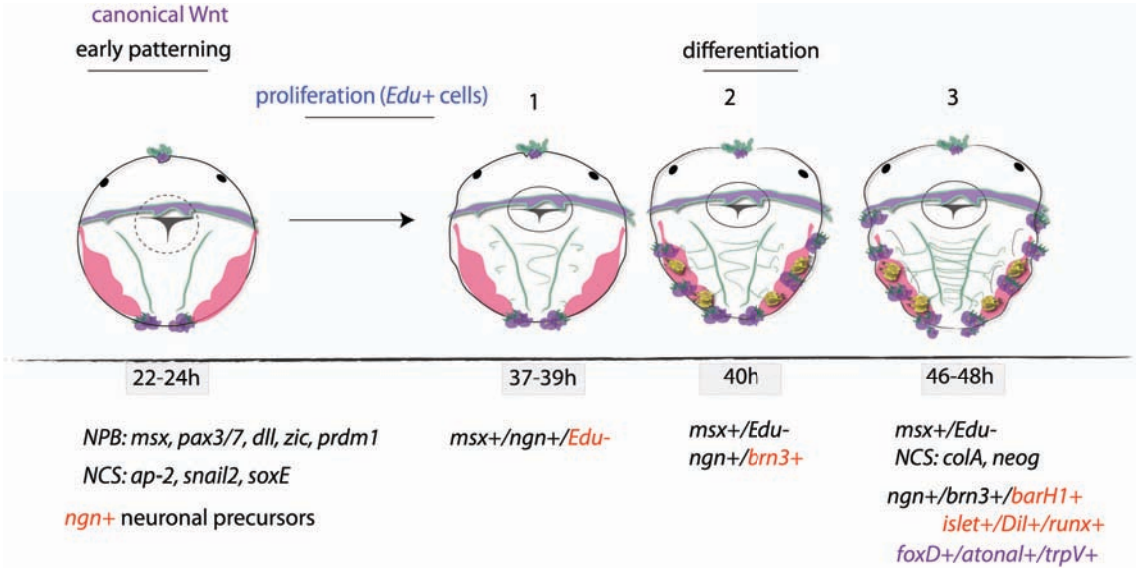


Figure 32. Temporal gene expression of terminal differentiation genes in the lateral neuroectoderm of *Platynereis* from 24h to 48h. This drawing schematizes the findings discussed so far. The sensory markers expressed per each time point are indicated in red. Between 22-24h *ngn*+ precursors arise in the lateral domain, where specific neural plate border genes (NPB) and neural crest specifiers (NCS) are expressed. The expression of some of the neural plate border genes is expanded in response to canonical Wnt signaling. Between 22-37h the lateral domain is highly proliferative (determined via Edu incorporation experiments). Between 37-39h (step 1 of sensory differentiation) the majority of the *ngn*+ precursors in the *msx* domain is Edu negative (Edu-). At 40h (step 2) Edu- cells in the cells in the lateral neuroectoderm starts to express *brn3*. At 48h (step 3) the expression of other specific neural crest genes (*colA, neog*) is observed in the lateral neuroectoderm. The cells now express *islet* and

barHI. Subsets of the lateral cells are the cilated cells of the ciliary bands. They express *foxD*, atonal and *trpV*.

II. Ectopical *ß-catenin* alters the trunk mediolateral patterning, expanding the lateral neuroectoderm

Next, I asked whether homology of the *Platynereis* lateral neuroectoderm with the dorsal-most part of the neural tube in vertebrates is supported by the presence of similar developmental mechanisms involving the same signaling systems in both populations.

In vertebrates, different Wnts have been shown to influence the D-V patterning of the neural tube, via both the canonical (involving *ß-catenin*) and non-canonical pathway(Muroyama et al. 2002), (Dickinson et al. 1995), (Dorsky, Moon, and Raible 1998), (Deardorff et al. 2001)(Hari et al. 2002). The induction of neural crest at the dorsal neural tube is dependent on canonical Wnt signaling (Dickinson et al. 1995). Therefore, I set out to investigate the effect of canonical Wnt signaling in *Platynereis*, consistent with a possible role in patterning this territory.

Orthologs of the *wnts* are expressed during neuroectoderm development in *Platynereis* (Janssen et al. 2010). Some of the canonical representatives including *wnt1*, *wnt6* and *wnt8* are expressed laterally, as in vertebrates (Janssen et al., 2010).

In order to investigate the role of canonical Wnt signalling during patterning of the *Platynereis* neuroectoderm, I exposed the developing larvae between 24h-48h to different concentrations of 1-*Azakenpaullone*. This is a specific antagonist of glycogen synthase kinase-3ß, that controls the physiological degradation of excess *ß-catenin*. These types of treatments have already been successfully used in *Platynereis*, and lead to the stabilization of nuclear *ß-catenin*, mimicking the activation of canonical WNT signaling (Schneider and Bowerman 2007; Tomer et al. 2010; Marlow et al. unpublished). After determining an effective concentration range for the drug, only low concentrations were considered, and the treated animals were analyzed via WMISH at 48h.

II.1 Effect of *ß-catenin* ectopic activation on the lateral neuroectoderm

Similarly to the vertebrate scenario (Muroyama et al. 2002),(W. Yu et al. 2008) I observed an expansion of the trunk lateral neuroectoderm of *Platynereis*. For instance, patterning genes such as *olig* (fig.33 A-A'') and *dll* (not shown) and proneuronal genes such as *ash1* (fig.33 D-D''), *ash2* (not shown) are expanded toward the ventral territory. In the control larvae I observed two distinct territories positive for the proneural genes *ash1* and *ash2*: one medial and one lateral (in the peripheral nervous system precoursors), a situation similar to the vertebrates. Conversely, in the treated larvae, distinct domains are no longer visible (compare black dashed lines indicating the extension of the domains in D-D''). Instead, a homogenous pro-neural domain extending from lateral to medial is present.

Surprisingly, and again similar to the vertebrate scenario, the lateral marker *pax3/7* (fig.33B-B'') is also expressed in the midline of treated animals, a ventral domain that is always *pa3/7* free in wild type animals (fig.33B). I observed a similar phenotype for *zic*, although in this case I could consistently observed only one *zic+ ectopi*c cell in the midline(fig.33C-C'').

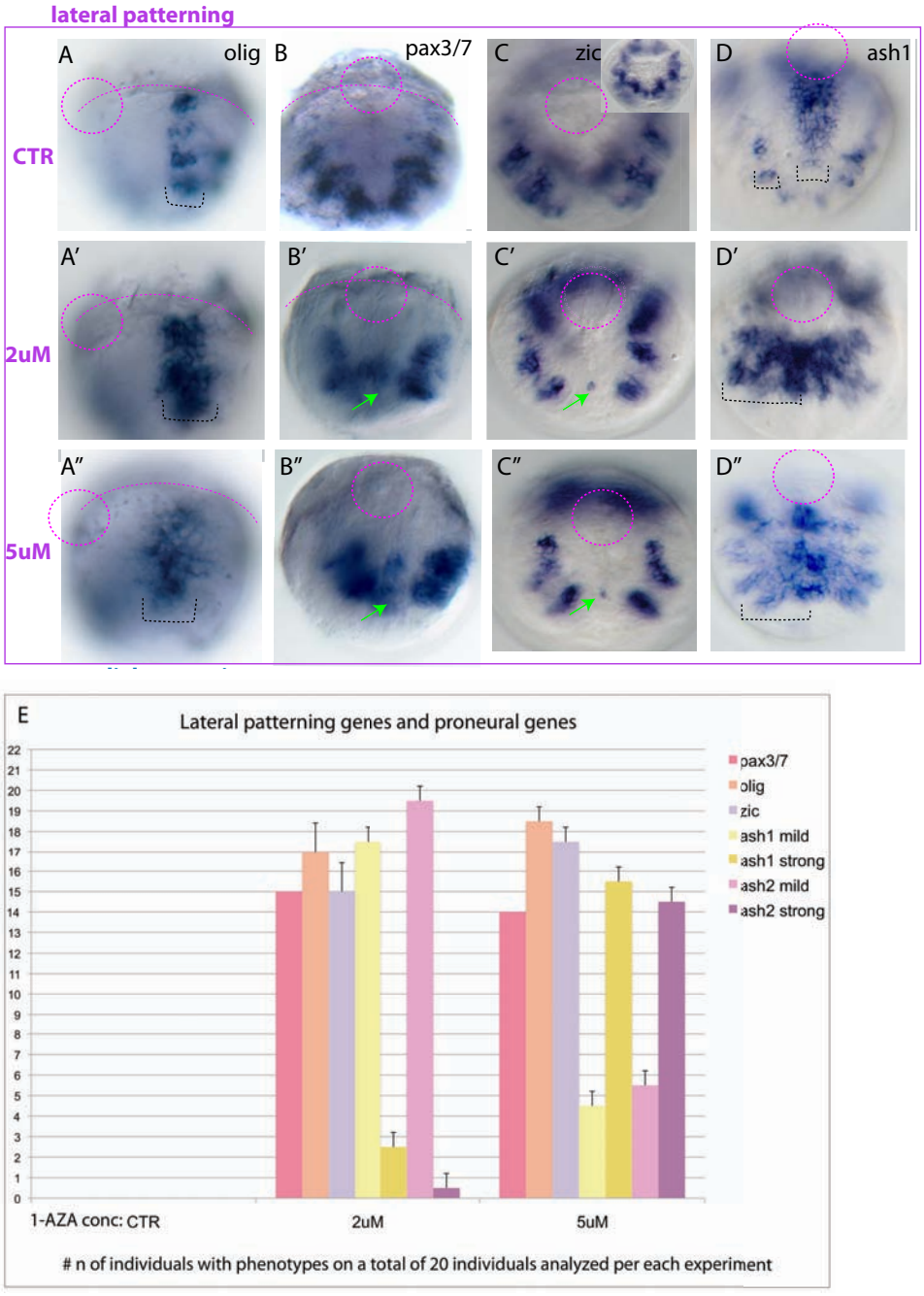


Figure 33. The effect of ectopic *ß-catenin* activation between 24-48h on the lateral domain. For each gene the control, and the treated larave at 2uM and 5uM are shown. The name of the gene analyzed is indicated along the top. Black dashed lines indicate the extension of the domain within the context of the neuroectoderm, dashed pink lines indicate the ciliary band and dashed pink circles, the stomodeum. Pictures in A-A′ are lateral views. Green arrows in B′,B′′, C′ and C′′ indicate the midline expressing *pax3/7* (B′,B′′), and a small number of cells in the midline expressing *zic* (C′,C′′). Inset in C shows the mesodermal expression of *zic*, that is lost as well in the treated animals. **E:** Number of embryos with phenotype observed out of 20 embryos counted per each condition. For ash1 and ahs2, a strong and a mild phenotype were observed. The strong phenotype refers to a strong midline expression. Bars indicate a standard deviation between 2 independent experiments where two different batches of embryos were used.

II.2 Effect of *ß-catenin* ectopic activation on the medial neuroectoderm and conserved sfrp/Wnt antagonism

Additionally, I observed that in the animals treated with *1-Azakenpaullone,* the medial-most domain (*pax6, nk6+)* was reduced (fig.34 A-A'',B-B'', but the pax6 brain expression appears to be unaffected, as previously shown in Tomer et al., 2010). Nevertheless, the medial-most *nk2.2* domain was expanded towards the lateral domain (fig34 C-C''), as observed in vertebrates. This is similar to the expansion of nk2.2 domain in the vertebrate neural tube in absence of pax6 (a S. Kim et al. 2001), (Lei et al. 2006).

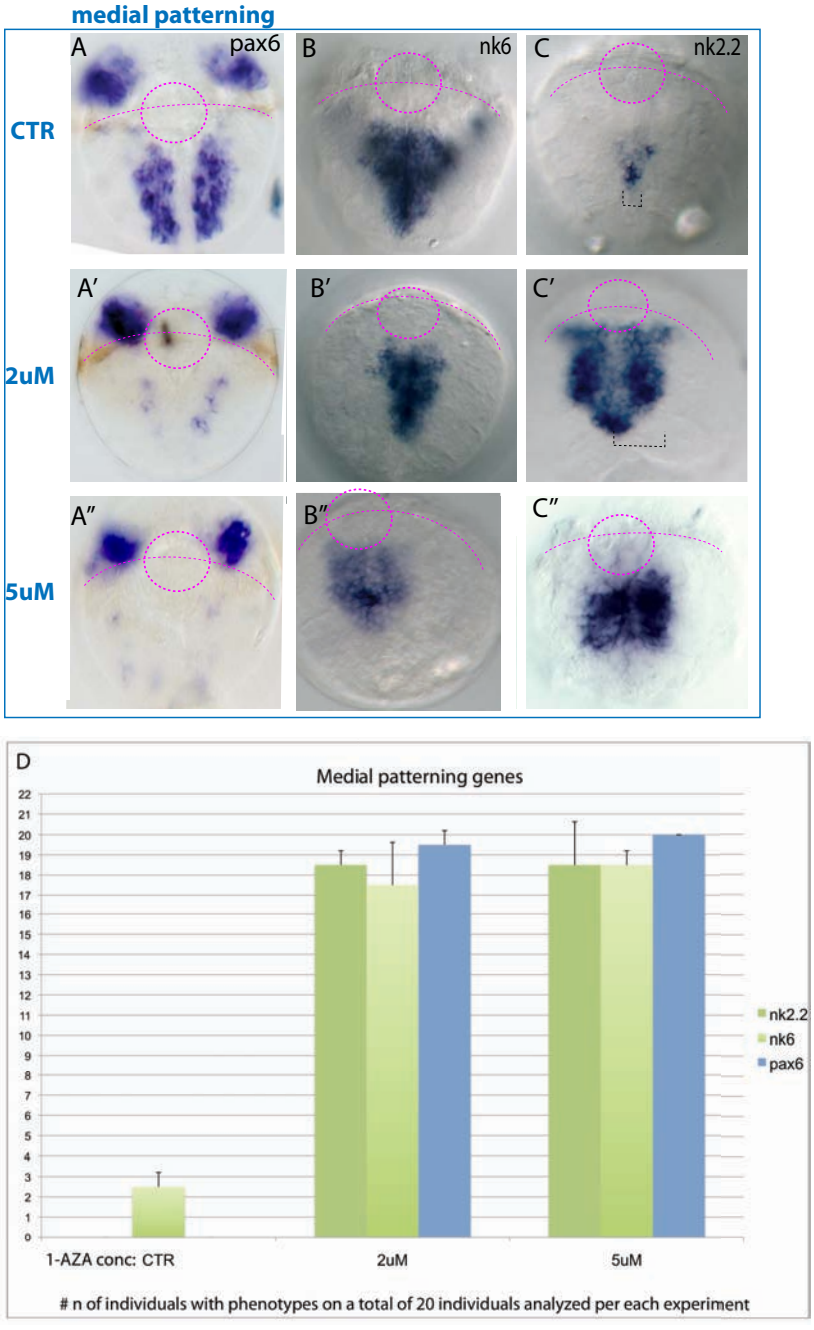


Figure 34. The effect of ectopic *ß-catenin* activation between 24-48h on the medial domain

It has been recently demonstrated that the boundary between *pax6* and *nk2.2* is controlled via inhibition of Wnt, by sFRP2 (a S. Kim et al. 2001), (Lei et al. 2006) (Introduction, paragraph II.1.2). sFRP2 is a secreted Wnt inhibitor expressed in the *pax6* domain and is responsible (through the activation of Tcf4) for the downregulation of *nk2.2* in the boundary domain. The results obtained with the *1-Azakenpaullone* in *Platynereis* were consistent with what observed in vertebrates.

Hence, in order to broaden our understanding of the effect of canonical Wnt on the trunk neurodevelopment of *Platynereis* and to fully understand the phenotypes that I observed, I investigated the expression of one of the *Platynereis* sFRPs (a putative sFRP1/5 ortholog, the one that was observed to be expressed in the trunk by Dr. Heather Marlow, who cloned all the sFRP genes in *Platynereis* and kindly provided the probe, Marlow et. al. unpublished). I found that it is expressed in the *pax6* domain by 34h and clearer at 48h (fig.35A, D, compare with fig.34A).
This expression appears to be mutually exclusive with the expression of the *wnts*. For example, *Platynenreis wnt1* expression initiates at the blastopore (fig.35C), and then extends laterally (fig.35F), as is also observed in vertebrates (Agalliu et al. 2009). The *Wnt4+* domain extends from the blastopore to the more lateral domains by 34h (fig. 35B), and similarly to the vertebrate ortholog, it is also expressed in the dorsal-most domain (inset in fig.35B and fig.35E).
These expression data are consistent with a possible role for Wnt in patterning the medial-most neuroectoderm, and a role of the putative sFRP1/5 ortholog in maintaining a Wnt-free territory in the pax6 domain.

Consistently, prelimary data (not shown) suggest that after treatment with *Azakenpaullone,* the expression of *sfrp1/5* in the trunk is downregulated, in accordance with the expansion of *nk2.2* and with the vertebrate scenario (a S. Kim et al. 2001), (Lei et al. 2006).

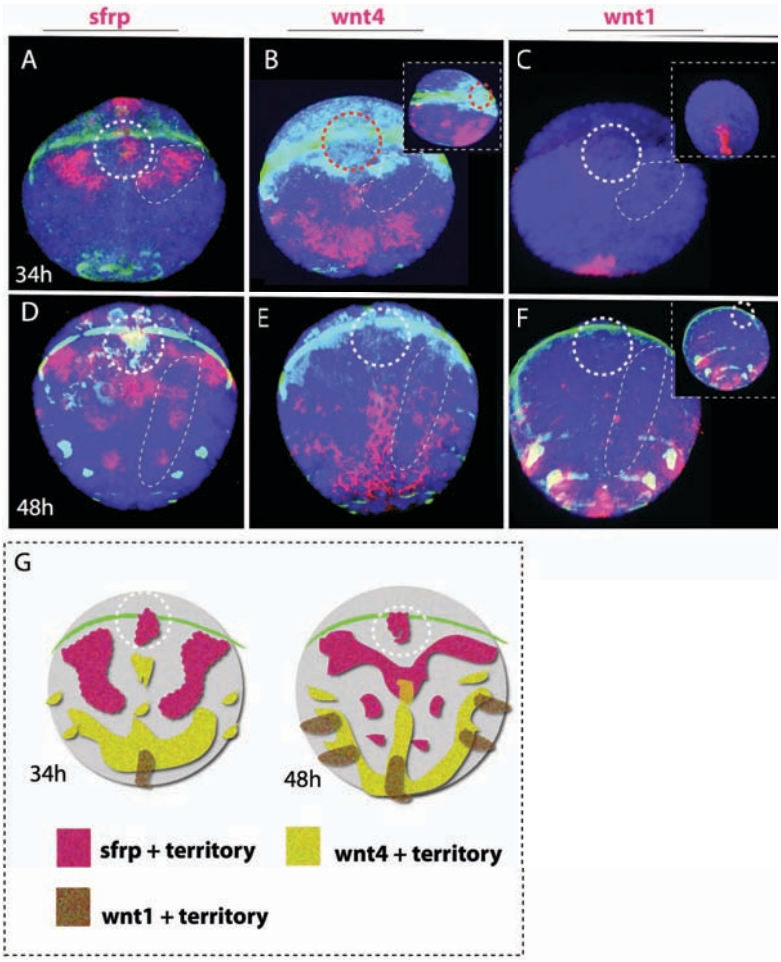


Figure 35. Expression of *sfrp*, *wnt4* and *wnt1* at 34h and 48h. A, D: *sfrp*. **B, E:** *wnt4*. **C,F:** *wnt1*. A lateral views in B and F are shown in the insets. In C, a posterior view of *wnt1* is shown in the inset. Dashed white outline indicates the *pax6/sfrp* + domain. **G:** schematic drawing of the distinct territories where *sfrp* and *wnts* are expressed at 34h and 48h.

Fig.36 summarizes the results obtained with *1-Azakenpaullone*.
In the wild type *Platynereis* embryos there are two different sources of Wnt (one ventral and one dorsal).
Several *wnts* are expressed in the dorsal most territory, such as *wnt1* and *wnt4*. Neural plate border genes such as *pax3/7, olig and zic* are expressed (Results, paragraph I.3) in this area, (green in fig.36A). This dorsal fate is promoted after ectopic stabilization of *ß-catenin*, as is also observed in vertebrates (fig.36B).

I also observed that medially, the Wnt – territory is juxtaposed to a Wnt + territory. The Wnt – area is *pax6/sfrp* + (red in fig.36A). Conversely, *nk2.2* is expressed in a Wnt + territory more medially (yellow in fig.36A).
It is likely that Wnt ligands are secreted and diffuse from the midline and from the lateral-most domain and that the intermediate *pax6* domain is kept Wnt free by the activity of diffusing sFRP protein. The drug treatment experiments shown here suggest that this mechanism contributes to the preservation of distinct domains along the D-V axis.
Indeed, after ectopic stabilization of *ß-catenin* (mimicking canonical Wnt signaling), the *pax6* territory is lost specifically in the trunk, and *nk2.2* is ectopically expressed in this

territory, as is also the case in vertebrates (yellow area in fig.35B).

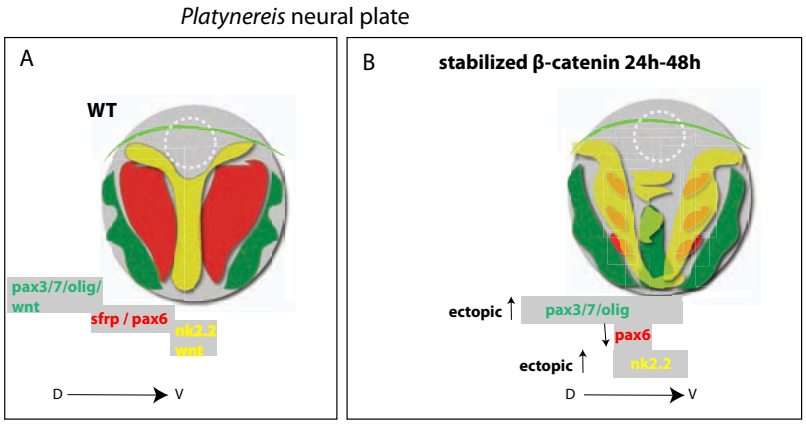


Figure 36. Schematic representation of the global effect of stabilized *ß-catenin* on the patterning of the neural plate in *Platynereis*. See text for explanation.

Collectively, these data show that canonical Wnt acts to promote the lateral fate in both annelids and in vertebrates. Therefore it is likely that this is a shared feature between the annelid lateral domain and the vertebrate dorsal precursors.
Furthermore, these data show that Wnt controls the formation of the medial-most motoneuron domains. As this is similar to the mechanism through which canonical wnt signaling shapes the mediolateral domains of the vertebrate neural tube, it is likely that wnt-mediated mediolateral pattering was established at the dawn of bilaterians.

III. The trunk sensory cells form part of the peripheral nervous system of the juvenile

In vertebrates at least three waves of proliferation an neurogenesis of the early migrating neural crest cells contribute to form the sensory neurons of the dorsal root ganglia along the spinal cord (Marmigère and Ernfors 2007; Lallemend and Ernfors 2012, Lallier 1991; Nicole M Le Douarin and Dupin 2003), (fig.14). I found that annelid peripheral sensory neurons originate from a territory molecularly homologous the one that gives rise to the neural crest in vertebrates, the neural plate border (chapter I of Results, fig.32). Similarly to vertebrates, the patterning of this territory is influenced by canonical Wnt signalling (paragraph II.1 of Results, fig.36). In this domain *ngn+* precursors proliferate and express *brn3*, *islet* and *runx* in a temporal sequence (fig.32).

Next, I asked how this sensory lineage contributes to the formation of the juvenile peripheral ganglia that have been described morphologically in different annelids. To answer this question I followed the development of the cells arising at the lateral neuroectoderm using WMISH for sensory markers combined with Edu, live imaging techniques, and high resolution immunohistochemistry (which I optimized in this study).

Already at 64h (and even more clear after three days of development) the larva is elongated and the three peripheral appendages (called 'parapodia') are well formed (fig.37A-B-C, indicated with the yellow outlines).

The peripheral parapodial nerves are also distinguishable (red arrow in fig.37C). I could observe expression of *brn3* and *islet* in the lateral domains; an intermediate domain, where likely interneurons are formed, is also present.

III.1 The second wave of proliferation in the lateral neuroectoderm: cell division via interkinetic nuclear migration at 3 dpf.

To follow the behaviour of the lateral trunk cells and the formation of the peripheral ganglia I used live imagine techniques. After injection of mRNAs for nuclei and membrane labelling (H2AmCherry and mYFP), I performed time-lapse movies (detailed protocol in Material and Methods). I found that at 3 days of development neuroblasts start to divide again in the peripheral neuroectoderm. This wave of division occurs via a canonical interkinetic nuclear migration (Del Bene 2011). As shown in fig. 37 from F to J and in the scheme in I the nucleus of the progenitors migrate from the basal layer to the apical layer of the neuroepithelium, where then mitosis occurs, perpendicular to the apical-basal axis such that the daughter cells are then located basally. This is a widespread mode of division in vertebrate nervous system, also present in *Drosophila* and *Nematostella* (E. J. Meyer, Ikmi, and Gibson 2011).

Interestingly, I did not observe proliferation in the first trunk segment that bears chaete. This observation is in agreement with the fact that this segment always remains smaller until it transforms into part of the head (Steinmetz, phD thesis, 2006) and with the observation that the sensory precursor cells are not found in this segment at 48h.

Edu labelling between 70-72h confirmed the data obtained with the time lapses: many cells are indeed labelled with Edu (proliferating) in the second (II) and third (III) segment (fig.37D), while the first (I) segment is almost entirely Edu -. Moreover, according to the interkinetic nuclear migration mode of division observed with live imaging, the Edu+ precursors are located apically in the progenitor compartment in the ganglion. Conversely, the differentiating neurons are basal and express *brn3* (inset showing the II left appendage in fig.37 D) and islet (not shown)

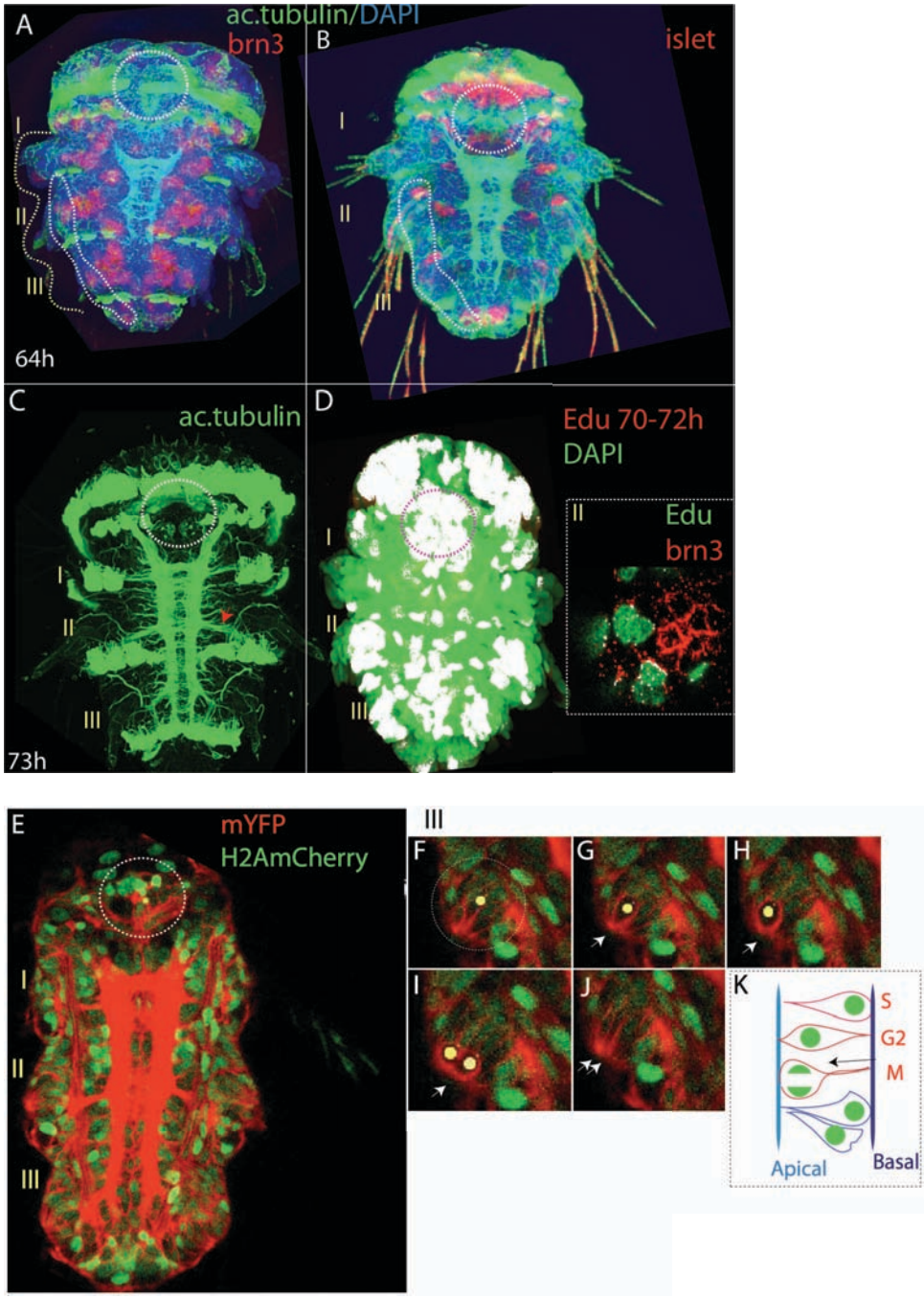


Figure 37. Proliferation in the peripheral neuroepithelium at 3 days (72h). A, B: WMISH of *brn3* (A) and i*slet* (B) at 64h. The putative ganglia precursors are indicated in the area defined by the white outline in II and III segment. The appendages are indicates with yellow outlines and roman numbers (I, II, III). **C:** immunofluorescence staining the axonal scaffold at 73h. **D:** Edu pulse between 70-72h, in white is the co-localization between DAPI (green) and Edu labelled cells (red). The inset shows the *brn3*+ staining in the II left parapodium obtained with WMISH after Edu pulse between 70-72h. **E:** z-projection of the animal injected with membrane (red) and nuclear markers (green) and used for the live imaging. **F-J:** analysis of the division of neuroblasts in the peripheral ganglia occurring via interkinetic nuclear migration. One precursor (yellow dot) is followed over the time throughout the division. More extensive analysis if found in Appendix, fig. b3. **K:** a representation of division via interkinetic nuclear migration is shown. Apical and Basal layer of the neuroepithelium are also indicated as well as the cell cycle phase. Precursors are in red and differentiating neurons in blue. The time lapse was perfomed at the confocal, with a z-step of 1.25 um and a time interval of 8'.

These data show that during the transition from the larval to the juvenile nervous system newborn sensory neurons are added to the first larval pioneering neurons that I described for earlier stages (Results, chapter I).

III.2 The peripheral nervous system in the juvenile

Between 3-5 days, the peripheral nervous system continues to develop further and at 4 days peripheral and central ganglia are clearly visible (fig. 38 A).

As shown in fig.38 the peripheral nervous system is most likely composed of neurons belonging to peripheral ganglia. These neurons are located in the worm appendages (parapodia) and are the sensory neurons that expose cilia on the surface (fig.38C, drawing in F). They likely comprise the first pioneer sensory neurons born around 40h and the offspring of the second wave of neurogenesis, occurred around 3 days of development.

Next, I set out to confirm that the cells in the peripheral ganglia are sensory neurons and therefore test whether they project afferent nerves to the ventral nerve cord. To do this I immobilized the juvenile worm (protocol in Materials and Methods), and I gently injected the DiI dye into the ventral cirrus, through which the cilia of the peripheral sensory cells are exposed to the surface. After few minutes I imaged the injected alive animal at the confocal.

Doing this I was able to label the peripheral cells, and the diffusion of the DiI through the axons of the injected cells allowed me to visualize afferent nerves that projected from the periphery to the ventral nerve cord through the peripheral nerves (fig.38D).

It has been described that motoneurons from the central ganglia send efferent projections to the muscles in annelids, including *Platynereis* (Müller 2006; Denes et al. 2007). Accordingly, with high resolution immunofluorescence I could even observe for the first time neuromuscular junctions on the asciculum muscles (fig.38E); muscles that control the parapodia movements. These data suggest that in annelids a proper peripheral nervous system is present; composed of sensory cell bodies and efferent projections from motoneurons.

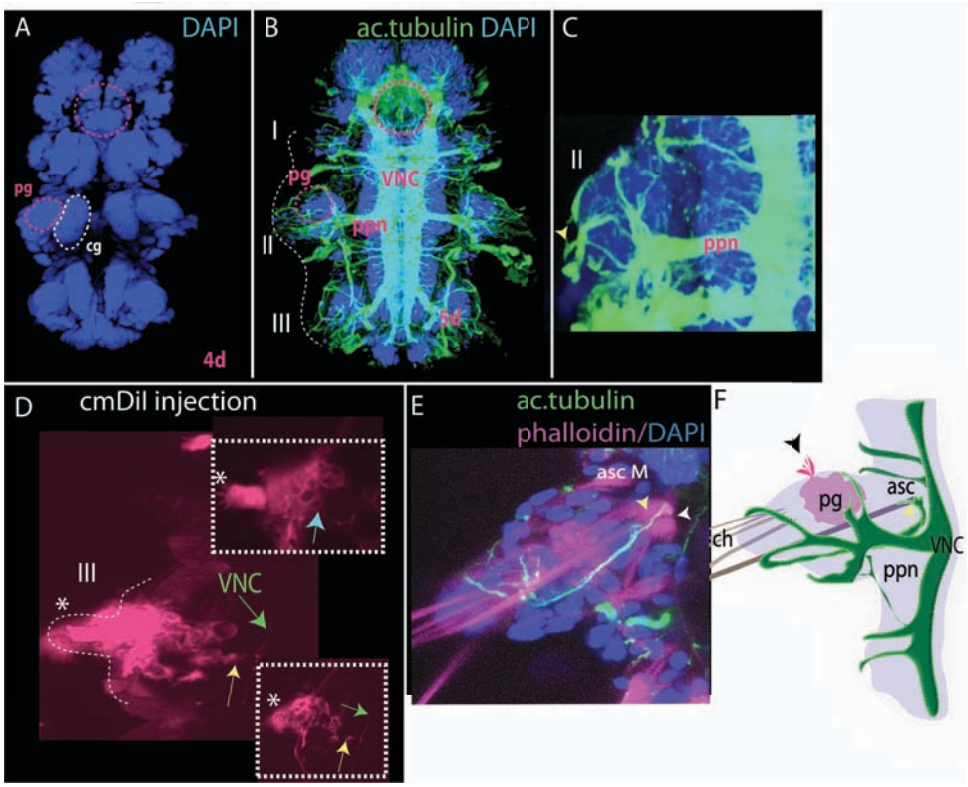


Figure 38. The peripheral nervous system of the juvenile (5days). A DAPI staining at 4 days showing central (cg) and peripheral ganglia (pg)**. B:** immunofluorescence at 5 days showing the axonal scaffold. **C:** close up on the II parapodium of immunofluorescence at 5days. The yellow arrow indicates the cilia outside. Ppn: peripheral parapodial nerve, VNC: ventral nerve cord. The pink circle in B indicates the position of the ganglion at 5 days. **D:** confocal stack of a live animal injected with DiI in the III parapodium. The site of injection is shown with the white asterisk. The insets show a Z-projection of few stacks. Passively labelled axons of the ventral nerve cord (green arrow) and of the peripheral nerves (yellow arrow) are visible. **E:** immunofluorescencence showing the axonal scaffold (green) and the muscles (pink) of the II parapodium. Afferent nerve makes a neuromuscular junction on the asciculum muscle (ascM. indicated with white arrowhead, the neuromuscular junction is indicated with yellow arrowhead).**F:**schematic drawing showing part of the peripheral nervous system located in the parapodium. Pg: parapodium ganglion, asc: asciculum, ch: chaete. Epidermis is in blue, nervous system in green and muscles in pink.

III.3 The molecular fingerprint of the peripheral nervous system

-Sensory neurons of the parapodial ganglia

From 5 days onwards peripheral ganglia in the appendages (parapodia) are well formed. The sensory cells in each ganglion express sensory differentiation markers such as *brn3*, *islet*, *barHI* (fig.39 A- E).

I then looked for sensory effector markers and I found that at this stage also the cells in the ganglia express the ortholog of *trpV* channel (*trpV1*, fig.39F), a sensory effector marker (Gunthorpe et al. 2002; J. Kim et al. 2003; Koltzenburg 2004; Kahn-Kirby and Bargmann 2006; Hjerling-Leffler et al. 2007)..

I found another *Platynereis trpV* orholog that I called *trpV2*. This channel is phylogenetically related to the *osm-9* receptor of the nematodes (phylogenetic analysis in Appendix, figD.2), and it seem restricted to a subpopulation of the ganglionic sensory neurons (fig.39G).

These cells utilize most likely glutamatergic transmission since they express the vesicular tglutamate transporter (*Vglut*, fig.39H) similar to the Rohon Beard cells, the neural crest derived sensory neurons (Fernández-López et al. 2012; Landry et al. 2004). Based on these molecular datasets I cannot discriminate the nature of the sensory modality. It has been indeed shown that *la* TRPV can act as mechanoreceptor, thermoreceptor, as well as a chemoreceptor (Mutai and Heller 2012; Colbert, Smith, and Bargmann 1997; Bargmann 2006). Hence, I cannot exclude that different kind of stimuli are perceived from the same sensory cell.
Consistently with the expression data of the sensory markers at earlier stages, in the juvenile the sensory ganglia are formed only from the II segment onwards. Interesting this is where *hox1* and *hox4* are co-expressed, a domain that in vertebrates demarcates the caudal neuroectoderm (Pourquié 2009), where t-NCc and RBc originate.

-*ColA*+ cells in the periphery

I found also another peripheral population of cells in *Platynereis*. Indeed, I noticed that at 5days few *colA* cells are found above the peripheral nerves (orange arrowheads in fig.39I). To investigate this further I then sectioned the adult ventral nerve cord and performed an ISH for *colA*.

Surprisingly I found that the peripheral nerves of the adult are all surrounded by cluster of *colA*+ cells not in a continuous manner, similar to the Schwann cells in vertebrates (Woodhoo and Sommer 2008), (Nave 2010).

These vertebrate supporting cells derive from the early migratory neural crest (Marmigère and Ernfors 2007)(fig.14) and surround the peripheral axons. Some of these cells form myelin sheath around the axons, which allow fast conduction of the signal . Furthermore they have trophic functions for the neurons and produce supportive nerve extracellular matrix (Nave 2010). Accordingly they produce collagen (Antonio et al. 2006).

It is not clear if protostomes possess vertebrate-like supporting cells or myelin (although some myelin-like substance has been found in molluscs and annelids, (Schweigreiter et al. 2006; Roots 2008), but the presence of neuroglia (Vagnetti and Farnesi 1978), (Baskin 1971) that produces fibrous processes has been described in many members of the protostomes, including annelids.

This is the only molecular data available for annelid *colA* expression in the nervous system. Hence, although this requires further studies, the *colA*+ cells around the axons might represent supporting cells homologous to the vertebrate Schawnn cells, yet another neural crest derived cell type.

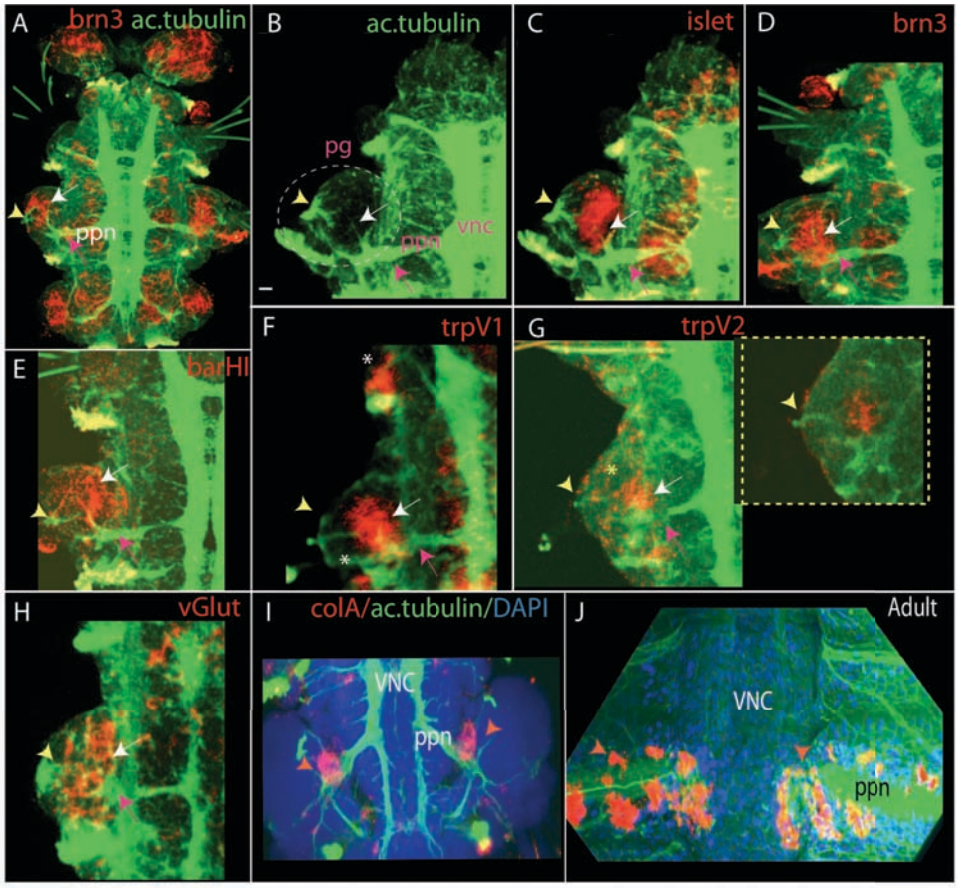


Figure 39. Molecular fingerprint of the peripheral ganglia in the juvenile. A: ac.tubulin staining showing the position of the parapodial ganglion in the white dashed circle, as for the other pictures the cilia protruding from the ganglion are indicated with a yellow arrowhead, the peripheral parapodial nerve (ppn) with a pink arrow, and the arborization in the ganglion with a white arrow**. B-G:** The pictures are all WMISH showing gene expression in the ganglion of the II parapodium. *Islet* (C), *brn3* (D), *barHI* (E), *trpV1* (F), t*rpV2* (G), *vGlut* (H). Asterisk in E indicates expression in the ciliary band. Inset in G shows one Z position for trpV2 where the cilia protruding from the trpV2 cluster are well visible. **I:** WMISH for *colA* at 5 days showing the expression in cells above the peripheal nerves (orange arrowheads), most likely supporting cells. **J:** longitudinal view of adult sections, showing the expression of *colA* in cluster of cells, along the adult peripheral nerves (orange arrowheads).

IV. Searching *Platynereis* cell types homologous to other vertebrate neural crest derivatives

The neural crest gives rise to a variety of different cell types with specific molecular fingerprints. In addition to the peripheral sensory neurons and supporting cells also the autonomic ganglia, enteric neurons, melanocytes, and cartilage originate from neural crest (Groves and Bronner-Fraser 1999), (N. Le Douarin and Kalcheim 1999), (Gammill and Bronner-Fraser 2003).

Therefore, to deepen our understanding on the evolution of the neural crest, an exhausting description of the cell types originating from the ancestral population at the neural plate border-like territory was needed. I started to investigate the presence of such cell types in the marine annelid. In addition to the sensory neurons and to the putative supporting cells that I described in the previous paragraphs, I found that putative visceral motor and sensory neurons, enteric neurons, and pigment cells are present and expressed a conserved set of transcription factors and effector genes.

IV.1 *phox*2+ cells

In vertebrates *phox2* is a master regulator in the development and maintenance of visceral nervous system. Usually *brn3* and *phox2* are mutually exclusive, the first controls the somatic fate, the second the visceral fate. The visceral neurons comprise the viscero-sensory neurons in the the placode derived cranial ganglia, the brachiomotor neurons (in the hindbrain), and the motoneurons of the autonomic ganglia that neural crest derived (Pattyn et al. 2000; D'Autréaux et al. 2011; Hirsch et al. 1998).

It has been shown that lamprey neural crest gives rise to DRG sensory neurons, but does not form autonomic ganglia (Häming et al. 2011). This is clearly a gnathostome acquisition.

In *Platynereis* the *phox2* was cloned by Dr. Alexandru Denes. I found that it is expressed broadly at 48h in the trunk, and I identified three different domains from medial to lateral (fig.40). PrImR analysis revealed that the medial most domains express *VACht* (fig.40B), a cholinergic marker, and it might represent a subset of visceral motoneurons. Later in development, I observed that *phox2* is in deep neurons of the central ganglia (yellow arrow in fig.40D). Although from the Z-projection it appears co-expressed with *brn3*, virtual cross sections show that the *brn3*+central neurons are located superficially (fig.39 A, C) while the *central phox2*+neurons neurons are deeper (fig41, A,B). Therefore they are different subsets of neurons.
Just below *the central phox2*+neurons an intricate net of axons surrounds and innervates the gut (pink arrow in the inset in fig.40D). *Phox2*+ central neurons might be contributing to some of these projections. A more detailed molecular fingerprint of these neurons is needed to understand how these neurons compare to the brachiomotor-visceromotor neurons of the hindbrain and sympathetic neurons.

Interesting a lateral *phox2*+ domain (co-expressed with *olig)* is also present (yellow outline in fig.40C).
In the juvenile these cells are located in the parapodial ganglia (white arrow in fig. 40E, likely superficial as the *brn3*+ neurons, fig.41A', B'). This most likely means that a subset of the peripheral sensory neurons of the ganglia might have a visceral identity. In order to shed light on this a double WMISH for *brn3* and *phox2* at 5days is needed, this will help to rule out if they represent two distinct subsets of peripheral neurons.

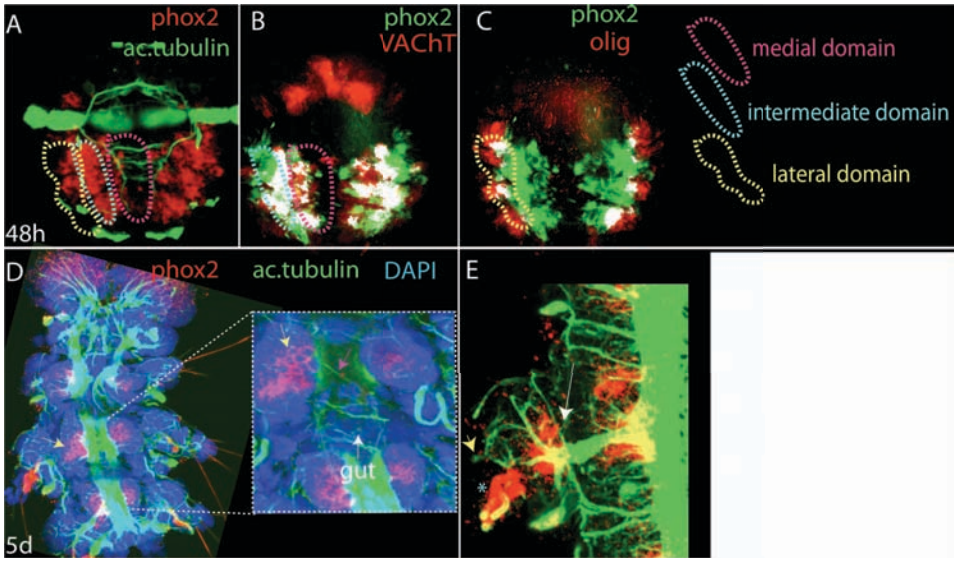


Figure 40. Analysis of *phox2* expression at 48h and 5 days. **A:** expression of *phox2* at 48h, Z-projection of a confocal scan. **B, C:** co-localizations of *phox2* with *VAChT* (B) or *olig* (C) generated with PrImR. Three different expression domains are indicated with different coloured dashed lines. **D:** expression of *phox2* at 5days, deep central ganglia *phox2* + are indicated with yellow arrow. The inset shows a close up of the deep ganglia just above the gut. Gut innervation is shown with pink arrow. **E:** Expression of *phox2* in the peripheral ganglia (white arrow). Cilia protruding from the ganglion are shown with the short yellow arrow. Blue asterisk indicate ISH background in the spinning gland.

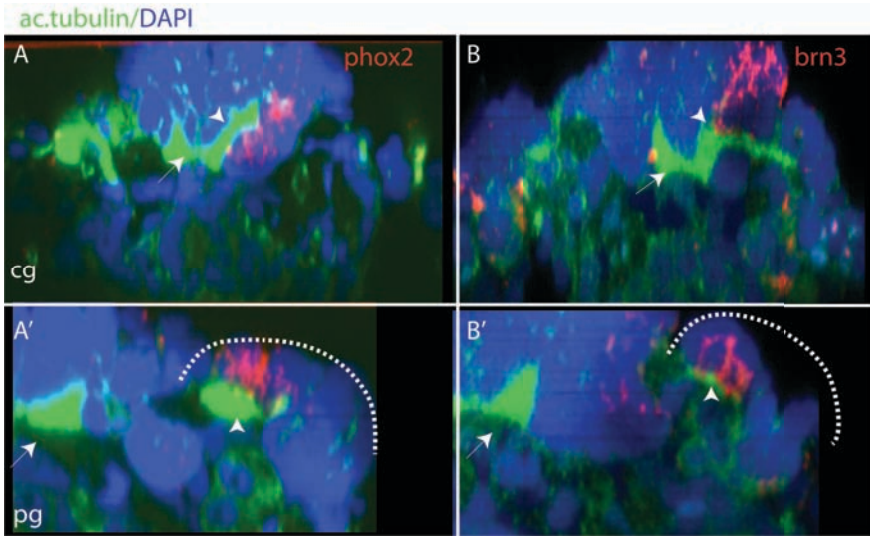


Figure 41. Virtual cross section of *phox2* and *brn3* ISH at 5 days. **A-B:** virtual cross sections of *phox2* (A) and *brn3* (B) at the level of the ventral nerve cord. Phox2 neurons are located in the central ganglia (cg) below the peripheral axons. **A'-B':** close up of the virtual cross sections of *phox2* (A') and *brn3* (B') at the level of the peripheral ganglia (pg). White arrows indicate the ventral nerve cord, white arrowheads indicate the peripheral nerve used as a reference, dashed white lines indicate the peripheral ganglia

IV.2 *Ash1*/*soxE* +, *trpA*+ and serotoninergic + cells in the gut

In vertebrates, enteric neurons derive from neural crest cells and innervate directly the gut at different A-P level. In zebrafish enteric neurons express *phox2b*, *sox10* and *mash1*. (Elworthy et al. 2005)

In *Platynereis* I observed the expression of *ash1* and of the ortholog of sox10 (*Platynereis soxE)* in cells of the foregut (fig.42 A,B,C) and few cells around the hingut (fig.42 F,G). It is likely that in *Platynereis* these are neurons because, already at 3 days, *syt* (fig.42H) is expressed in the hindgut population, and expression of *syt* occurs in the foregut region at around 2 weeks (not shown). Despite this conservation, I did not detect the expression of *phox2*, usually also marking enteric neurons. Interestingly, in the basal vertebrate lamprey there is no domain of co-expression between *phox2* and *ash1* (Häming et al. 2011)., suggesting that this is not an ancestral situation even in vertebrates. I also observed that the ortholog of the transient receptor potential *trpA* ortholog is expressed in the midgut (fig.42E), as reported in vertebrate gut , where it is involved in regulating gastrointestinal motility and visceral sensation(Motter and Ahern 2012) .

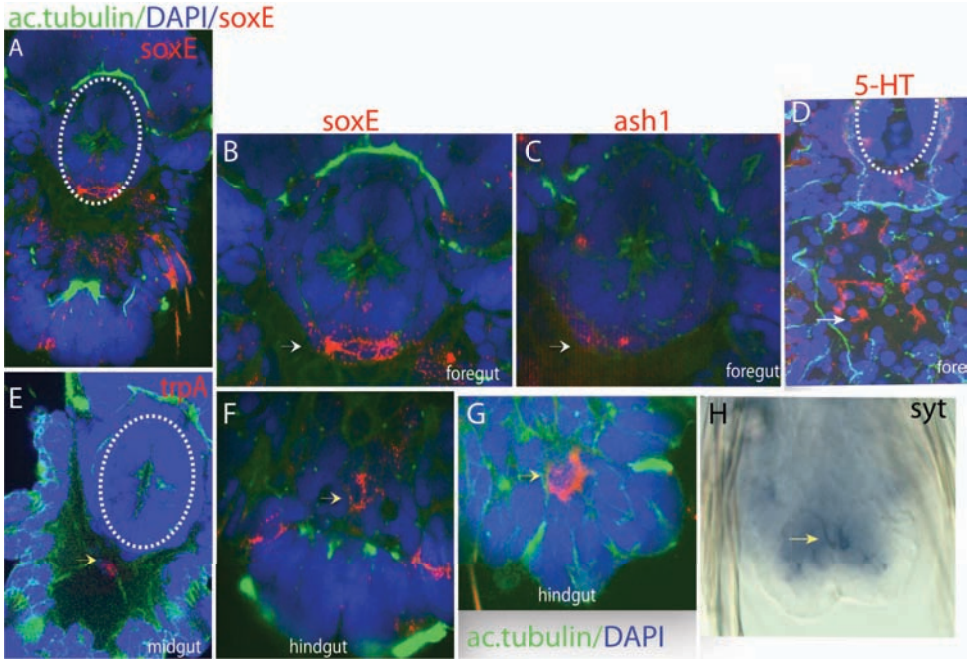


Figure 42. *SoxE,ash1, trpA and serotonin* in the gut. A *soxE* at 3 days. **B:** close up of A on the foregut expression. **C:** *ash1* in the foregut.**D:** localization of serotoninergic neurons in the midgut at 2weeks visualized with the immunofluorescence for 5-HT. **E:** *trpA* in the midgut. **F:** *soxE* in few cells of the hindgut.**G:** *ash1* in the hingut. **H:** *syt* in the hindgut.

Additionally I also found a population of serotoninergic + cells in the midgut of *Platynereis* (fig.42D). These cells might be evolutionary linked to the enterochromaffin cells that populate the vertebrate gut and that interestingly are not neural crest derived (N M Le Douarin and Teillet 1973; Alenina, Bashammakh, and Bader 2006). The enterochromaffin cells might be reminiscent of an ancestral population predating the emergence of the neural crest cells and their contribution to the enteric nervous system.

IV.3 *Mitf* pigmented cells precursors in the mesoderm

In vertebrates neural crest cells migrate dorsally and give rise also to the pigmented cells (melanocytes) of the skin from *sox10+* precursors (Aoki et al. 2003) (Sauka-Spengler and Bronner-Fraser 2008b). These cells express the bHLH transcription factor *mitf* that regulates the development and differentiation of all melanoblasts, controlling the expression of melanin synthesizing enzymes such as the tyrosinase proteins *tyr* and *tryr-p* (Curran et al. 2011; Curran, Raible, and Lister 2010), (Hou, Panthier, and Arnheiter 2000).
Hence, to test the presence of similar cells in *Platynereis* I analyzed the expression of the *mitf* ortholog (probe courtesy of Dr. Maria Antonietta Tosches). I found that *mitf* is expressed in mesodermal domains of the trunk (fig. 43A red arrows, white arrows in B, C), in a domain where also the *Platynereis* ortholog of *sox10* is expressed (fig. 43D,white arrows).
Dr. Pavel Vopalensky found that this territory is also positive for the expression of an ortholog of *tyr/ tyr-p* genes (not shown), similarly to the expression observed in vertebrate melanoblasts. In this domain pigment cells arise later in development, confirming that these cells might represent melanoblasts.

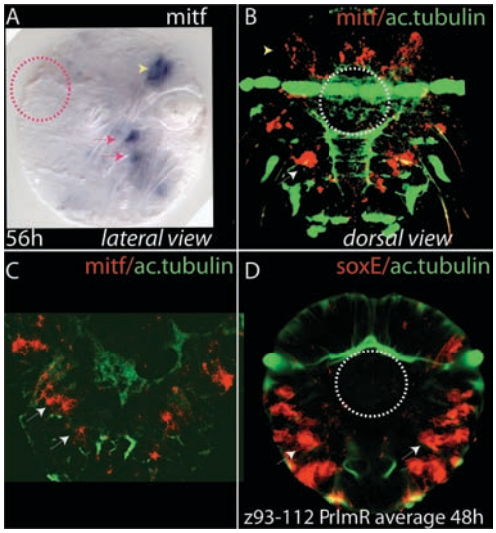


Figure 43. Expression of *mitf* and *soxE* in the mesoderm. A: bright field of *mitf* expression at 56h. The pink arrows indicate the *mitf+* cells in the trunk; the yellow arrowhead indicates the pigment cells of the larval eyes. The same expression is observed at 48h (not shown). **B:** confocal scan of *mitf* at 56h. **C:** close up of the mesodermal expression of B. **D:** *soxE* average obtained with PrImR at 48h. This is a projection from z-93 to 112 showing mesodermal expression. This might correspond to the *mitf+* domain.

Pigment cells arising from mesodendodermal tissue are wide spread in invertebrates, such as echinoderms (Gibson and Burke 1985). Indeed, based on partial conservation of the gene regulatory network and migratory behaviour, pigment cells have been proposed to be tunicate neural crest-like cells (Jeffery, Strickler, and Yamamoto 2004), (Jeffery 2007).
Conversely, in *Platynereis* I observe that cells expressing the majority of the genes belonging to the neural crest gene regulatory network are found in the lateral neuroectoderm, similarly to the vertebrate neural crest and that give rise to the sensory neurons. I also found a population of melanoblasts similar to the neural crest derived melanoblats. Although unlikely (judging from the time lapse movies, not shown), it remains to be determined if these *Platynereis* putative melanoblasts derive from the neuroectodermal population of cells of the lateral trunk (see chapter V of Results).
It would be also helpful to analyze the molecular development of the adult pigment cells

that arise later and determine if they arise from the cells of the lateral neuroectoderm.

IV.4 The *colA+* cells outside the nervous system

A specific feature of the cranial neural crest cells is that they give rise to the cartilagenous skeleton of the head. In his 'new head theory', Northcutt postulated that the evolution of the head cartilage contributed to the shift from a filter feeding to a predator life style that represents one of the most important steps in vertebrate evolution (Gans and Northcutt 1983).

Many invertebrates, including basal chordates such as amphioxus, possess a collagenous skeleton, which apparently does not derive from neural crest-like cells.

In *Platynereis* I found that a group of cells outside the nervous system express *colA* (fibrillar collagen, black arrowhead in fig.44B), as in the amphioxus gill slit skeleton and notochord.

I followed the development of these cells, and I found that they are specified very early in putative mesodendodermal territory (20h, fig.44A), they converge in the midline from anterior to posterior (fig.45B,C) and form a rod-like muscular structure dorsal to the ventral nerve cord (they are stained with palloidin, fig.44A. The presence of actin fibers was also confirmed via transmission electron microscopy, not shown[5]). We named this structure 'axochord'.

To study the features of this structure in more detail I established a protocol for scanning electron microscopy after skin removal at 5 days (fig.46 see Material and Methods). This protocol maintains the morphology of the cells and structures as close as possible to the physiological one. With this protocol I found that the cells which make up the axochord (the *colA+* cells) have a peculiar cell morphology, fibroblast-like (black arrowhead in fig.46).

Interestingly, I also observed a sheath, which enveloped the nervous system of the worm (blue arrow fig.46B, C). A similar neuroglial sheath has been described in different annelids and it is most likely formed by secreted extracellular matrix (Vagnetti and Farnesi 1978), (Baskin 1971), therefore it is likely that the *colA+* (comprising also the axochordal cells) contribute to the formation of this sheath. In Nereis this tissue has been referred as 'fibrous neuroglia' (Baskin 1971), but molecular features still need to be elucidated.

Interesting At 72h the axial *colA +* cells express *foxD* (fig.44C), the annelid ortholog of foxD3, one of the neural crest specifiers crucial for the formation of pharyngeal and craniofacial cartilage(Arduini, Bosse, and Henion 2009; Stewart et al. 2006) . The ortholog of foxD3 in amphioxus is expressed in the notochord (J.-K. Yu, Holland, and Holland 2002), similar to the expression of foxD3 in the vertebrate notochord that precedes the expression in the neural crest cells (Steiner et al. 2006).

[5] This experiment was performed with Thibaut Brunet and Oleg Simakov

The Platynereis axochord also expresses the notochordal marker *brachyury*(Vujovic et al. 2006) (not shown, data obtained in collaboration with Thibaut Brunet, a PhD student in our lab) and of signalling molecules such as *netrin* and *slit* (fig.44 D,E). The axochord is also present in the adult (*colA* ISH, fig.46G), and scanning electron microscopy shows that it is embedded in the sheath which envelops the nerve cord (fig.46E, F).

At the moment a detailed functional characterization of this structure is carried in collaboration with Thibaut Brunet, in order to determine how the annelid axochord compares to the vertebrate notochord.

In addition the pharyngeal muscle around the stomodeum also expresses *colA* (fig.44F) , as well as another annelid specific paralog, *colA2*(fig.44G). This is likely comparable to the amphioxus *colA+* pharyngeal arch mesoderm.

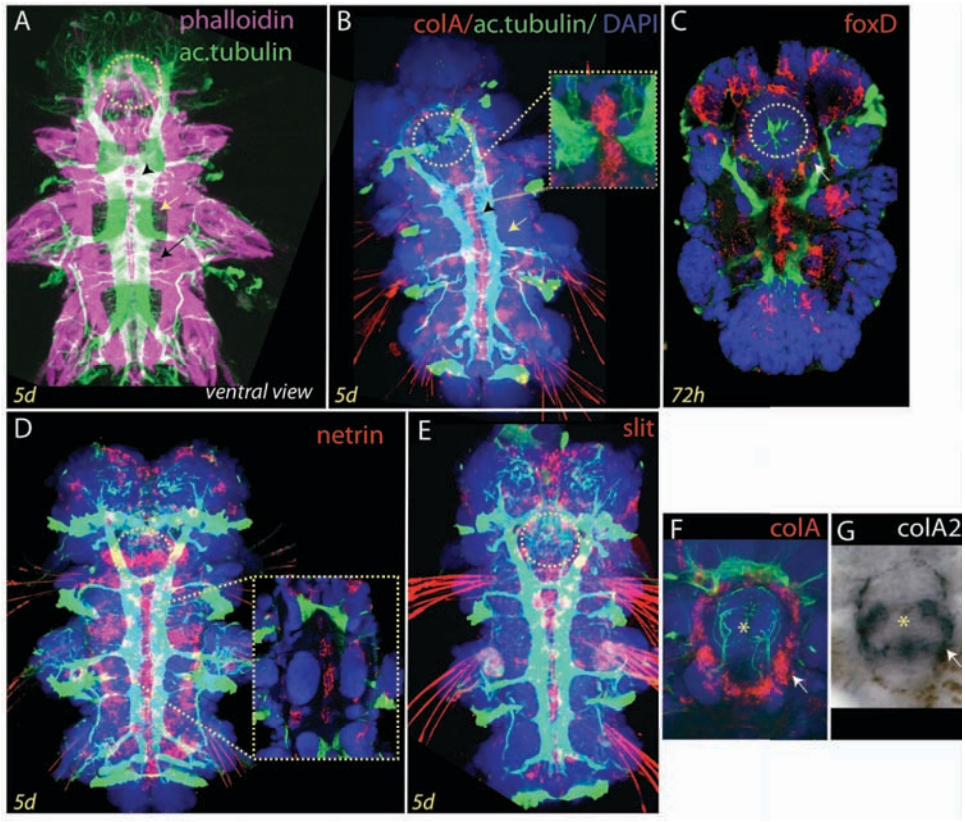


Figure 44. The *colA+* axochord. A: immunofluorescence at 5days, acetylated tubulin (green) shows the axonal scaffold and phalloidin (pink) the musculature. **B:** *colA* expression at 5days. The inset shows the position of the colA+ cells deep between the ventral nerve cords. In A and B yellow arrows indicate the ventral nerve cord, black arrowheads the axochord and black arrows the oblique muscles. **C:** *foxD* expression in the axochord at 72h. White arrow indicate the expression in the pharyngeal mesoderm.**D-E**: *netrin* (D) and *slit* (E) expression at 5days. The inset in D shows the position of the *netrin+* cells. **F-G:** *colA (F)* and *colA2 (G)* expression in the pharyngeal mesoderm at 5 days.

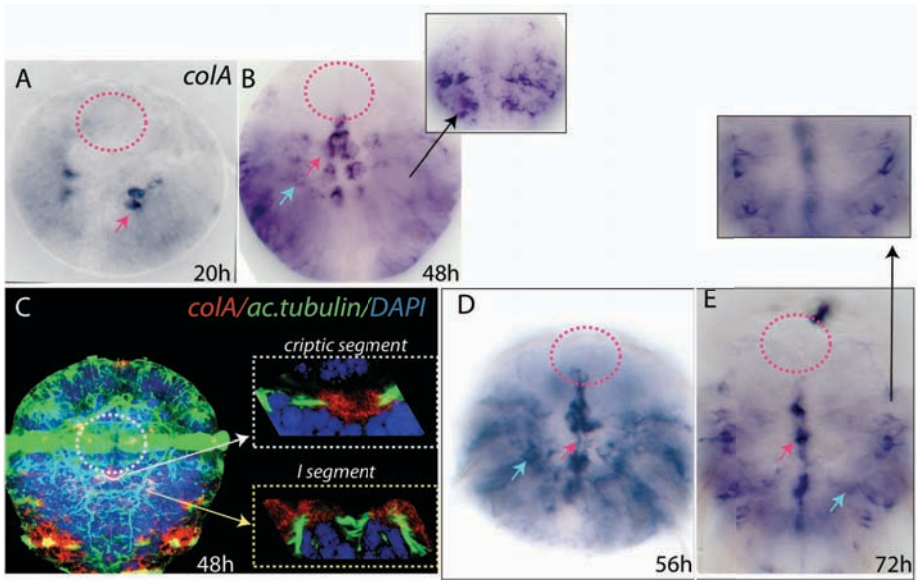


Figure 45. *ColA* expression between 20-72h. A: *colA* expression at 20h. **B:** *colA* expression at 48h. The red arrow indicates the axochordal precursors; the blue arrow indicates the mesodermal expression. Only the anterior most ones are joined in the midline at this stage. The inset in B indicates the expression in the neuroectoderm.**C:** confocal Z-projection of the *colA* expression at 48h.The inset with the dashed white line in C shows the anterior most *colA* cells joined in the midline in between the axons of the VNC. The inset with the dashed yellow line shows the cells in the posterior II not jet joined. **D:** *colA* expression at 56h.At this stage the colA+ medial cells form already a rod-like axial structure. **E:** *colA* expression at 72h. Expression in the neuroectoderm is still visible (inset indicated with the black arrow)

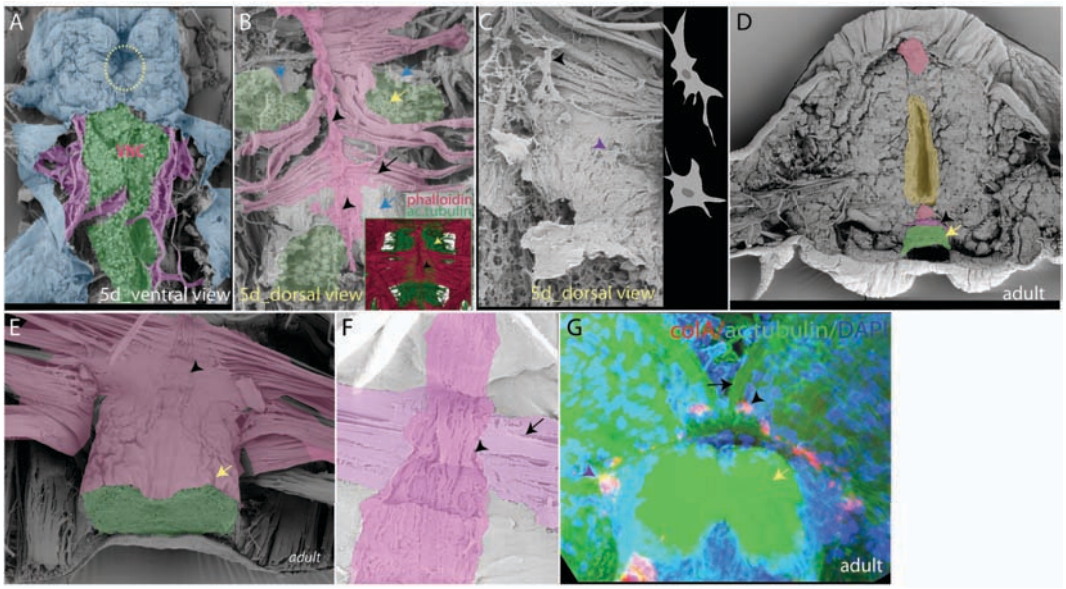


Figure 46. Pseudocolored scanning electron micrographs of *Platynereis* juveniles after skin removal and expression of *colA* in the adult axochord. A: ventral view**.** The neurons of the ventral nerve cord as well as muscles are visible. Epidermis is in blue, nervous system in green and muscles in pink. **B:** dorsal view. Close up on the axochord structure indicated with the black arrowhead. The oblique muscles attached to the axochord are indicated with a black arrow. The sheath is indicated with blue arrows. The underlying neuroectoderm is indicated with a yellow arrow. In the inset phalloidin (purple) is staining the same structure from a dorsal view. The ventral nerve cord is also visible (green). **C:** another view on the axochord from dorsal. In this picture the fibroblast-like morphology of the cells in the midline is more visible, similarly to that of other cells on the sheath (purple arrow). A drawing schematizes this morphology. **D:** scanning electron micrograph of an adult section. In red the blood vessels are shown, the gut is in yellow, the nerve cord in green and the axochord in pink**. E:** the same section as in D, but cut opened. The gut, the gametes and the vessels have been removed. The flat fibroblast-like morphology of

the axochordal cells is well visible. F: close up on the axochordal cells seen in E. **G:** Z-projection of a confocal scan of *colA* expression in an adult cross section. Expression is observed also in other cells around the ventral nerve cord (purple arrow), likely the same as in C.

In vertebrates the head cartilage, comprising also the pharyngeal arch cartilage originates from the neural crest cells. The presence of an axial muscle structure in annelids and a pharyngeal mesoderm expressing the mRNA of molecules implicated in neural crest migration, head cartilage formation and notochord formation, such as colA and foxD is very interesting. It supports the hypothesis that a genetic module acting in the colloagenous skeleton predated the emergence of the neural crest derived skeleton (Votano, Parham, and Hall 2004). The data presented here suggest that this module was already established at the base of Bilateria.

Collectively the data point to an uninspected complexity of the cell types inventory of the annelid trunk nervous system and suggest that many of the cells that in vertebrates are neural crest derived were already present at the dawn of Bilateria (see Discussion).

V. Is migration involved in the formation of the peripheral nervous system? An open question and prelimary results

The most important feature of the vertebrate neural crest is the extensive migratory behaviour. This way the neural crest populates different locations and differentiates in several cell types.
Therefore, once identified the *Platynereis* cell types homologous to those that in vertebrates originate from the neural crest, I asked whether some of these precursors showed migratory behaviour. To address this point I started pioneering experiments analyzing different stages of development via live imaging of larvae injected with mYFPand H2AmCherry. These experiments showed cell division (fig.37E, Appendix fig.a3,b3), and single cell migratory behaviour. Indeed I observed single migrating cells in the *brn3* medial territory between 48h-65h (fig.47). But, these cells are outside the neural plate border-like domain that I identified so far, and no other migratory behaviour was visible. Nevertheless, the analysis of the data was extremely difficult due to the dense neuroectoderm.
Moreover with this approach it is nearly impossible to follow long range migration of individual cells and understand their fate; this because all the cells are labelled, and due to proliferation and to axon growth, the neuroectoderm becomes very thick and makes impossible to track individual cells.

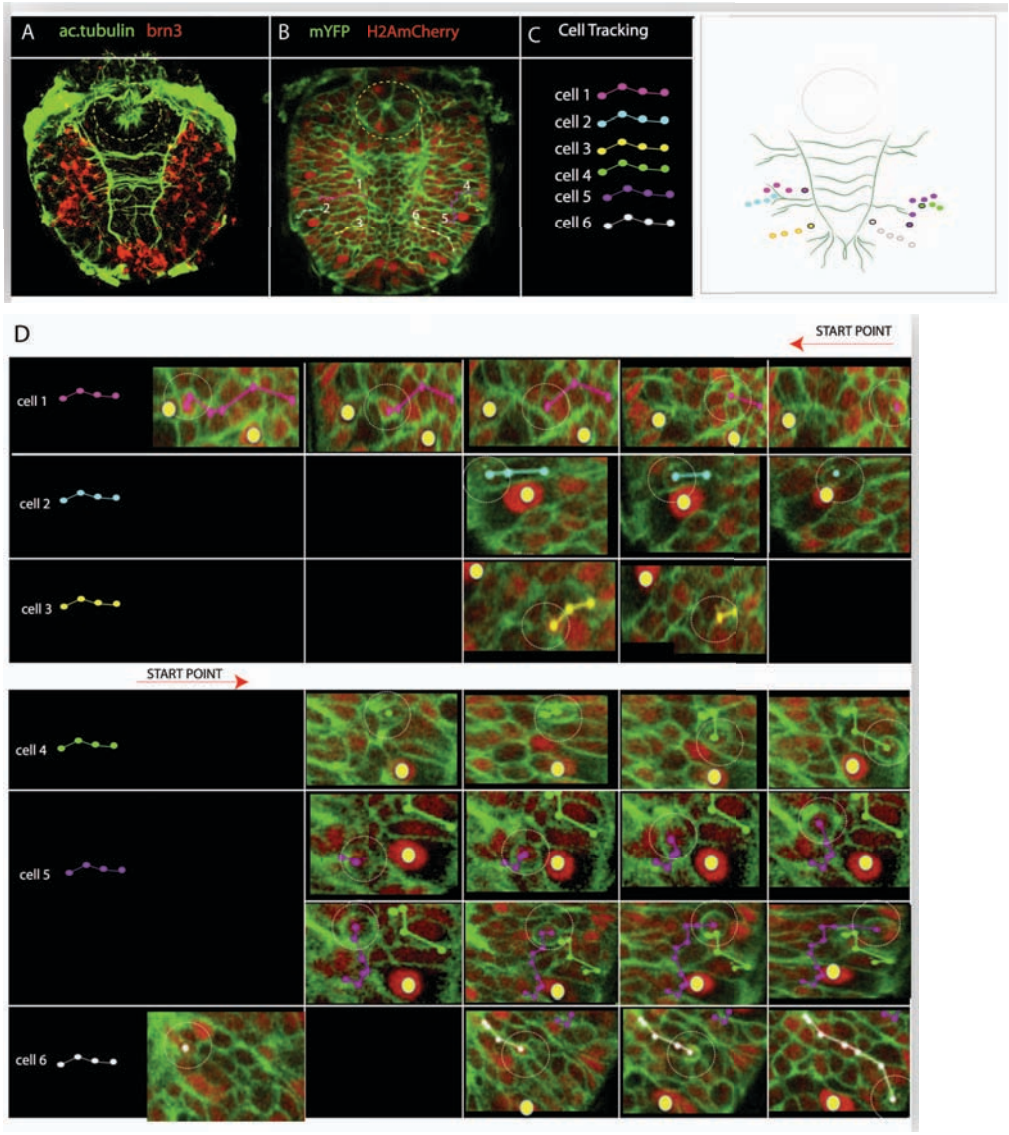


Figure 47 Manual tracking of the migratory cells in the *brn3*+ neuroectoderm of *Platynereis* between 48-65h. Single migrating cells were observed in the neuroectoderm of *Platynereis*. Each cell and its path is labelled with different colours. **A:** expression of *brn3* mRNA at 48h. **B:** z-projection of few time point of the original time lapse to shows the position, the start point, the tracking and the end point of the migratory cells. The end point is the position where the analysis was stopped because it was impossible to track the cell further. **C:** drawing showing the tracked cells on the left and on the right the position, start point, the tracking and the end point of the migratory cells in the trunk. **D:** manual tracking per each cell. The tracked cell is highlighted with a white circle. Position of stationary cells (such as the ciliate cells of the ciliary bands for cell 4 and 5) are indicated per each time point with yellow big dot as a reference.

Hence, to test the migratory behaviour, I also decided to perform DiI injection of the cells of the neural plate border in the trochophore and then analyzed the DiI + cells later in development (fig.48). After injection of DiI in the lateral domain, with this technique I did not observe migration. I observed the presence of DiI+ cells in the ciliary band the day after the injection (white asterisk in fig,48D) . This could be due to contribution of some anterior most lateral neuroectodermal cells to the ciliary band. An active uptake of DiI

from the surrounding medium cannot be excluded, since there is always licking from the needle during injection.

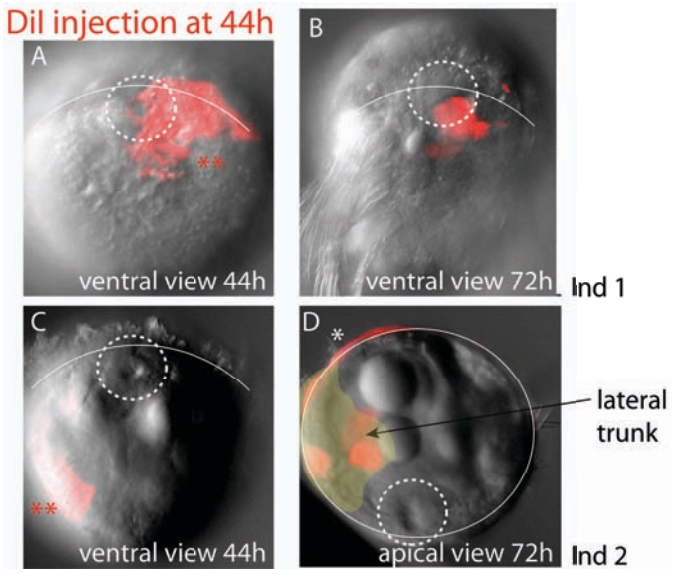


Figure 48. Injection of DiI in the neuroectoderm at 44h and observation at 72h. Injection at different sites was performed to compare the possible migratory behaviours. **A:** DiI was injected in the neuroectoderm of 44h individual 1 comprising part of the stomodeum and anterior midline. Asterisks indicate the site of injection. **B:** individual 1 microphotographed at 72h. **C:** In individual 2 the DiI was njected in the lateral neuroectoderm. **D:** individual 2 microphotographed at 72h. The pictures are taken at the Zeiss M2 epifluorescent microscope on the alive animals. Unfortunately in this case it was impossible to orient ventrally the animal. D is therefore an apical view, but the individual has been analyzed also from ventral view and it was clear that the DiI+ cells are located along the injected site of the trunk.

These data are not conclusive because the experiments with this technique were extremely difficult to reproduce. This was likely due to the variability of the precise site of injection as well as the intrinsic viability of the embryos after the experiment.
Although not likely, with these experiments I could not exclude 100% that some of the cells of the lateral neuroectoderm have migratory behaviour.
To address this I plan to use the KikGR (Kikume Green-Red) photoconvertible green-to-red fluorescent protein (courtesy of Dr. Atsushi Miyawaki from the RIKEN Brain Institute, Japan), (Nowotschin and Hadjantonakis 2009; Habuchi et al. 2008; Votano, Parham, and Hall 2004) This plasmid that I obtained encodes for a green-to-red photoconvertible protein that compared others, shows high stability in time after photoconversion.

The mRNA for KikGR has been used to follow cell migration in different contexts, including the neural crest), (Nowotschin and Hadjantonakis 2009; Habuchi et al. 2008; Votano, Parham, and Hall 2004). In ivertebrates, it has been injected in sea urchin larvae, mesenchimal cells were photonverted and the migration of photoconverted cells has been successfully documented after 4 days of development (Wei, Angerer, and Angerer 2011).

The same kind of experiment in *Platynereis* would offer a different approach to test whether migratory cells from the lateral neuroectoderm contribute to other derivatives apart from the sensory ganglia described in this study.

2. A canonical neurotrophic signalling in bilaterians

VI. The neurotrophic molecules in *Platynereis*

The development of the neural crest derived sensory neurons is dependent on the neurotrophic signalling, which is mediated by Trk receptors and their ligands (the neurotrophins) . For instance, different subtypes of peripheral sensory neurons derive from the neural crest and express different combinations of the Trk receptors and neurotrophins in the last steps of their differentiation (Lallemend and Ernfors 2012).
The neurotrophic signaling is required for their development, axonal projections, and the continued survival of these neurons (Lallemend and Ernfors 2012), (E J Huang and Reichardt 2001), (Eric J Huang and Reichardt 2003).
Interestingly, neurotrophic signaling was long considered a vertebrate innovation , and its appearance was linked to the evolution of the neural crest in the vertebrate lineage (Wittbrodt 2007).
Therefore, after I determined that the formation of sensory neurons is a conserved feature of the cells at the neural plate border in bilaterians (and therefore likely an ancestral trait), I asked whether the neurotrophic signaling was acting in these cells at the base of Bilateria.
When I started this study, no invertebrate Trk receptor was described, except for the one in amphioxus (Benito-Gutiérrez et al. 2005). Different putative orthologs were occasionally proposed in protostomes, however none of them was a canonical receptor (Pulido et al. 1992),(Winberg et al. 2001),(Beck et al. 2003), . It was therefore postulated then that this signaling pathyway was a chordate innovation (Benito-Gutiérrez, Garcia-Fernàndez, and Comella 2006). Hence, I needed to find out whether a *Platynereis* Trk ortholog existed and determine its domain composition, to learn if it is a canonical receptor. During my PhD I characterized the molecules implicated in the signaling cascasde, and I am now am beginning to investigate the function of this signaling in *Platyneries.*

VI.1 The annelid ortholog of the high affinity neurotrophic receptor Trk is expressed in the nervous system

When I started looking for *Platynereis* orthologs of Trk receptor only partial data from the transcriptome project (just started at the time from Dr.Tomas Larsoon) and an EST collection was available. Nevertheless, I found one hit corresponding to a small piece of the intracellular domain of the *trk* receptor. Next, performing several rounds of RACE PCRs, I was able to isolate the full length of the *Platynereis* Trk receptor. Surprisingly *Platynereis* Trk possesses all the canonical domains (details in the next paragraph). I was not able to find more then one Trk receptor.

Next, I examined the expression pattern of *Platynereis trk* during development. I was unable to observe consistent trunk expression of trk in the trocophore larva without background. This could be because the level of the transcripts are very low and impossible to detect with the WMISH, or because the receptor is not expressed at these

stages. Conversely, I found that in the juvenile *trk* is broadly expressed in the nervous system.

In vertebrates different trk receptors (A,B,C) are expressed in different parts of the nervous system, but every cell of the nervous system expresses at least one of the receptors. Therefore it is not surprising that the only annelid Trk receptor is expressed broadly in the nervous system. Indeed several *trk+* neurons are found in the trunk where central ganglia form (black arrow fig.49A,B). Anterior big neurons of the trunk are also *trk* + (yellow arrow in fig.49A). *Trk* is expressed also in the periphery where the sensory ganglia are located (white arrow in fig.49B). At this stage *trk* is expressed in a cluster of cells in the brain, these cells comprise the circadian center (Arendt et al. 2004) and the photoreceptors (inset in fig.49B).

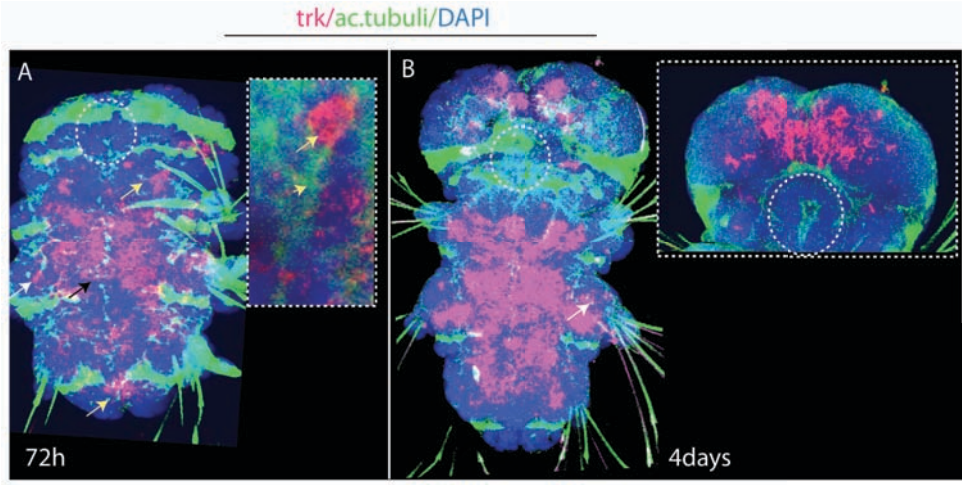


Figure 49. Expression of *Platynereis trk* receptor in the juvenile. A,B: WMISH at 72h (A) and at 4 days (B). Black arrow indicates expressison in the neurons of the trunk central nervous system. White arrows indicate expression in peripheral neurons. Yellow arrows indicate expression in the primitive neurons of the trunk. Inset in A shows one of the big neurons in the anterior neuroectoderm, yellow arrowhead indicates the large axon of the neuron. Inset in B shows the expression of *trk* in the brain.

VI.2 Cloning of Trk, neurotrophin and p75 *Platynereis* orthologs, domain prediction and sequence alignment reveals canonical features

As mentioned in the first paragraph I obtained the full-length sequence of the Trk receptor via sequential race PCR on a cDNA library created mixing different stages (details in Material and Methods).

In the same way I also obtained the full-length sequence of the co-receptor p75. After a blast search with different vertebrate neurotrophin ligands I found the *Platynereis* neurotrophin.

Next, in order to compare the *Platynereis* neurotrophin components to the vertebrate/invertebrate counterparts, I performed a sequence analysis, using different tools for domain prediction such as *Smart domain* (Schultz et al. 1998) and *ProSite (Expasy,* (Sigrist et al. 2010) predicted domains for each molecules are found in fig. 50, 51,52 Appendix figb4). I also performed protein sequence alignment using *Clustal X* (Jeanmougin et al. 1998)and *Muscle (EBI)*(Edgar 2004)

For a better comparison, after search in the genomic data, I have cloned and sequenced also the orthologs from *Capitella Teleta*[6], another polychaete annelid with a sedentary life cycle . These analyses surprisingly revealed canonical molecular signatures in each of the proteins analyzed.

Because several putative orthologs of Trk receptor and neurotrophin have been wrongly annotated among invertebrates, I also generated phylogenetic trees (details in Material and Methods) using full length and partial domains of the molecules to resolve the relationship of the *Platynereis* molecules with the vertebrate/ invertebrate counterparts. The results will be explained below.

-The *Platynereis* Trk receptor

I found that *Platynereis* Trk *(pdu*Trk*)* contains a canonical intracellular TK (tyrosine kinase) domain (fig.50A) with conserved molecular signatures, such as the binding domain for Src proteins (number 1 in fig.50), the ATP binding site (2) , the catalytic domain of the Tk receptoes with the Aspartate (3) and the autophosphorylation domain (4).

Platynereis Trk contains even the docking site for the PLCγ [P(VIS)YLD(IV)L(GE)], that, once activated, catalyses the formation of DAG (Diacylglycerol) and IP3 (Inositol Triphosphate) from PIP2 (Phosphatidylinositol 4,5-Bisphosphate). This pathway is implicated in cytoskeletal rearrangement, long term potentiation and neuronal plasticity(Stephens et al. 1994).

Since crustaceans also (*Daphnia pulex* Trk : *DpulexTrk*) possess the PLCγ docking site (Wilson 2009), it is likely that this domain was lost in amphioxus (Benito-Gutiérrez et al. 2005).

Remarkably, and contrarily to the orthologs that have been isolated so far in other protostomes (except for *Dpulex*Trk), *pdu*Trk has also highly stereotypical extracellular domains. Two LRR (leucin rich domains) are present, followed by two predicted IgG (immunoglobulin domains), that in vertebrates are responsible for the binding to the neurotrophin.

From the phylogenetic tree done using the full length of the the Trk receptors is clear that among the RTK receptors *pdu*Trk belongs to the Trk family.

[6] The cDNA was kindly provided by Dr. Heather Marlow

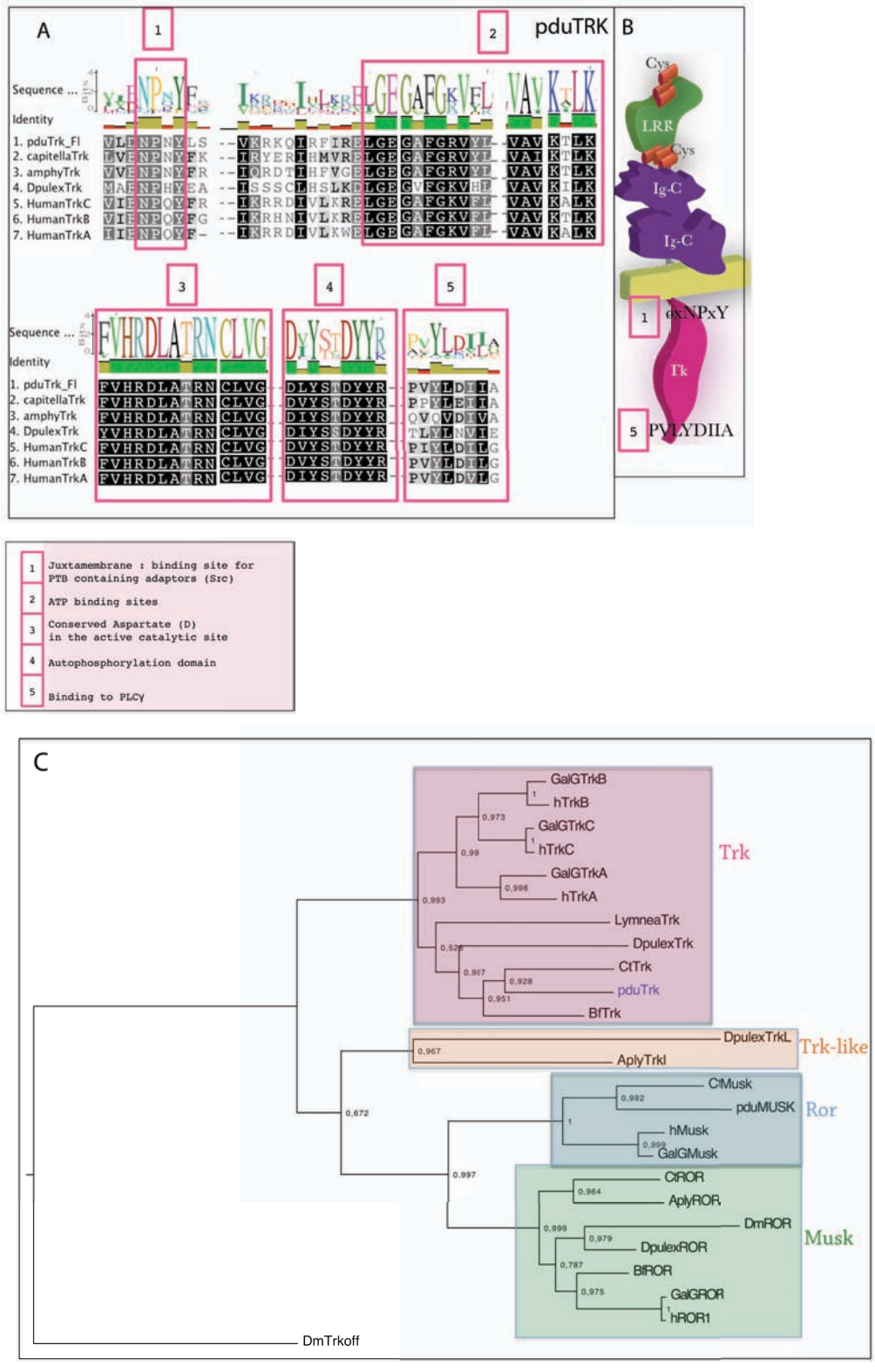


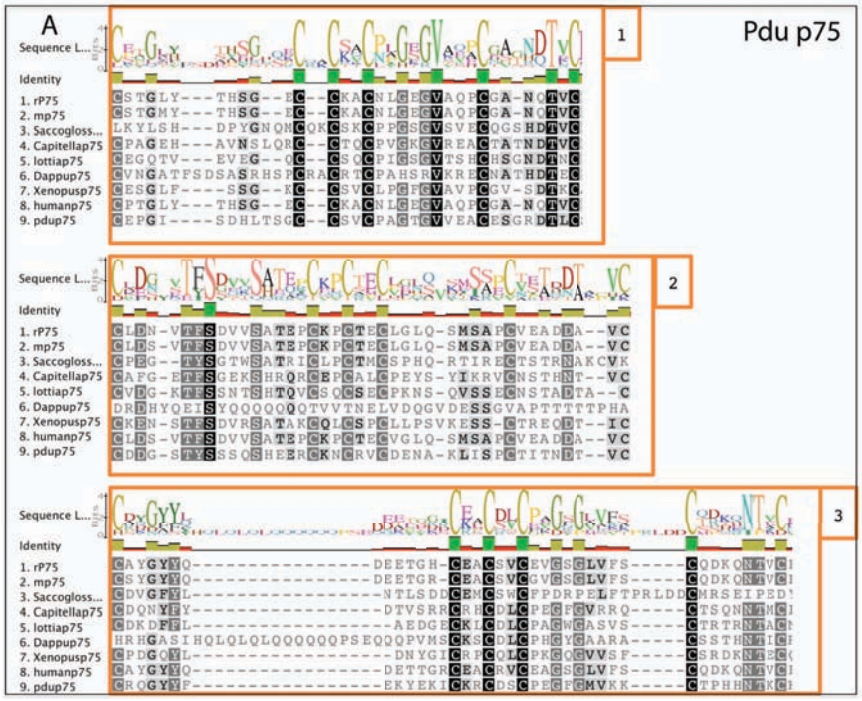
Figure 50. Protein alignment of the Tk domain and phylogenetic analysis of the full length of *Platynereis* Trk (pduTrk). A: multiple sequence alignment of the TK intracellular domain of the Trk receptors. Important signatures are numbered and described in the legend on the bottom left of the alignment. **B:** schematic drawings of the domains composing *Platynereis* Trk. The leucin reach domains (LRR), the immunoglobulin domain(IgG) and the tyrosine kinase domain (TK) are shown. Two signatures (1-5) are also mapped on the juxamembrane (1) and intracellular (5) domain. **C:** phylogenetic analysis of *Platynereis* Trk in comparison to other vertebrate and invertebrate Trk and other members of the RTK superfamily (Ror and MuSK). The alignment was obtained with

Muscle, the tree was generated with PhyML,100 bootstrap replicates, LG substitution model. ML (ML bootstrap values) are indicated. GalG (*Gallus gallus*), h (*human*), Dpulex (*Daphnia pulex*), Lymnea (*Lymena Staginalis*), Aply (*Aplysia*), Dm (*Drosophila melanogaster*), Bf (*Brachiostoma floridae*), Ct (*Capitella teleta*).

-The *Platynereis* p75 co-receptor

The low affinity co-receptor p75 binds to the complex Trk-NT in vertebrates (J Huang and Reichardt 2003, fig.24). Using race PCR approach I have cloned *Platynereis* p75 co-receptor. As the vertebrate counterpart, the extracellular portion of *pdu*p75 possesses 4 canonical CRD (cysteine rich domains) with 6 Cysteine each (fig.51 A, B). This is not the case for the crustacean p75 (*Dpulex* p75) that has only 3 CRDs (Wilson 2009).

The intracellular portion of *pdu*p75 contains a canonical death domain, known to activate the caspases implicated in cell death. Phylogenetic analysis shows that *pdu*p75 belongs to the TNFRSF family (tumor necrosis factors receptor superfamily) and clusters with the other p75 molecules (fig. 51C).



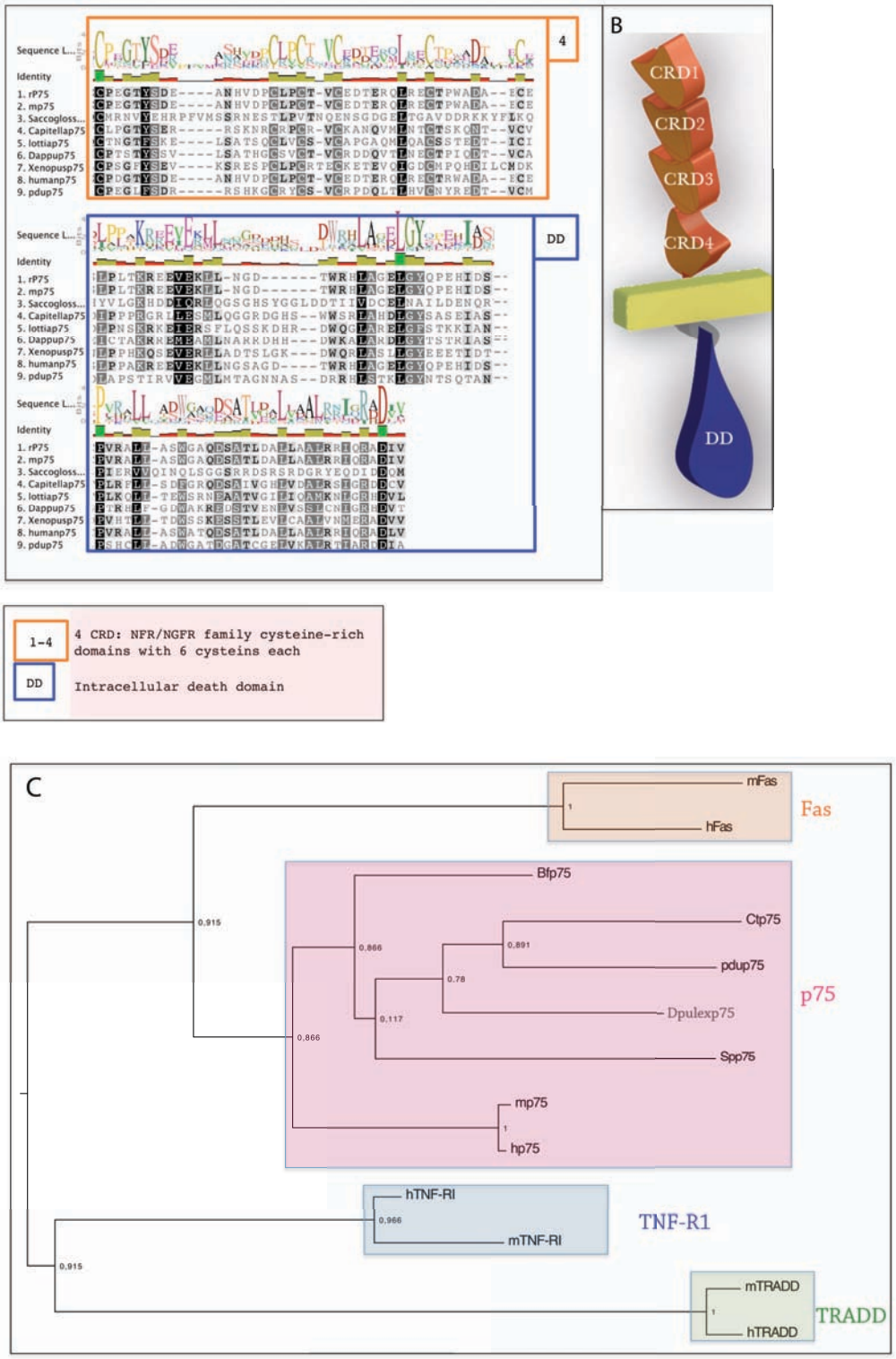


Figure 51. Protein alignment and phylogenetic analysis of the full length of *Platynereis* p75 (*pdu*p75). A: multiple sequence alignment of p75 co-receptors. The four Cys rich domains (CRDs) and the death domain (DD) are numbered and described in the legend on the bottom left of the alignment. **B:** schematic drawings of the domains composing *Platynereis* p75. **C:** phylogenetic analysis of *Platynereis* p75 in comparison to other vertebrate and invertebrate p75 and other members of the TNFRSF family. The alignment was obtained with Muscle, the tree was generated with PhyML, 100 bootstrap replicates, LG substitution model. ML (ML bootstrap values) are indicated. m (*mouse*), h (*human*), Dpulex (*Daphnia pulex*), Bf *(Brachiostoma floridae)*, Ct *(Capitella teleta)*, Sp *(Strongylocentrotus purpuratus).*

-The *Platynereis* putative ligand*: pdu*NT

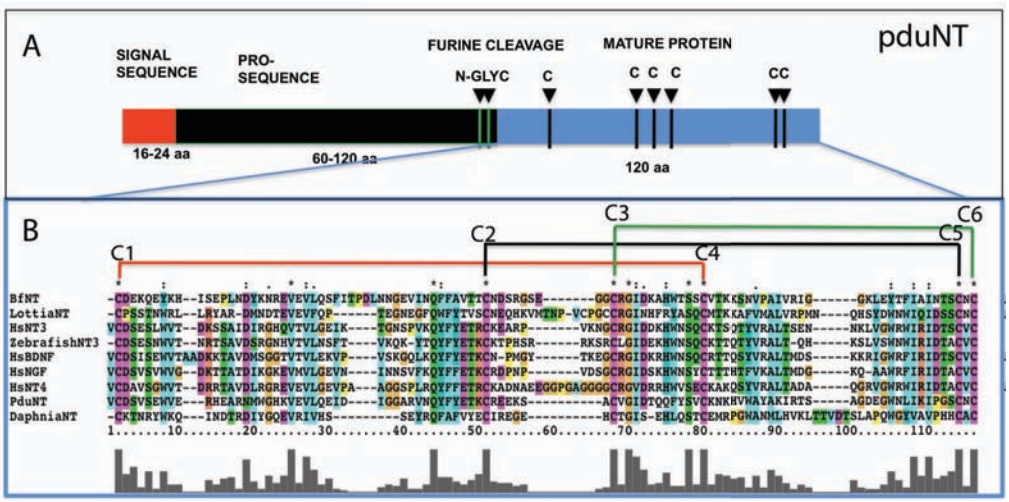
Because the receptor and the co-receptor are present in *Platynereis* I also searched for the presence of a ligand. Likewise the extracellular domain of Trk receptors and the other RTK receptors, the sequences of their ligands undergo fast evolution. This makes quite difficult to search for homologous ligands in the genomes and the phylogenetic comparison.

Nevertheless I found one hit in the *Platynereis* transcriptome for a neurotrophin-like molecule.

As the vertebrate counterparts, this *Platynereis* NT (*pdu*NT) is composed of a signal peptide and a pro-neurotrophin domain containing N-glycosilation site, important for the secretion of the molecule. After the predicted protease cleavage site there is the core of the protein composed of cysteines (fig. 52A). Amino acids comparison shows that this Cys core (so called *cys Knot*) of the molecule is highly conserved (fig.51B).

Many extracellular proteins and hormones such as transforming growth factor-b (TGF), glycoprotein hormone and platelet-derived growth factor (PDGF) contain the so called *cys Knot* domain. This is formed by 6 cysteines that are essential for the formation of disulfide bondings and loops, the typical consensus stretch of ammino acids of the cysteine knot superfamily is: C1-(X)n-C2-X-G-X-C3-(X)n-C4-(X)n-C5-X-C6. (Vitt, Hsu, and Hsueh 2001), (Butte 2001).

As previously a mentioned, the phylogenetic tree of neurotrophin molecules are difficult as for the ligands because it is known that ligands are the most fast evolving components of signaling pathways. Nevertheless, a NJ tree of the conserved *cys Knot* domain shows that *Platynereis* putative Trk ligand candidate (*pdu*NT) belongs to the neurotrophin family. PDGFb also contain a cys Knot and has been used as outgroup, since it belongs to another family of ligands.



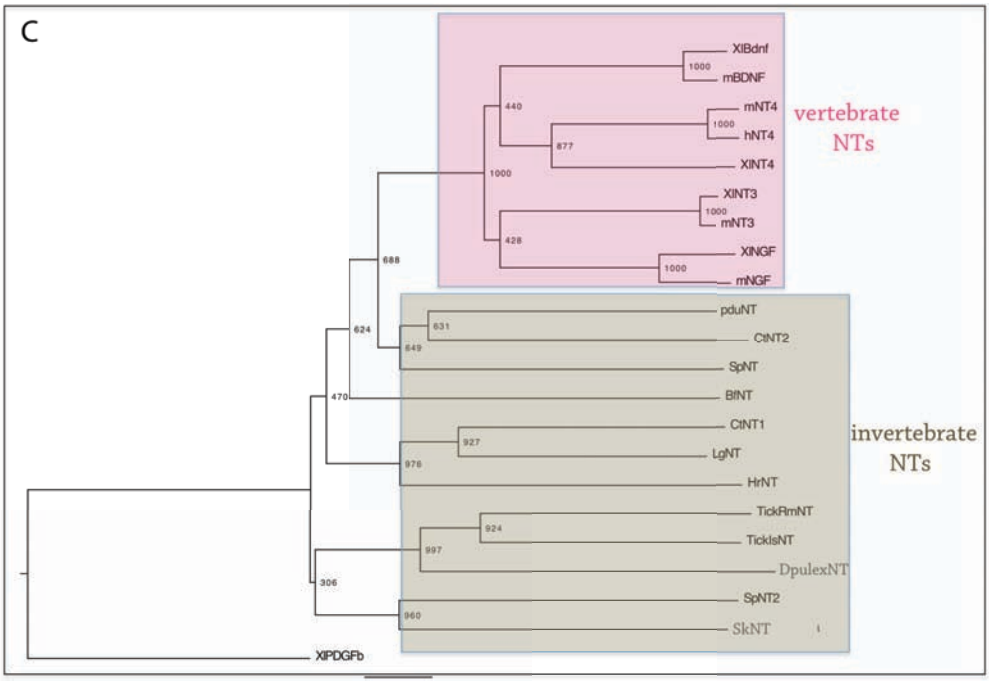


Figure 52. ***Platynereis*** **Neurotrophin and phylogenetic analysis A:** schematic representation of the domains composing *Platynereis* Neurotrophin (*pdu*NT). N-Glyc: N-glycosilation site. C: cysteines. **B:** multiple sequence alignment of the Cys Knot domain of different neurotrophins. The predicted disulphide bonds among the Cysteines are shown with different colours. **C:** phylogenetic analysis of *Platynereis* NT in comparison to other vertebrate and invertebrate NTs. PDGF is used as outgroup. The alignment was obtained with Muscle, bootstrap values of the NJ trees (100 bootstraps) are indicated. Xl (*Xenopus laevis*), m (*mouse*), Dp (*Daphnia pulex*), Lg (*Lottia Gigantea*), Bf *(Brachiostoma floridae),* Ct *(Capitella teleta),* Hr *(Helobdella robusta),* Sk *(Saccoglosus kovalensky),* Sp *(Strongylocentrotus purpuratus),* the other species'names are as in fig.50.

VI.2.1 *In silico* 3D structure prediction

Using protein homology modeling algorithms such as *CPHmodels 3.2*(Nielsen et al. 2010) and *M4T (*Multiple Mapping Method with Multiple Templates, (Fernandez-Fuentes et al. 2007; Rykunov et al. 2009). I then performed 3D structure prediction of the *pdu*NT (fig. 53) and visualized the predicted structure using *Chimera* (developed by the Resource for Biocomputing, Visualization, and Informatics, funded by the National Institutes of Health, NIGMS 9P41GM103311, (Pettersen et al. 2004).

These algorithms perform a PSI blast to identify similar proteins and then obtain a secondary structure based alignment, and then based on this, a 3D model is built. These tools have evident limitations, because they base their modelling on existing datasets for homologous proteins. Nevertheless for *pdu*NT the programs are able to predict the prototypical folding given by the *cyst Knot*.

In vertebrates three disulphide bonds are present: between C1-C4, C2-C5, C3-6 (fig. 53A, B). This arrangement leads to the formation of 3 distinct domains, two antiparallel beta-sheets between C1-C2 and C4-C5 (fingers) and a heel in between. In vertebrates it is known that this particular 3D conformation exposes the hydrophobic amino acids on the surface, and they mediate the homodimerization with other neurotrophin monomers (Butte 2001).

.

Once obtained the 3D model of the *pdu*NT I also predicted the secondary structure of the extracellular domain of *pdu*Trk (fig. 54A). According to the prediction *pdu*Trk extracellular domains are assembled in canonical LRR and IgG domains, as the vertebrate counterpart (fig. 54B). These data *in silico* are only predictive and need to be validated experimentally. Nevertheless, these predictions indicate that the extracellular domain of *pdu*Trk is able to fold according to the predicted domains that are known for the vertebrate counterpart and bind the neurotrophin.

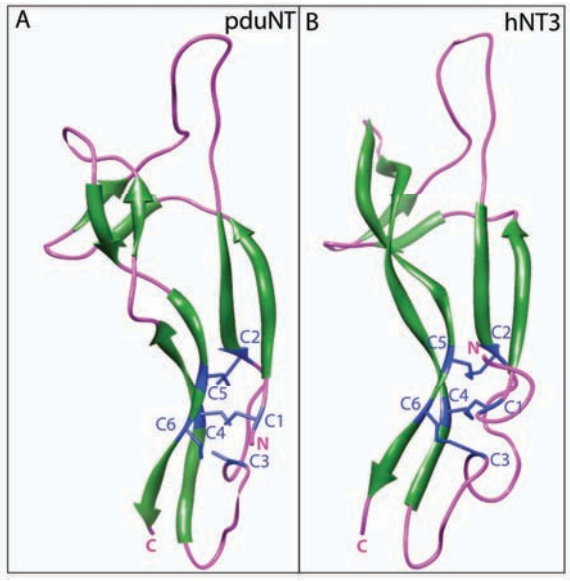


Figure 53. *In silico* 3D structure prediction of *Platynereis* mNT (mature form) monomer compared to the human mNT-3. A: pduNT 3D predicted structure. The picture was creating in *Chimera* using the model produced via *CPHmodels 3.2*. **B:** human NT-3 3D structure. The picture was created in *Chimera* using the experimentally determined structure of human NT-3 (DOI:10.2210/pdb1nt3/pdb). The cysteins involved in the disuphide bonds are indicated, as well as the N-terminus (N) and C-terminus (C) (in pink).

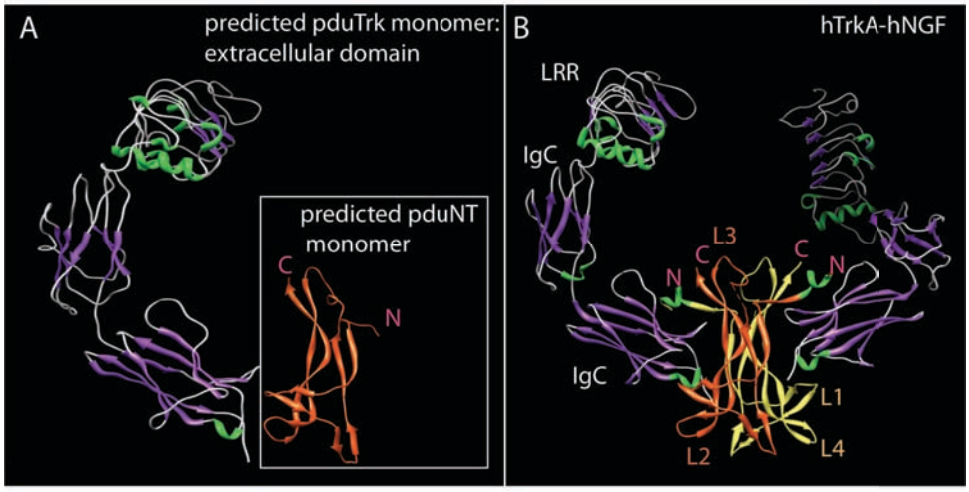


Figure 54. ***In silico*** **3D structure prediction of the extracellular portion of** ***Platynereis*** **Trk compared to the extracellular domain of the human human TrkA binding to NGF. A:** predicted 3D structure of the extracellular domain of *pdu*Trk. The picture was creating in *Chimera* using the model produced via *M4T*. In the inset the predicted monomer of pduNT is shown. **B:** 3D structure of the complex between the extracellular domain of TrkA and the NGF. The picture was created in *Chimera* using the experimentally determined complex (DOI:10.2210/pdb1www/pdb). The domains are indicated (see text for details), as well as the N-terminus (N) and C-terminus (C) (in pink).

VI. 3 The expression pattern in the brain

At 48h *Platynereis neurotrophin* and *trk* are expressed in the brain (fig.55A-C,G). The cells producing NT are part of the apical organ complex, they are likely the *trpA* + sensory neurons (Appendix, fig.b5). Faint expression in the cells of the ciliary band is also observed. The mRNA of the *trk* receptor is indeed specifically expressed deeper in the cells of the dorsal brain (fig.55D-F,H); this cluster is still present later in development (fig.49) and comprises the ciliary photoreceptors (fig. 55F).

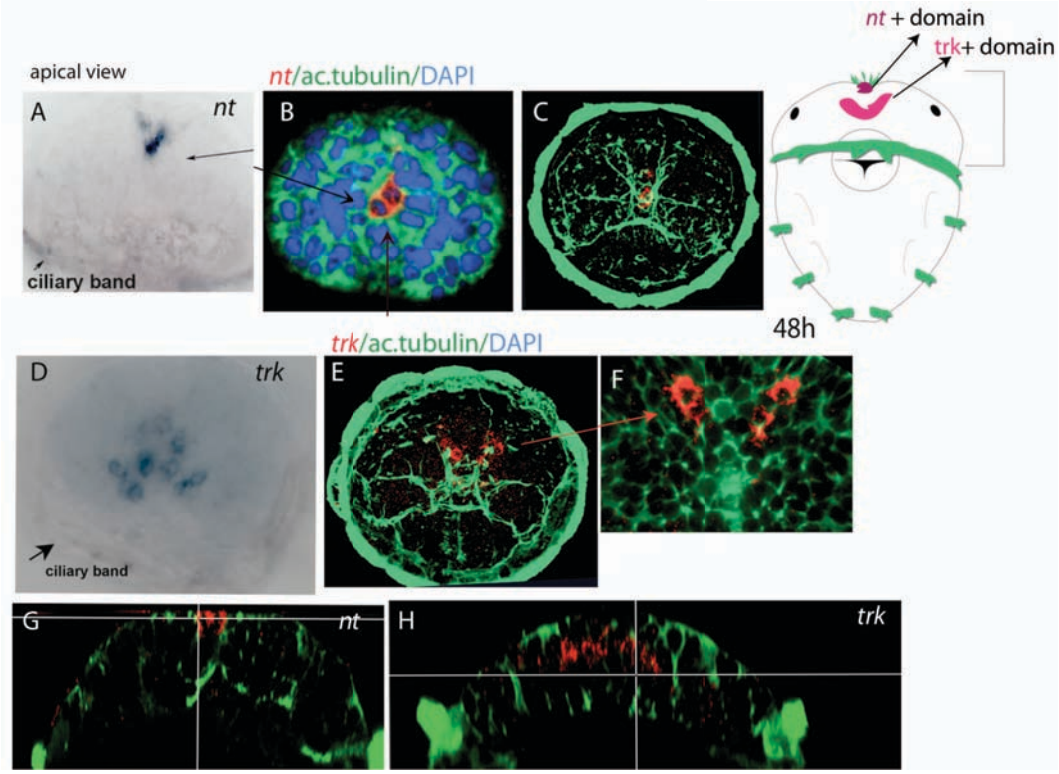


Figure 55. Brain expression of *pdu*trk and *pdu*Nt at 48h. A: WMISH. Bright field picture of nt expression in cells of the apical organ. **B:** Z-projection of superficial layers of a confocal scan of the same individual in A.**C:** Z-projection of the entire confocal scan for the brain. **D:** Bright field picture of trk expression in the brain**. E:** Z-projection of the entire confocal scan for the brain. **F:** a subset of the *trk*+ cells is shown. **G-H:** virtual cross sections obtained with *Imaris* of the original confocal scans for nt (G) and trk (H).

VI. 4 The expression pattern in the trunk

In the trocophore stage only *pdu*p75 is consistently detected in the trunk (fig.55). It is expressed at the midline and broadly in the posterior part of the neuroectoderm, most likely involved in neuronal development as in vertebrates. This expression pattern makes plausible that *pdup75* acts independently of neurotrophin in this context. Similar expression has been observed at 3 days (data not shown).

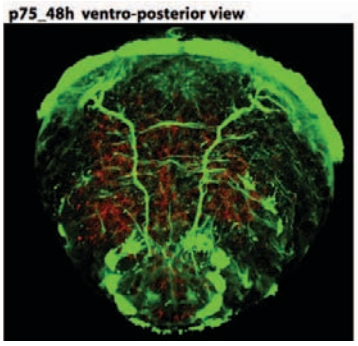


Figure 56. Trunk expression of *pdu*p75 at 48h: Z-projection of the entire confocal scan for the trunk. Ac.tubulin in green and NBT-BCIP precipitate in red.

At later stages *pdu*NT is expressed in the trunk (fig.57). Expression in the ciliary bands is detected and new expression domains are observed. The cells of the neuronal midline produce the neurotrophin indeed (also *pdu*p75 is still expressed in these cells).

Interestingly, muscles, which are developing at this stage, express *pdu*NT. *Pdu*Trk in instead broadly expressed in the trunk nervous system (fig.49).

The expression of *pdu*Trk in neurons and *pdu*NT in neuronal midline and muscles suggests a role in axon pathfinding. In vertebrates the receptor is expressed in the developing neurons that need to grow the axons and reach their targets, while the ligand acts as an attractant for the growing axons and it is therefore produced by the target tissues; in this case the developing muscles (E J Huang and Reichardt 2001; Buj-Bello, Pinon, and Davies 1994; Liebl et al. 1997; Ockel, Lewin, and Barde 1996; Hory-Lee et al. 1993; Davies 1994; Tessarollo 1998) Experiments which interfere with the signaling of this pathway are still needed in order to test this hypothesis.

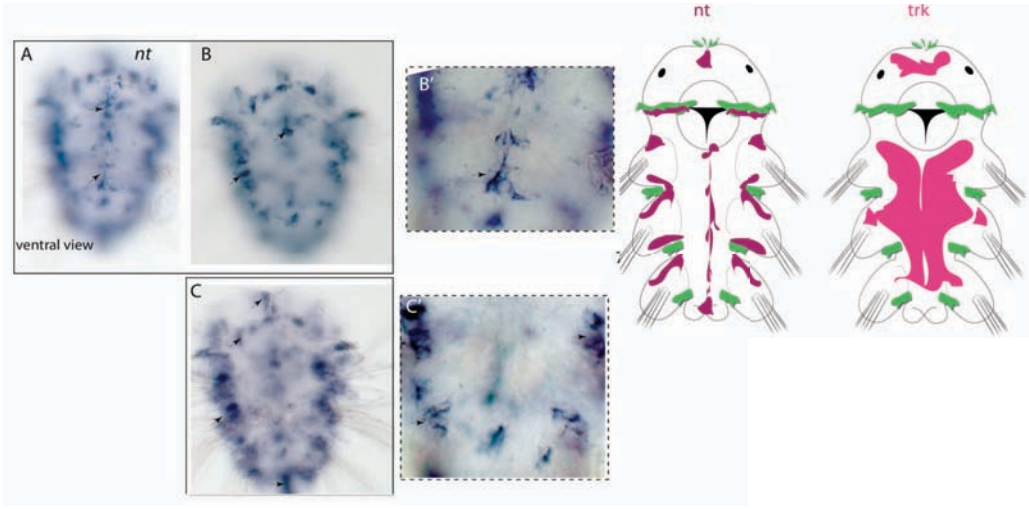


Figure 57. Trunk expression of *pdu*Nt at 72h. A-C': Bright-field picture of nt expression. A-B-C are pictures at different Z-levels. In C labelling in the apical organ is indicated with a black arrow. **B':** superficial neuronal midline expression is visibile. **C':** deep muscle expression is visible. The drawing on the upper right schematizes the expression of trk and neurotrophin at 72h, considering also fig.46.

-The expression pattern in the late juvenile

After 5 days the juvenile worms undergo metamorphosis, after which the life style is completely changed (see paragraph I.1.2, Introduction, fig.2).

The worm settles, stops swimming by means of the ciliary bands and becomes benthic. In this phase the stomodeum (foregut) is increasing as well as the jaw size.

In order to characterize the neurotrophic signaling during these stages of development I then selected individuals according to the signs of metamorphosis indicated above. At this stage the cells expressing *pdu*Nt, *pdu*Trk and *pdu*p75 are found dorsally (fig.58A,A',B,B' and fig.59). These cells comprise most likely ectodermal sensory neurons found also in other polychaetes (Dorsett 1964). Accordingly they also express markers of *Drosophila* peripheral sensory organs such as *barH1* (Higashijima et al. 1992) and *sensless (sens,* (Nolo, Abbott, and Bellen 2000; Jafar-Nejad et al. 2003) *(*fig.58C,D*).*

Furthermore, I performed scanning electron micrographs on the juvenile to analyze the presence of cilia protruding outside the cuticle, different kind of cilia have been indeed morphologically described with this approach (Purschke 2005),(Hausen 2007).

I could observe cilia of different sensory structure both in the head and in the trunk. In the trunk, long cilia are presents on each segment, protruding from the peripheral cirri (Appendix, fig.b6E-E'). This is the position where the cilia of the sensory cells of the peripheral ganglia protrude. In addition to this, I also observed cilia protruding from the dorsal skin (Appendix, fig.b6F-I); however, it remains to be determined if these cells correspond to the *trk*+ ones.

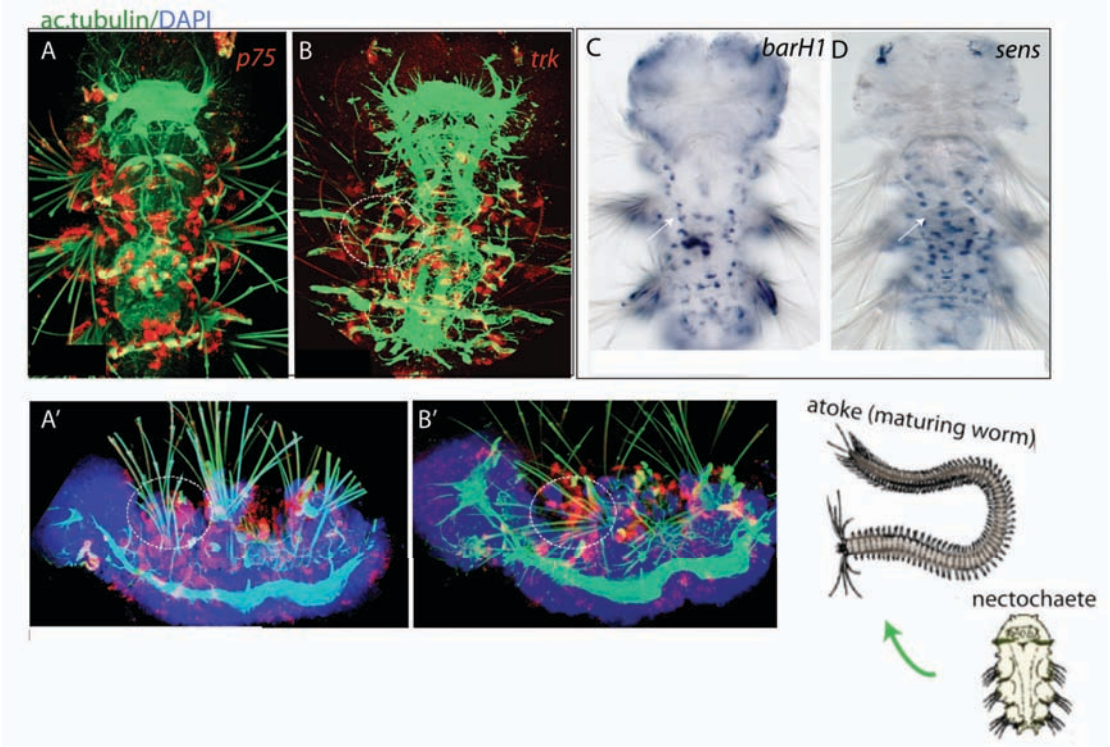


Figure 58. Expression of *pdu*Trk and *pdu*P75 in the juvenile after metamorphosis. A-B': dorsal (A-B) and lateral (A',B') view of Z-projection of the WMISH for *p75* (A,A') and *trk* (B,B'). **C,D:** dorsal view. Bright field picture of the dorsal expression of *barHI* (C) and *sensless* (D).

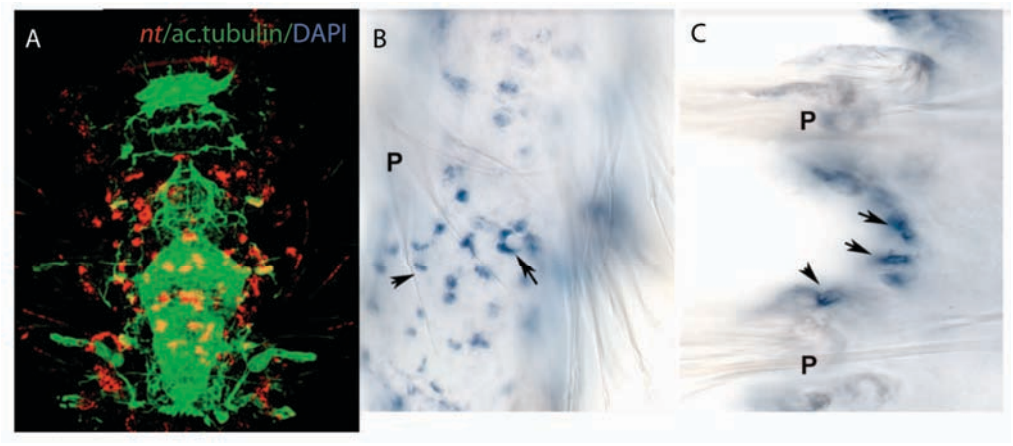


Figure 59. Expression of *pdu*NT in the juvenile after metamorphosis. A : dorsal view. Z-projection of the WMISH for Nt. **B:** dorsal view. Bright field picture, close up on the dorsal *Nt* + cells (black arrow). **C:** ventral view. Close up of the *Nt* + lateral cells (black arrows) between the parapodia (P)

VI.5 Production of a polyclonal antibody directed against *Platynereis* Neurotrophin (detailed protocol in Materials and methods)

Next, I set out to produce a polyclonal antibody to detect the presence of the neurotrophin ligand in the nervous system because the WMISH showed the cells expressing the neurotrophin mRNA, but these are not necessarily all the cells where neurotrophin is actinging as ligand. In vertebrates neurotrophin is secreted and then binds Trk and p75 on growing axons of the neurons.

VI.5.1 Expression of pro-neurotrophin (proNT) and mature neurotrophin (mNT) in *E.coli*

In collaboration with the Protein expression and purification core facility at EMBL I tried to express the proNT and the mNT in expression systems based on *E.coli* and *Baculovirus*. It was experimentally determined that the *E.coli* expression system, based on the formation of the fusion protein between Sumo and my protein, was the most efficient. Therefore the protein was expressed in *E.coli* and then was purified on Nickel (affinity columns (fig.60).

Originally these experiments were supposed to lead also to the native recombinant proteins, to be used to induce the neurotrophin signaling *in vivo* and in *vitro*. Therefore the full-length version of the proteins was produced. The protein was repeatedly confined to the inclusion bodies of *E.coli*, therefore resulting in misfolded proteins. Several attempts were made to refold the protein, and although some gave promising results on small scale, the large-scale experiment was difficult to achieve.

The protein product was then used for antibody production.

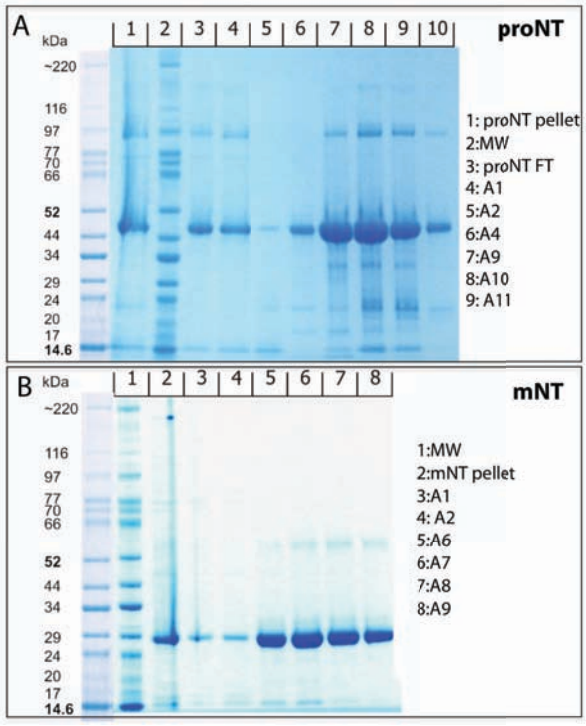


Figure 60. Expression of proNT and mNT proteins in *E.coli*.A: Acrylammide gel stained with Comassi Blue. Recombinant proNT before (lane1) and after purification on Nichel nickel column. Different fractions collected are indicated with A. FT: flow through. The expected size of the proNT fused to the tag (sumo) is 49 kDa. **B:** Recombinant mNT before (lane2) and after purification on column. Different fractions collected are indicated with A. FT: flow through. The expected size of the mNT fused to the tag (sumo) is 29 kDa.

VI.5.2 Production and purification of the polyclonal antibody

The purified pro-NT and the m-NT were then used to immunize 4 rabbits in total (two rabbits per each form of the protein : rabbit # 75 and #32 immunized with the proNT and rabbit #86 and #74 immunized with the mNT). The serum after each bleed (three in total) was tested via immunofluorescence and western blot, using as control the serum of each rabbit prior to immunization (preIm in fig.61). The serum 75III (III bleed from rabbit 75) was then chosen because it recognized a band of around 35kDa on protein extracts obtained from *Platynereis* larvae (lane 5 in fig.61). The same was done for the serum 86III.

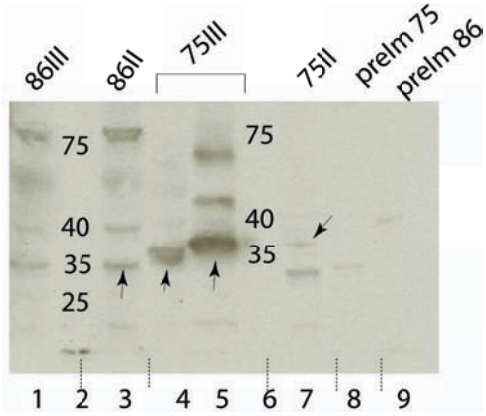


Figure 61. Analysis of the sera containing polyclonal antibodies:[7] The different bleeds were tested via immunoblot. Lane 1,3,5,7,8,9 contain protein trunk extract of *Platynereis* larvae. Lane 4 contains 100 ng of the

[7] The dot lines in the bottom of the figure indicate where the PVDF membrane was cut before incubation with the I antibodies. The membrane was then recomposed based on the protein marker (visible on the membrane). Doing this the lanes are not 100% on the same level in the final picture, this is visible with the marker in lane 2 and 6. Lane 3 was also lower compare to lane 4. Different size of the protein in lane 4 and 5 could be due to known protein modification (such as glycosilation) in the protein from the extract of the larvae (lane 5).

purified proNT used for the immunization of the rabbit #75. Lane 2 and 6 contains the protein marker. The sera tested are indicated above the picture. *preIm:* pre-immunization serum. Compared to the II bleed (lane 7) an enrichment of the reactivity of the serum 75 against the protein around 34KDa is observed in the III bleed(lane 5).

VI. 5.3 Immunofluorescence and detection of *Platynereis* NT protein in the juvenile

Both purified antibodies (75III and 86III) were tested via immunofluorecence. In general, normal protocols for immunohistochemistry fail to detect the endogenous neurotrophins in vertebrates. This happens for several reasons: the immunogenic epitopes of the neurotrophins might be masked due to its association with the receptors, or the protein might be expressed at very low levels. Also over-fixation might lead to a similar result.
I tried different protocols to achieve the detection of the neurotrophin in *Platynereis* , and finally I have optimized a protocol using the Zamboni fixative (Zamboni and Stefanini 2012), that gave good results. This fixative is milder compared to the classical ones (contains less then 2% of PFA), and it has been shown to be extremely good in keeping the neuronal morphology as well as the 3D conformation of the epitopes (see Materials and methods).
With this protocol I was able to detect the endogenous neurotrophin in *Platynereis* using the purified 75III and 86III antibody (fig.62). Serum 86III gave similar results and recapitulated better the mRNA expression seen in the early juvenile worm (fig.57).
The protein is detected in the neuronal midline (fig.62C, white arrow), which might correspond to an attractive point for commissural neurons as *in Drosophila* (where a neurotrophin-like is also expressed(Zhu et al. 2008). The protein is also detected in the ectoderm and muscle cells (yellow arrow). Accumulation of the protein at the nerve endings (fig.62D) is also visible; consistent with the hypothesis that neurotrophin might guide nerves during their development.

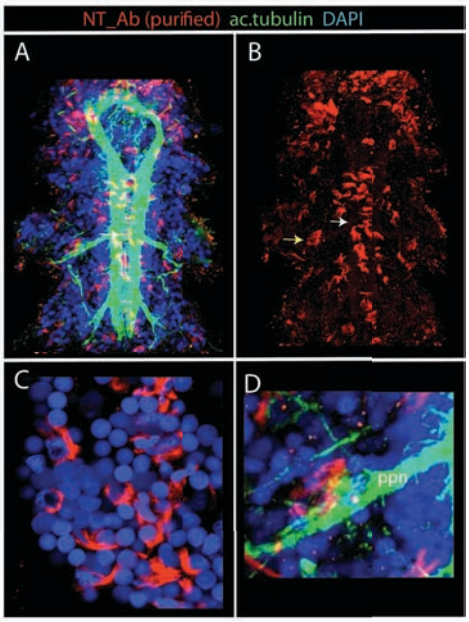


Figure 62. NT protein detection in the juvenile. A: Z-projection of a confocal scan of the immunofluorecence with the purified antibody 86III. B: only the channel detecting the neurotrophin is shown. **C:** close up of the neuronal midline (white arrow) where accumulation of the neurotrophin protein is found. **D:** close up showing accumulation of neurotrophin at of the peripheral nerve endings of the II left parapodium, ppn: peripheral parapodial nerve.

VI. 6 Towards determining the function of the annelid neurotrophic signaling

Based on these results that uncover some aspects of the neurotrophic signaling in *Platynereis* I started to develop some tools to be able to interfere with *Platynereis* neurotrophic signaling and understand its function. These experiments are still on going and this might represent one of the big task of my future, nevertheless some preliminary work was achieved and I will briefly summarize it.

***-In vitro:* is *Platynereis* neurotrophic signaling able to activate canonical survival pathways?**

In order understand the activity of *Platynereis* neurotrophin signaling I need to test that the ligand is able to bind to the receptor and co-receptor and mediates an intracellular response, involving phosphorylation of Akt (see paragraph IV.3.2 of the introduction).
These kinds of experiments are done in standard mammalian cell cultures in vitro. They have been performed for the amphioxus Trk and the Lymnea Trk (Benito-Gutiérrez et al. 2005),(Beck et al. 2003), but in both cases a chimeric receptor was built, so that the extracellular portion was a vertebrate one and was responding to the vertebrate ligands. These experiments have proven only that the intracellular TK domains of amphioxus and Lymnea Trk are able to activate the AKT signaling.

I decided to create expression constructs for the endogenous *Platynereis* Trk, p75 and neurotrophin. These constructs encode for a fusion proteins designed as in fig. 63, this way the expression of the proteins in cell culture will be followed performing immunoblots against the tags.
In all cases the sequence for a vertebrate signaling peptide was introduced at the 5′ to be able to secrete the protein in the mammalian cells.
After transfecting the cell culture with the different combinations of receptor, coreceptor, and ligand expressing constructs protein extracts from the cells will be collected and will be analysed via immunoblot.

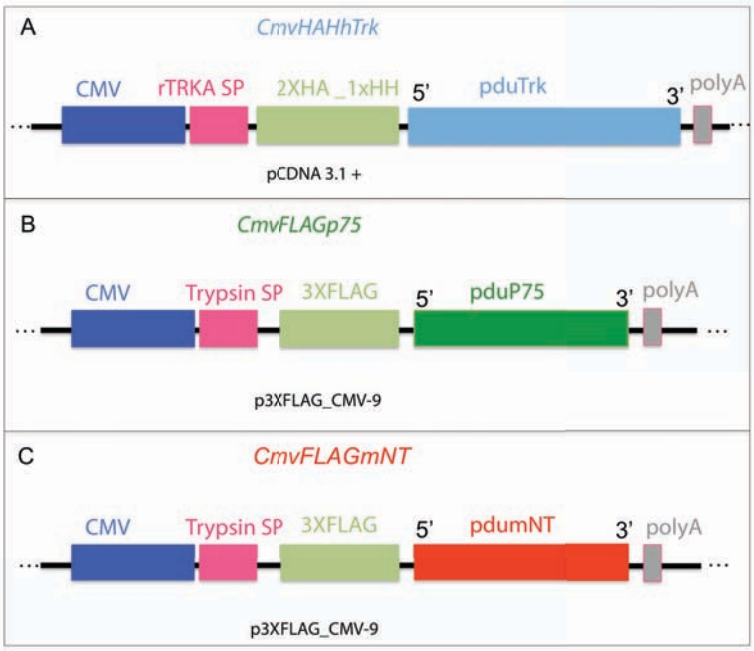


Fig.63. Mammalian expression constructs for pduTrk, p75 and NT

A: CmvHAHhTrk construct. The most important features are indicated. CMV: cytomegalovirus promoter. rTRKA SP: signal peptide coding sequence (cs) of TrkA (courtesy of Dr Elia Benito Gutierrez). 2XHA_1XHH: 2x hemoagglutining tag, 1x histidin tag. polyA: human beta-globin polyadenilation signal. **B,C:** CmvFLAGp75 construct (B) and CmVFlagmNT (C). Trypsin SP: signal peptide of Trypsin. The original vectors are indicated in the bottom of each panel, p3x3XFLAG_CMV-9 was coutesy of Dr. Flavia D'Alessio, pDCNA 3.1 + was coutesy of Dr. Jan Medenbach.

-*In vivo* approaches to investigate axon pathfinding:

In order to test the function of the neurotrophin signaling in vivo different approaches have been started. On one side I generated a construct containing the cds of the mature form of the neurotrophin (fig.64). The original plasmid was courtesy of Dr. Heather Marlow and contains a T3 polymerase-binding site in order to perform in vitro transcription (Marlow, Roettinger, Boekhout, & Martindale, 2012; Roure et al., 2007). After producing the construct I have generated the mRNA.

I will inject the mRNA into the zygote, grow the larvae and then assess the formation of axonal scaffold.

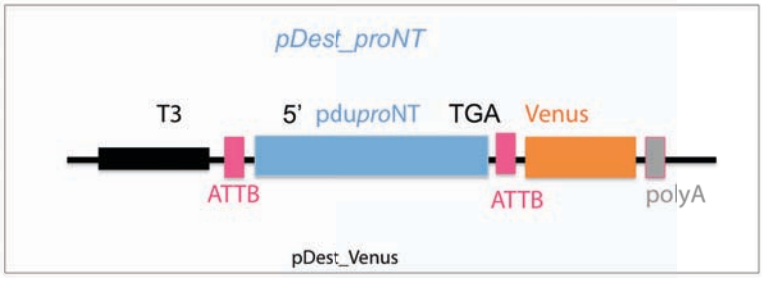


Fig.64. pDest_pro-NT construct. The most important features are indicated. T3 : binding site for polymerase T3. ATTB: gateway homologous recombination sites B created after LR reaction between entry clone and destination vector. Venus: Venus coding sequence. The original vector is indicated in the bottom of each panel, it contains a pRN3 backbone (Marlow, Roettinger, Boekhout, & Martindale, 2012; Roure et al., 2007), it was courtesy of Dr. Heather Marlow.

- If the neurotrophin signaling is involved in axon pathfinding then the *pdu*Trk receptor should localize at the nerve endings. To be able to visualize the protein I decided to produce also Trk antibody. To do this I have isolated an immunogenic peptide of Trk receptor that should cross-react also with other invertebaate Trk (on going).

- Once the cell types expressing the *trk* receptor and the full length sequence of Trk have been determined, in order to test further the function of the neurotrophic signaling in *Platynereis* I have also generated an ATG directed morpholinos for pduTrk.
Because *Platynereis* genes have an high SNP (single nucleotide polymorphism) rate (as observed by Dr. Maria Antonietta Tosches and Oleg Simakov) I first performed a SNP analysis of the upstream genomic region of Trk (fig.65), using genomic DNA from 10 individuals. Therefore I could design a morpholino on a non-polymorphic area indicated in fig.64. This will be injected into the zygote, and axon pathfinding will be examined.

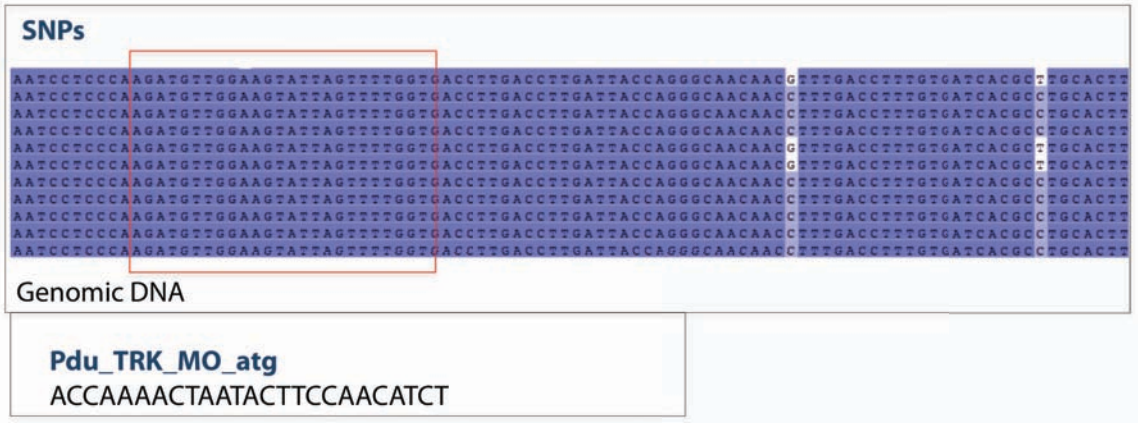


Fig. 65 SNP analysis of the genomic region surrounding the start codon of the ATG. DNA sequence in each row was obtained by different individuals. The red box indicates the region where the morpholino was designed.

VII. Search for a cnidarian neurotrophic signaling

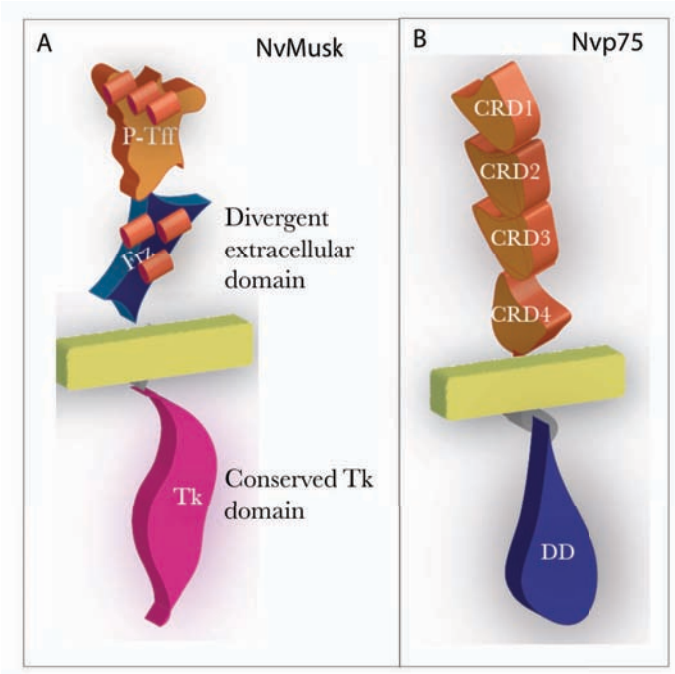
Once determined that the neurotrophic signaling is most likely a Bilateria synapomorphy, I then asked whether it is an invention of the Bilateria.

To determine this I searched for orthologs in the genome browser of *Nematostella vectensis*: a basal Cnidaria (fig.1).

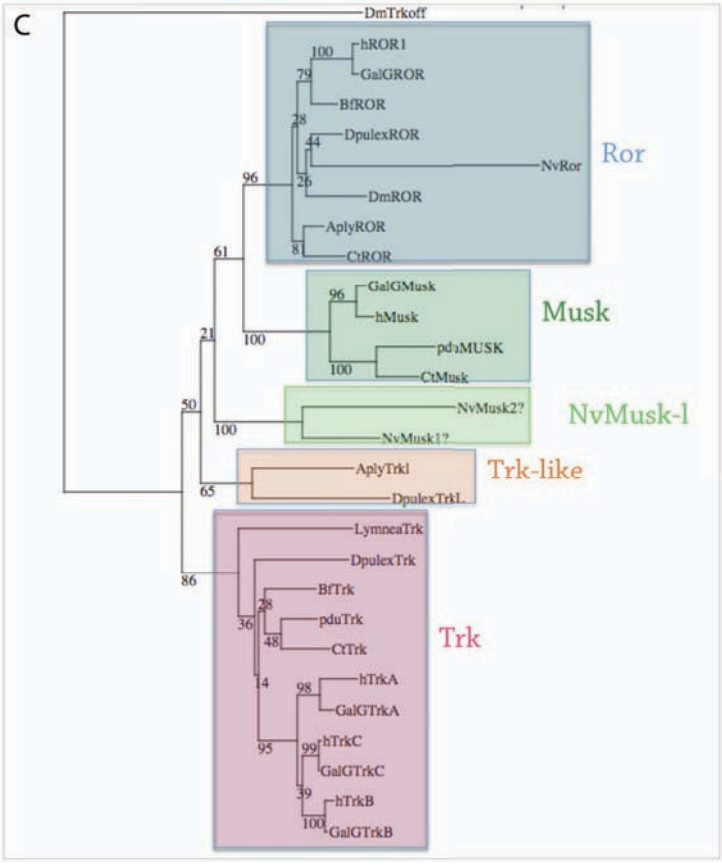
No canonical Trk receptor was found. Nevertheless I found a conserved TK kinase on scaffold 95. Next, I performed a race PCR on cDNA race library (kindly provided by Dr.Heather Marlow), and obtained the full length of the receptor and analyzed the domain composition (fig.66A). This receptor is not an ortholog of Trk, but a MuSK-l receptor. The extracellular portion is composed of domains present in other members of the Trk superfamily (see introduction, paragraph IV.3, fig.23). I also found another *Nematostella* MuSK-l candidate.

Next, I performed phylogenetic analysis [8] (fig.66C). The analysis suggests that originally a MuSK-Ror receptor gave rise to the *Nematostella* MuSK-l receptors, the MuSK and the Ror receptors (Sossin 2006).
Interesting I also found annelid specific MuSK receptors (in *Platynereis dumerilii* and *Capitella teleta*). From the phylogenetic analysis it is clear that the annelid MuSKs receptors belongs the MuSK family.
These observations indicate that the split between Ror, Trk, and MuSK occurred at the base of bilaterians (see Discussion).

Consistently with the absence of a canonical *Cnidaria* Trk receptor, I was not able to find a neurotrophin ortholog. I instead found a conserved p75 in *Nematostella* which is consistent wth the observation that p75 is the less divergent member of the neurotrophic signaling in invertebrates and therefore may represent the original component of the signaling present at the dawn of Eumetazoa. The function of these molecules need to be determined



[8] This was done with the help of Dr.Tomas Larsson


C
DmTrkoff
hROR1
GalGROR
BfROR
DpulexROR
NvRor
DmROR
AplyROR
CtROR
Ror
GalGMusk
hMusk
pduMUSK
CtMusk
Musk
NvMusk2?
NvMusk1?
NvMusk-l
AplyTrkl
DpulexTrkL
Trk-like
LymneaTrk
DpulexTrk
BfTrk
pduTrk
CtTrk
hTrkA
GalGTrkA
hTrkC
GalGTrkC
hTrkB
GalGTrkB
Trk

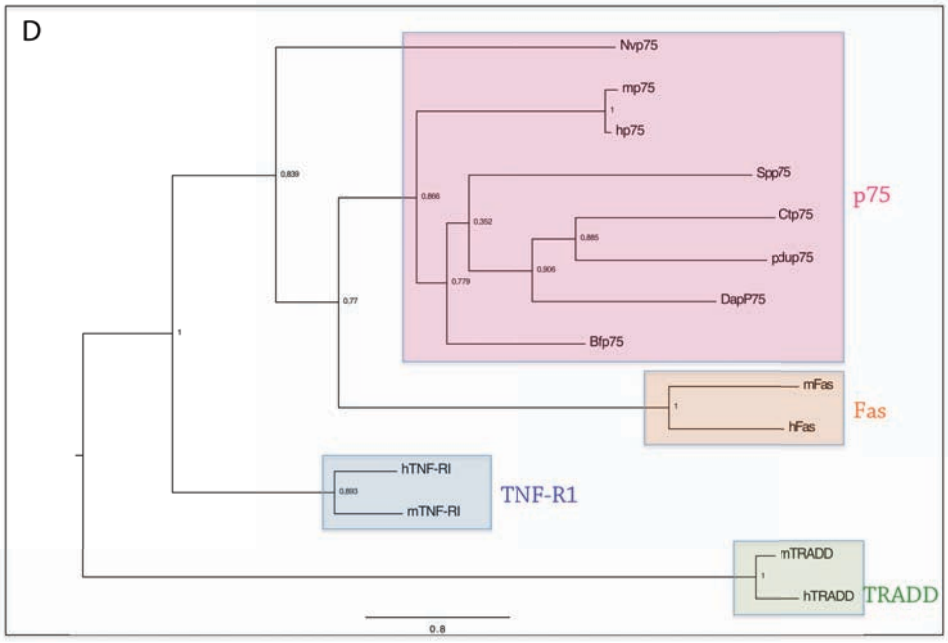


Figure 66. Schematic representation of the domain composing *Nv*MuSK-l receptor, *Nv*p75 and phylogenetic analysis of the full-length proteins. A: Tk: tyrosine kinase domain, Kr: kringle domain, P-Tff: trefoil domain. **B:** see fig.49B.**C:** phylogenetic analysis of *Nematostella* MuSK-l in comparison to other vertebrate and invertebrate members of the RTK superfamily (Ror and MuSK). **D:** phylogenetic analysis of *Nematostella* p75 in comparison to other vertebrate and invertebrate members of the TNF superfamily. The alignments were obtained with Muscle. Gaps were trimmed with Gblocks. The tree was performed using PhyML by Dr. Tomas Larsoon, 100 bootstrap replicates, 8 gamma rates and the LG substitution model. For the species included see fig. Nv (*Nematostella victensis*).

VII.1 Expression pattern of *Nv*MuSK-l and *Nv*p75[9]

Given the absence of the canonical components of the neurotrophin signaling cascade in *Nematostella*, it is difficult to speculate that p75 (the only conserved component) was involved in the signaling initiated by *Nv*MuSK-l. Moreover the ligand of this receptor is not known.

Therefore, in order to gain more information about the signaling a first step was to investigate the expression pattern of *Nv*MuSK-l and *Nv*p75.

Nematostella larvae have a rather simple body plan and nervous system, with not apparent centralization of the nervous system, harbouring neurons in both endoderm and ectoderm. Accordingly to a role in the nervous system, I found that *MuSK-l* is expressed in the pharyngeal endoderm, in the tentacles and in the directive mesenteries, where neurons are present. Also ectodermal cells express *MuSK-l* (white arrow, fig. 67).

Surprisingly *p75* is expressed specifically in the directive mesenteries, sharing therefore the domain with *MuSK-l* (fig.68)

[9] The expression patterned has been analyzed with the help of Dr. Heather Marlow

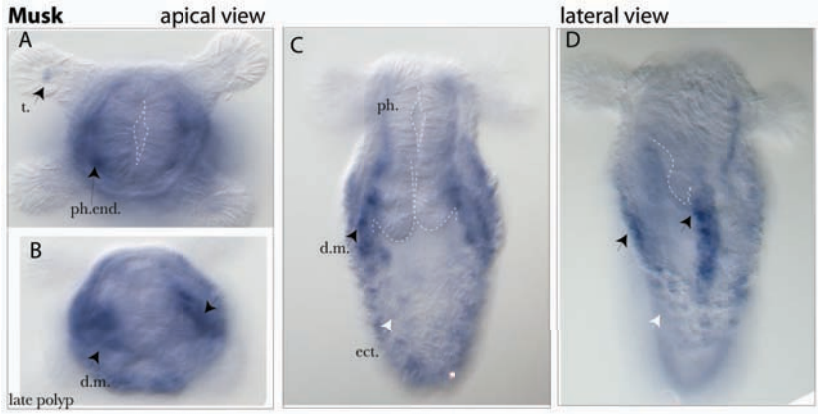


Figure 67. Expression of *Nv*MuSK-l in the late polyp of *Nematostella*. A-B: apical views. Ph.end: pharyngeal endoderm. D.m.: directive mesenteries. Ect: ectoderm E: schematic drawing of *Nematostella vectensis* polyps. Relevant features are indicated

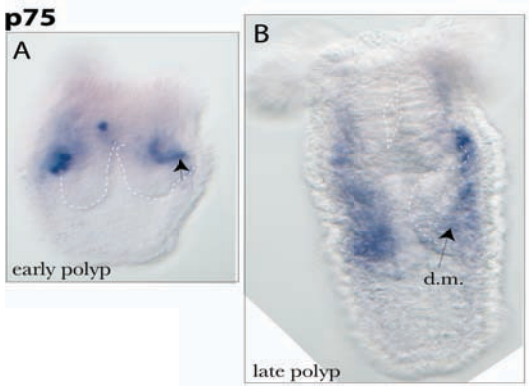


Figure 68. Expression of *Nv*p75 in the early (A) and late (B) polyp of *Nematostella*. D.m.: directive mesenteries

In vertebrates the Ror, MuSK and Trk receptors are expressed in many different neurons and at the neuromuscular junctions (MuSK) and have specialized trophic functions in the nervous system that are mediated by specific ligands (Zhang et al. 2004; Jing et al. 2010) (Eric J Huang and Reichardt 2003; Yoda, Oishi, and Minami 2003).
To be able to compare with vertebrates, a more detailed analysis is required in the future to establish the signaling pathways in *Nematostella* and *Platynereis* . Nevertheless, based on these preliminary results we can speculate that a simple trophic signaling was already present at the base of *Eumetazoa* when the first neuronal circuits arose, and was most likely contributed also by a Trk/MuSK/Ror ancestral receptor.

In summary, I found that *Platynereis* possesses canonical molecules belonging to the neurotrophic signaling; conversely to the vertebrates, the marine worm possesses only one receptor Trk, one co-receptor p75 and one putative ligand molecule : the neurotrophin. Each of these molecules is composed of vertebrate-like canonical domains. These data demonstrate that the presence of molecules of the neurotrophic signaling is not a vertebrate or chordate innovation (as previously postulated).
The receptor Trk is expressed in almost all the neurons of the nervous system, the ligand neurotrophin is expressed in both in the nervous system and in the muscle. This indicates that they might be involved in the neuronal development and in processes of axon

navigation, as in vertebrates. This is only an hypothesis and needs to be investigated further; for this purpose different tools for in vivo and in vitro experiments have been established.

The data presented in this section also show that a similar set of neurotrophin molecules is missing in Cnidaria, therefore it is likely a bilaterian invention. In *Nematostella vectensis* (a member of the Cnidaria) MuSK-like receptors (belonging to the same RTK superfamily as Trk receptors) and canonical p75 are found, where only the intracellular domain is a conserved. *Platynereis* also possesses canonical Musk receptors. It is likely that the canonical Trk receptor evolved only in Bilateria from an ancestral molecule that gave rise also to Musk receptors, Ror receptors and the *Nematostella* Musk-like receptors.

DISCUSSION

1. What did the evolutionary precursors of the neural crest look like?

With the work of my PhD I aimed to shed light on the evolution of the neural crest; one of the most plastic cell population in development, indeed neural crest cells are neuroepithelial cells, then they undergo ectodermal to mesenchymal transitions and migrate to give rise to many different types of derivatives: from neurons to cartilage. This work contributed to put forward plausible scenarios for the emergence of the neural crest, but gave rise also to many new questions, which remain to be answered. Here I will discuss the results, together with the future challenges in the field.

I. What is the correct approach to search for neural crest prototypes in invertebrates?

It is accepted that the neural crest as such represents a vertebrate innovation. Nevertheless, new cell types and structures do not arise *de novo*. It is more plausible to imagine that new cell types originate from precursors which likely re-elaborated their genetic components during the course of evolution. These evolutionary precursors might acquire novel gene regulatory network modules, deploy new signaling pathways (or change existing ones). Together with protein neo/sub-functionalization after gene duplication, these changes might be involved in conferring new functions to the cells.
Nevertheless, some molecular and cellular components (the most ancestral ones) should still be present in the neural crest precursors outside vertebrates, and might be useful to uncover latent neural crest evolutionary precursors in invertebrates.

Bearing this in mind, evolutionary biologists have searched for neural crest homologs in protochordates such as the lancelet amphioxus. This approach was based on the systematic analysis of the expression pattern of the genes involved in the neural crest gene regulatory network, inferred almost entirely on data from chicken and mouse.
In my opinion, although this approach has offered interesting outcomes, it has a big limitation.
The neural crest is an extremely plastic cell population; this implies that it evolved very fast and easily acquired new features. Therefore, it can be misleading using only the gene regulatory network known from birds and mammals for comparison purposes. Conversely, the cellular and molecular features of the anamniote and of lamprey (a basal vertebrate, (Sauka-Spengler and Bronner-Fraser 2008a) Nikitina, Tong, and Bronner 2011), neural crest are likely the most ancestral ones, and should be taken in account for an accurate comparison of the genes involved in neural crest development between vertebrates and invertebrates.

II. Rohon Beard-like cells are likely among the first cell types to originate from the evolutionary precursors of the neural crest

II.1 The annelid peripheral sensory neurons and supporting cells derive from lateral precursors expressing Rohon Beard/neural crest genes.

In this study I undertook a comparative approach to shed light on the evolution of the neural crest. Indeed, I also took into account the molecular development and the cell fate of the anamniote and lamprey neural crest , that likely reflects a more ancestral scenario in vertebrates.

Previous work in *Platynereis* has demonstrated that the mediolateral patterning of the nervous system predated the evolution of chordates (Denes et.al 2007). This discovery has revealed also that the conservation of the precise grid of transcription factors is then translated in a deep conservation of cell types, for example the *hb9+* motoneurons originating from the ventral *pax6* domain.

In my study I found new evidences corroborating the idea that the lateral most portion of the *Platynereis* neuroectoderm (characterized by *pax3/7/ dll/ msx+*) is homologous to the neural plate border of anamiotes, an idea put forward in Denes et al., 2007.
Indeed, as in anamniotes, gene expression of crucial neural plate border and neural crest genes is observed in the annelid lateral neuroectoderm very early in development and it comprises the ortologs of many genes shared between Rohon Beard cells and neural crest (Rossi, Kaji, and Artinger 2009, table in fig.68).
The annelid expression data are highly comparable to the expression data of the neural plate border in lampreys and in anamniote vertebrates, such as fishes and frogs. Therefore, conversely to what was previously postulated based on data from amphioxus, I speculate that a specialized neural border-like domain expressing neural crest specific genes is a shared feature of bilaterians.

-*Platynereis* Prdm1a:

Recently it was demonstrated that Prdm1a is a master regulator of the neural crest and Rohon Beard cells in anamniote vertebrates, acting in the upstream level of the gene regulatory network shared between these two cell types (Rossi, Kaji, and Artinger 2009, Olesnicky, Hernandez-Lagunas, and Artinger 2010,table in fig.69).

Platynereis prdm1 is also expressed in the lateral neuroectoderm. It is specifically expressed in the first cells differentiating from this territory: the precursors of the multicilated cells of the ciliary bands (fig.26,27). These cells are known to be the first locomotory apparatus of the annelids, as well as of other ciliated marine larvae (both in protostomes and deuterostomes (Jékely 2011), I found evidences that they have also a sensory modality. Indeed they express *atonal*, a terminal differentiation marker for sensory neurons, as well as the sensory effector gene *trpV*1 (Mutai and Heller 2012; Colbert, Smith, and Bargmann 1997; Bargmann 2006), already two days after development. They most likely represent the first sensory neurons of the annelid peripheral sensory system.

Interestingly *Platynereis prdm1* is immediately downregulated as soon as the cells expose cilia onto the surface, that is when they differentiate. This is very similar to the situation in zebrafish, where *prdm1a* is also downregulated as soon as the Rohon Beard cells are specified (Roy and Ng 2004). The ortholog of *prdm1-a* is also expressed in the peripheral sensory organs of *Drosophila* (Ng, Yu, and Roy 2006) and the neural plate border cells in lamprey(Nikitina, Tong, and Bronner 2011). Prdm1 is instead required only during brachial arches formation in amniote vertebrates (Vincent et al. 2005, neural crest derived structures). The annelid data, compared to the vertebrate data, strengthen the idea that *prdm1* was master regulator gene acting at the lateral neuroectoderm already at the base of bilaterians.

-**The Wnt signaling**

Despite the conservation of the molecular fingerprint, belonging to the upstream core of the GRN (Dickinson et al. 1995, Dorsky, Moon, and Raible 1998, Deardorff et al. 2001, table in fig.68, Introduction paragraph III.3.1), I found that lateral neuroectoderm territory is expanded in response to canonical Wnt signaling, as for the vertebrate dorsal neuroectoderm (fig.33, Lallemend and Ernfors 2012). This suggests that canonical Wnt signaling was already required for the establishment of the lateral (dorsal) neuroectodermal identity before vertebrate evolution, and represent, together with the upstream core of the GRN, a stable module conserved during evolution.

-Serial waves of sensory neurogenesis

In *Platynereis* other neurogenic precursors (*ngn+*) arising at the neural plate border differentiate as sensory neurons (expressing *brn3, islet, runx and trpV* orthologs in a temporal manner, fig.33, As in vertebrates a second wave of neurogenesis produces even more sensory neurons and at the end the juvenile is equipped with sensory ganglia containing primary sensory neurons, which harbours sensory cilia outside the cuticle (Psn : peripheral sensory neurons in fig.69, 70).

-Specific neural crest genes (snail, soxE, colA, neogenin) in *Platynereis*

Surprisingly some of the genes specific only for the neural crest lineage and not expressed in the Rohon Beard cells are expressed in *Platynereis* lateral neuroectoderm such as *snail, soxE, neogenin* and *colA* (fig.29). These genes are all implicated in the delamination and migration of the neural crest (Khudyakov and Bronner-Fraser 2009), (Sauka-Spengler and Bronner-Fraser 2008b), a feature missing in Rohon Beard cells. Although using nuclei and membrane labeling it was possible to follow single migrating neuroectodermal cells in *Platynereis,* I could not observe any migratory behaviour in the lateral territory. This need to be re-evaluated with different live imaging approaches that allow to follow the behaviour of the *soxE/ snail+* cell population in the embryo. In absence of migratory behaviour, it is plausible to speculate that the neural crest specifier genes are involved in a different function in *Platynereis*. For instance, as for the *Platynereis* ortholog, in Xenopus and lamprey *snail* is also expressed early in neural plate border, far before the specification of the neural crest (Essex, Mayor, and Sargent 1993). The early snail function is not known in these animals, but it was proposed that snail might be involved in the changes of the cellular cytoskeleton occurring during neurulation, a process that is different in anamniotes compared to chickens, where accordingly there is no early neural plate border expression of snail. A similar role might be envisaged for *Platynereis snail*, although this claim needs functional data.

In *Platynereis* neuroectodermal *colA* expression starts later at 48h (fig.29) and then is found in cells around the peripheral axons (fig.39). It is possible that these are supporting cells that surround the peripheral axons and produce fibrillar glia. The presence of such cell types has been postulated in annelids and molluscs, but it is still unclear which is the developmental origin of these cells and which is their (evolutionary) relation to the vertebrate Schwann cells (Vagnetti and Farnesi 1978), (Baskin 1971), (Schweigreiter et al. 2006; Roots 2008). Interestingly, vertebrate Schwann cells derive from the same early neural crest cells that gives rise to sensory neurons (fig.14). Therefore they might share the precursor with the sensory neuron and this might also be the case in *Platynereis.*

	t-NC 	RBs 	Platynereis trunk lateral cells
INDUCTIVE SIGNALS	Wnt- Bmp	Wnt- Bmp	Wnt Bmp
NEURAL PLATE BORDER	pax3/7,msx, zic, dll,prdm1a, ap2a, snail *(Xenopus)*	pax3/7 zic, dll,prdm1a	pax3/7, zic, dll,prdm1a, ap2a
SPECIFIERS	soxE, snail, foxD3, ld, ap2a,c-myc, twist (cranial) **SPECIFICATION PROLIFERATION**	dll, islet, ngn2, ap2a	soxE, snail2 ap2a,ngn2, dll, ld
EFFECTORS	soxE, foxD3, colA, cadh7, npn, neog, twist (cranial) **MIGRATION**		soxE,colA, neog
DIFFERENTIATION	ngn2, islet, brn3, trkA/B/C, p75, trpV, runx, Vglut	ngn2, islet, brn3, trkC, p75, trpV, runx, mir 183, Vglut	ngn2, islet, brn3, p75,Trk?, mir 183, trpV, runx, Vglut

Figure 69. Table showing the molecular comparison between t-NCc, RBc and the annelid lateral trunk sensory neurons. T-NCc and RBc differ at the level of the specifiers. RBc do not migrate and accordingly do not express genes such as *sox10 (*involved in keeping the multipotency of the neural crest and in initiating the ectodermal to mesenchimal transition) or *foxD3* (involved in the delamination and migration). For mir183 see Christodoulou et al., 2010.

II.1.1 Developmental fate of the neural plate border cells at the base of Bilateria

Based on this study and on comparison with other animals (see Introduction, paragraph III.6), I propose that:
1) a specialized neural plate territory was already in place at the base of bilaterians, in agreement with the idea that the nervous system was already centralized.
2) Canonical Wnt signaling was already promoting this lateral territory identity.
3) A prominent part of the genes regulating neural crest development was already acting in this territory with genes such as *soxE* and *snail* likely to be involved in a more basal function (see previous paragraph).
4) Primary sensory neurons and supporting cells were among the first cell types arising in this position in the trunk and constituted part of the peripheral nervous system of the swimming larva.

These annelid data corroborate the idea that the pre-commitment of the vertebrate trunk neural crest cells to the sensory lineage during development might recapitulate their ancestral identity, which is retained, as already suggested, in anamniote larvae in the form of Rohon Beard cells (Rossi, Kaji, and Artinger 2009) (Northcutt and Gans 1983) (fig.70).
As 'vestigial' cell types, zebrafish Rohon Beard cells disappear in development when neural crest differentiate into dorsal root ganglia sensory neurons (Lamborghini 1987) (Kollros and Bovbjerg 1997) (Williams et al. 2000), which take over the same position in the body plan and the same function.

Several data support the scenario that I propose: the same ontological origin of the t-NCc and RBc in anamniotes (Lamborghini 1980), the shared gene regulatory network between t-NCc, RBc (Rossi, Kaji, and Artinger 2009) and annelid Psn (peripheral sensory neurons),

the presence of Rohon Beard-like cells with a partial conserved GRN predating the formation of neural crest at the neural plate border in all the phyla examined so far: tunicates dorsal sensory neurons (DESN), amphioxus Retzius cells (RZc), and even insects sensory organs to a certain extend (table and drawing in fig.70, see also Introduction, paragraph III.6).

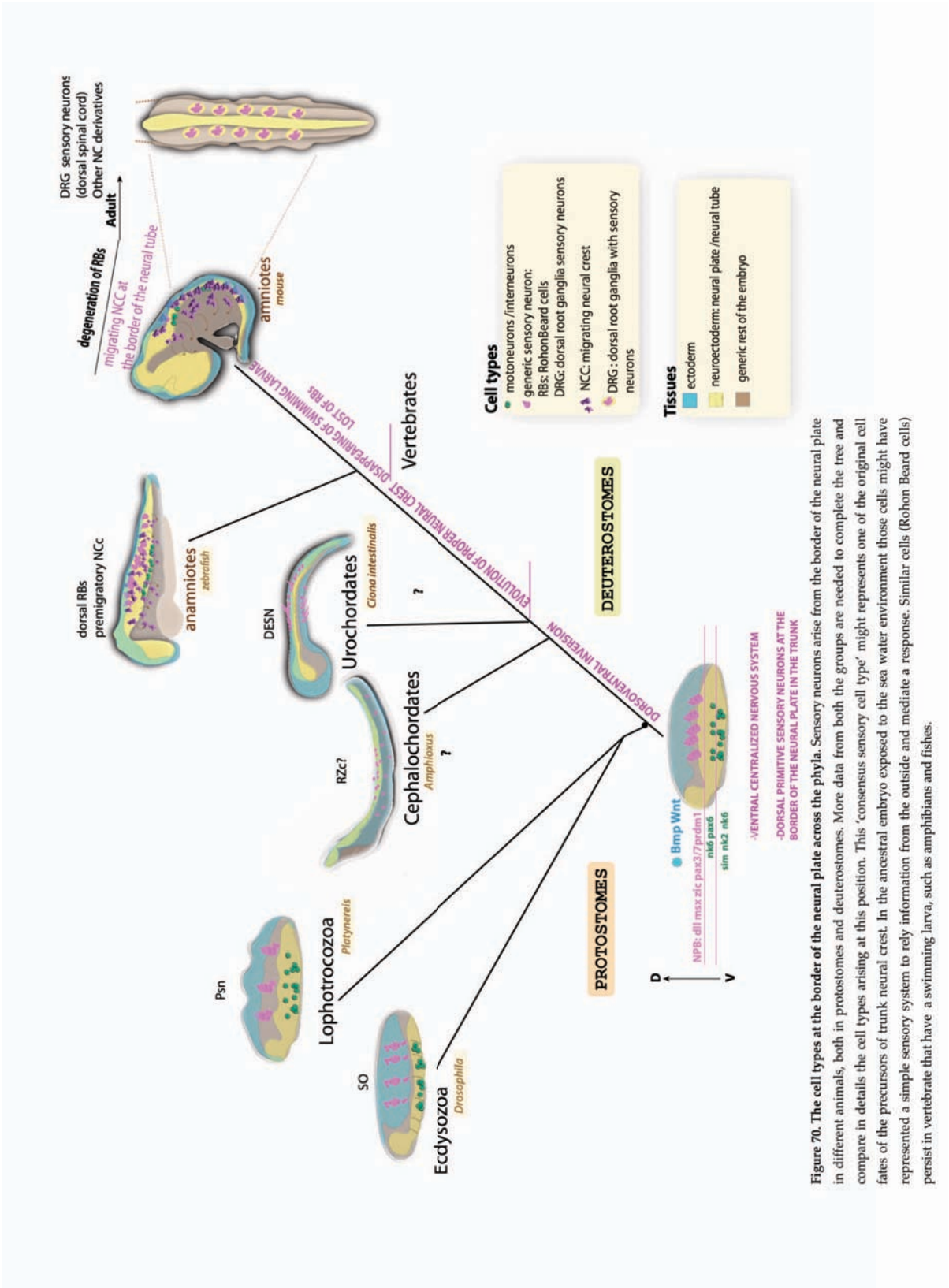


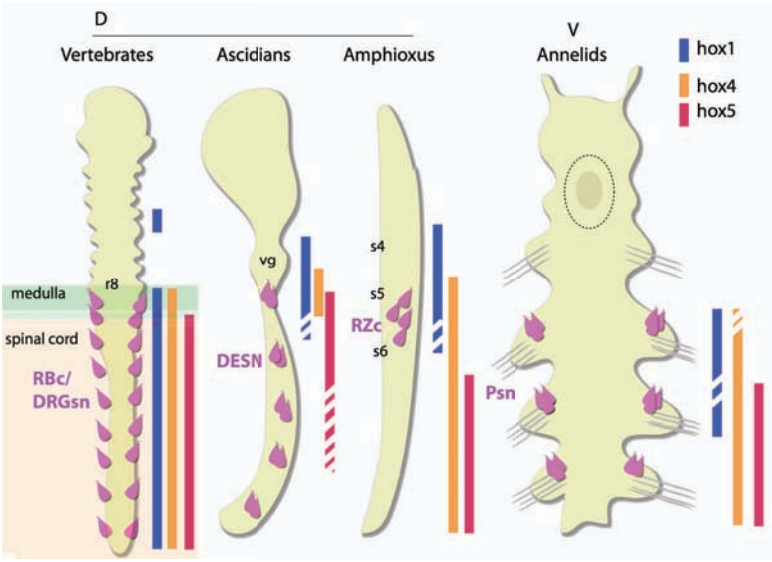
Figure 70. The cell types at the border of the neural plate across the phyla. Sensory neurons arise from the border of the neural plate in different animals, both in protostomes and deuterostomes. More data from both the groups are needed to complete the tree and compare in details the cell types arising at this position. This 'consensus sensory cell type' might represents one of the original cell fates of the precursors of trunk neural crest. In the ancestral embryo exposed to the sea water environment those cells might have represented a simple sensory system to rely information from the outside and mediate a response. Similar cells (Rohon Beard cells) persist in vertebrate that have a swimming larva, such as amphibians and fishes.

I found that peripheral sensory ganglia are present in *Platynereis* juvenile only starting from the second segment onwards (fig. 39, 71). This corresponds to the segment where *hox1* and *hox4* are expressed (Kulakova et al. 2007) .

The *hox1*/*hox4* + II segment might represent a region homologous to the caudal hindbrain, comprising the medulla oblongata (Pourquié 2009) , where the first RBc arise in vertebrates (fig.70).

In agreement with this, work done by Mette Handberg-Thorsager shows that *hox4* continues to be expressed in the posterior segments, also in the newly formed ones, together with *hox5*. Accordingly, sensory ganglia continue to develop in the newly formed segments, where *hox4* and *hox5* are expressed (fig.71). This is very similar to the scenario in the vertebrate spinal cord territory, where *hox4*/*hox5* are expressed.

Sensory neurons, with part of the conserved dorsal gene regulatory network shown so far (table in fig.71), develop both in protostomes and deuterostomes, along similar A-P coordinates. This suggests that such a *'hox code'* together with a D-V code instructed the development of trunk lateral sensory neurons at the dawn of bilaterian, more than 500 MYA.



	Deuterostomes			Protostomes	
	V	As	Amp	An	I
dll	✔	✔	✔?	✔	✔
msx	✔	✔	✔?	✔	✔
pax3/7	✔	✔	✔?	✔	✔?
snail	✔	✔	✔?	✔	✔
prdm1a	✔	?	?	✔	✔
soxE	✔	?	X	✔	X
colA	✔	?	?	✔	?
brn3	✔	✔	?	✔	?
ngn	✔	?	✔	✔	✔
islet	✔	?	✔	✔	?
trpV	✔	?	?	✔	✔
vGlut	✔	✔	✔	✔	✔

Figure 71. Trunk sensory neurons arising from the lateral neuroectoderm/ neural plate border is a shared feature of bilaterians. The position of the RB-like trunk sensory neurons along the A-P axis in different animals. *RBc:* Rohon Beard cells, *DRGsn*: dorsal root ganglia sensory neurons, *DESN:* dorsal ectodermal sensory neurons,*RZc:* Retzius cells, *Psn:* peripheral sensory neurons. *R8:* rhombomere *8*, *s*: somite, *vg:* visceral ganglion. The table on the left summarizes the expression of the genes belonging to the t-NCct-NCc/RBc GRN in the trunk sensory neurons in vertebrates (V), ascidians (As), amphioxus (Amp), annelids (An) and insects (I).

II.2 The emergence of *bona fide* neural crest in vertebrates

The evolution of *bona fide* neural crest involved likely different steps that 'upgraded' the features of the original cell population emerging from the dorsal neural tube. Modifications in the gene regulatory network might have been responsible for this (Sauka-Spengler and Bronner-Fraser 2008a). The formation of new binding sites in the regulatory regions and protein neo/sub-functionalization after gene duplication might have recruited new genes at the neural plate border. Elaboration of signaling systems might have been involved as well, conferring also migration and differentiation cues.

II.2.1 Neural crest derived cell types and the gene regulatory network point of view

Cells at the dorsal neural plate likely give rise to sensory neurons in all the invertebrates analyzed so far (fig.70,71) and this might be an ancestral fate of the lateral neuroectodermal cells in bilaterians.
But, vertebrate neural crest gives rise also to different type of cells, such as melanocytes, enteric and sympathetic neurons and cartilage (table in fig.11).
How far can we trace back these cell types?
In *Platynereis* I found many of these derivatives with a conserved molecular profile.
Platynereis melanocytes precursors (*mitF+,tyr+*) likely originate from the mesodermal *soxE+* territory (fig.43). Furthermore, although the projections of these neurons are not yet known in *Platynereis, phox2+* motoneurons originate from the medial neuroectoderm(fig.40). I also found putative enteric neurons with conserved molecular fingerprint (*ash1+, soxE+)* in the gut of *Platynereis (fig.42)*.
Serotoninergic cells are also present in the midgut. The latter might be related to the enterochromaffin cells in vertebrates, that are present along the gastrointestinal tract and do not originate from the neural crest, but most likely from the endoderm (Le Douarin and Teillet 1973; Alenina, Bashammakh, and Bader 2006).
Serotonin producing cells have been found in the gut of basal vertebrates such as hagfish and lampreys (Goodrich et al. 1980). The presence of serotonin producing cells in the gut of *Platynereis* makes it likely that they constitute an ancestral population of gut neurons predating the emergence of neural crest. These results are summarized in fig.72.
Finally, I have no evidence so far for a contribution of the lateral precursors to form the enteric neurons and the pigmented cells, as occurs in vertebrates. If this is confirmed, then it is likely that conserved cell types were already part of the cell inventory at the base of Bilateria, and predated the emergence of the neural crest that then started to contribute to them, via gene co-option.
We can still see this happening in the vertebrate neural crest, it is likely indeed that the contribution of the neural crest to the sympathetic cell lineage is a gnathostome innovation, neural crest of basal vertebrates such as lamprey contribute to dorsal sensory neurons and enteric neurons, but fail to produce autonomic ganglia.

II.2.1.1 Collagenous skeleton in *Platynereis* and the evolution of the neural crest derived cartilage

The head cartilage is clearly a vertebrate innovation and contributed to the shift from a filter feeding behaviour to predatory life style at the base of vertebrates (Gans and Northcutt 1983). Similarly to the other neural crest derivatives we can envisage that a minimal cartilage module was already part of a pre-existing developmental process at the base of Bilateria, before the emergence of the neural crest.

Accordingly I found that the axial muscle structure in *Platynereis,* that we named 'axochord', strongly expresses *colA* (a fibrillar collagen type A) and the annelid ortholog of *foxD*, expressed in the cartilaginous derivatives of the neural crest cells (Arduini, Bosse, and Henion 2009; Stewart et al. 2006),(McCauley 2008).
Scanning electron microscopy done in this study suggests that these collagen producing-cells contribute to the sheath that enwraps the nervous system. We have also recently demonstrated that the axochord constitutes a strong attachment point for the oblique muscles and it is implicated in determining the movements of the appendages during swimming. Furthermore it secretes important signaling molecules such as *noggin, slit* and *netrin* (Lauri et al., unpublished). These data suggest that the axochord might act as a supporting structure, a patterning center for neuroblasts and axonal navigation, as the vertebrate notochord.

Other *colA* + cells constitute also the pharyngeal mesodermal sheath around the foregut of *Platynereis* . Amphioxus gill slits are also *colA*+(J. Yu et al. 2008), as the lamprey brachial basket (Ohtani et al. 2008). We can speculate that a collagenous skeleton predated the emergence of a neural crest derived skeleton.
Supporting this idea it is known that the trunk skeleton derive entirely from the trunk mesoderm (Votano, Parham, and Hall 2004), likely the cartilage ancestral ontology.

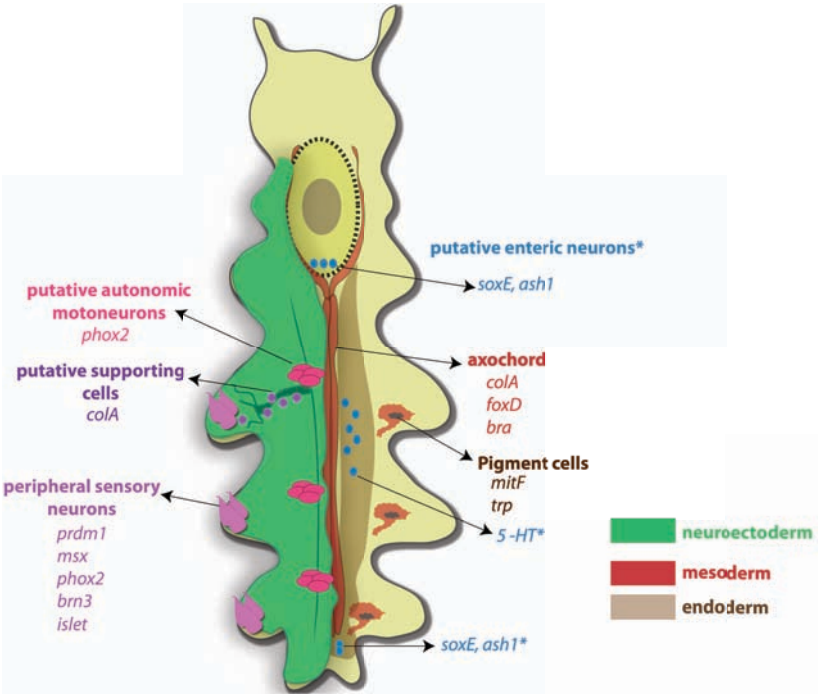


Figure 72. *Platynereis* possesses all the typical neural crest derived- cell types and gut serotoninergic neurons. The drawing summarizes the cell types found in *Platynereis*. Putative *phox2* + autonomic motoneurons populate deep central ganglia. Sensory cells and supporting cells with conserved molecular profile originate from the lateral neurectodermal *prdm1, msx* +. Putative enteric neurons are also present in *Platynereis* gut, some are *soxE, ash1*+, as in zebrafish. A subset of neurons in the midgut is also serotoninergic. Pigment cells arise from the mesoderm, likely from precursors *mitF, tyr*+ (genes expressed in neural crest derived vertebrate melanocytes). *ColA* and *foxD* are expressed in the axochord. The germ layers are also indicated with different colours.

The contribution of the neural crest to the head skeleton might have occurred via changes at the gene regulatory network and protein sub-functionalization. An example of this is given by amphioxus foxD gene. Similarly to the mesodermal expression of *Platynereis foxD, amphioxus foxD*(J.-K. Yu, Holland, and Holland 2002) is expressed in the notochord and in other mesoderm derived structures.
When the upstream region of *amphioxus foxD* is injected into the chicken it drives reporter expression only in the mesoderm and not in the neural crest (J. Yu et al. 2008). Together these data suggest that the original foxD was part of the a 'mesodermal genetic module' involved in the formation of the ancestral axial structure, from which the annelid axochord and the chordate notochord derives. We can imagine that gene duplication allowed protein neo-functionalization, and the 'newborn' foxD3 paralog was then recruited at the neural plate border in the neural crest cells. Together with this, changes occurred within the basic 'neural crest specifiers module', already acting at the neural plate border (soxE, snail, ap-2, colA); this contributed to the acquisition of the mesenchymal behaviour of the original neuro-epithelial cells.
Hence, a trunk collagenous skeleton, comprising also part of the gill slits might have predated the evolution of the head skeleton and neural crest.

Members of the SoxE family are required for skeletogenic cranial neural crest in vertebrates (Mori-Akiyama et al. 2003). Lamprey cranial skeleton expresses *soxE* genes

(Lakiza et al. 2011; McCauley 2008). Conversely, except for few cells in the notochord, the amphioxus *soxE* ortholog is not expressed in the pharyngeal mesoderm, but in nascent somites (J. Yu et al. 2008) . Similarly *Platynereis soxE* is expressed in mesoderm, but also in the neural plate border-like cells in the nervous system. Accordingly with an ancestral role for soxE in the nervous system, in vertebrates early expression of vertebrate *soxE* genes is observed during the early steps of the specification of the neural crest rather then during chondrogenesis (Marmigère and Ernfors 2007; Lallemend and Ernfors 2012).
These observations corroborate the idea that at the base of vertebrates neural plate border-like cells expressing *soxE* migrated out of the nervous system and skeletogenic cranial neural crest evolved by activation of a minimal mesodermal code(Rychel and Swalla 2007) (Cattell et al. 2011) .
Finally, lamprey lacks a cartilaginous tissue expressing all the genes of the neural crest derived cartilage known for gnathostomes (Cattell, Lai, Cerny, & Medeiros, 2011). A soft collagenous cartilage ('mucocartilage') in the oral/pharyngeal region is instead present and expresses genes such as *soxE, twist,* and *fibrillar collagen (co2a1a)* (Cattell et al. 2011). Additional gene- co-option (such as the recruitment of *runx* and *barx) in the* ancestral cranial neural crest giving rise soft catilage at the base of vertebrates might have driven the evolution of the head skull as seen in gnathostomes.

II.2.2 The signaling point of view:

II.2.2 .1 The Delta Notch signaling contributed to the divergence of t-NCc and RBc

It is possible to speculate that also the deployment of elaborated of signaling pathways contributed to the divergence of the neural crest from its original precursor. Delta-Notch signaling is a candidate.
In development this signaling usually occurs when inside an equivalence group a cell starts to express high level of Delta, all the neighbour cells will then receive this signal through Notch and differentiate in other cell types. This 'lateral inhibition' mechanism is utilised for the formation of the primary neurons, such as the Rohon Beard cells and the primary motoneurons in the early nervous system of anamniotes (Bruce Appel, Givan, and Eisen 2001).
It has been demonstrated indeed that the Rohon Beard cells expressing *delta* are the first choice at the border of the neural plate and that this is mediated via Delta-Notch signaling. Delta mutants show supernumerary Rohon Beard and primary motoneurons. They instead lack specifically trunk neural crest derivatives (Cornell and Eisen 2000),(R. A. Cornell and Eisen 2002) .

In *Platynereis* (Appendix, paragraph C, fig. c1), as in *Drosophila* and in *amphioxus* PNS (Lu, Luo, and Yu 2012), Notch inhibition leads to an increase of the number of neurons, comprising also the lateral trunk sensory neurons. We can speculate that the first neurons in Bilateria (comprising also Rohon Beard-like cells) were all positively responding to Delta signaling, and only later Notch was superimposed to generate different cell types, such as the neural crest in the dorsal neural tube. The

mechanism via which Delta-Notch controls the switch from Rohon Bead cells to neural crest is not known in anamniotes. Data in zebrafish show that this might occur decreasing the expression of proneuronal genes such as *ngn* in the precursors (R. A. Cornell and Eisen 2002), but this is still an open question. Studying these mechanisms in the future will maybe also shed light on how this occurred during evolution.

Delta-Notch might have been a common way that evolution used to segregate cell types. Similarly to neural crest and Rohon Beard cells, it was postulated the notochord and the floor plate share common midline organizer precursors in evolution as in development (Lewis and Eisen 2003). A similar Delta –Notch based mechamism occurs during the development of these structures and their differentiation. For instance an inhibition of the signaling leads to the development of more floor plate cells and reduced notochordal cells (B Appel et al. 1999).

II.2.3 Cranial and trunk neural crest: a different evolutionary origin or serial homology?

Interesting in zebfrafish Delta mutants the cranial neural crest derivatives are unaffected as the fin mesenchyme in the trunk (R. A. Cornell and Eisen 2002). It is now known that cranial and trunk neural crest have different genetic regulations and developmental potentials (see paragraph II.2.1.1). It is tempting to invoke a different evolutionary history for cranial and trunk neural crest. Interesting in *Platynereis* I found that the lateral trunk cells lack *twist* (that is expressed only in cranial neural crest), as lamprey neural crest cells (Sauka-Spengler and Bronner-Fraser 2008a). Furthermore the *Platynereis* lateral trunk cells originate in a position along the body axis that is determined by the same hox genes where Rohon Beard cells are formed.

We have very little comparative data to understand the evolutionary differences between cranial and neural crest.

Nevertheless, we can envisage that the trunk neural crest cells originated from the precursor giving rise also to the Rohon Beard-like cells. It is possible that cranial neural crest originated from a different precursor cell; another hypothesis is that also cranial neural crest originated from Rohon Beard-like cells present in the head region and serially homologous to the trunk Rohon Beard cells, via their gradual elaboration.

As it has been postulated, the presence of Rohon Beard-like cells that are neural crest derived in the vertebrate midbrain (in the mesencephalic nucleus of the trigeminal nerve, MesV) supports this hypothesis (Baker and Bronner-Fraser 1997; Pratt and Aizenman 2009). Indeed the cranial neural crest cells that give rise this midbrain sensory ganglion don't migrate, and might be more similar to the ancestral cranial neural crest, elaborated from pre-existing sensory neurons as in the trunk. This ganglion is involved in relaying the sensory information coming from the jaws and likely evolved only in the gnathostome lineage.

Accordingly, jawless vertebrates (basal vertebrates), such as lampreys and hugfishes lack the MesV ganglion, but Rohon Beard cells in continuity with the ones located in the spinal cord are present for the entire extend of the medulla and project through the trigeminal nerve (Butler and Hodos 2005b). This might be the ancestral situation at the base of vertebrates. In these rostral precursors of the brain stem, specific changes at the

gene regulatory network might have allowed the co-option of mesodermal modules to generate the head skull, the big vertebrate innovation see paragraph II.2.1.1.

2. Annelid Rohon Beard-like cells and the evolution of bilaterian trunk circuits

III. In bilaterians mediolateral regionalization produces Rohon Beard-like cells and motoneurons in distinct domains

III.1 Starting with multifunctional cells

It has been postulated that the first neurons (protoneurons) arose from a single multifunctional cell present at the base of Eumetazoa (Parker 1919, Mackie 1970,Arendt 2008, Jékely, 2011). These types of multifunctional cells are still present in eumetazoan larvae, such as sponges, cnidarians and annelids. Indeed, the ciliated cells of the ciliary band of *Platynereis* constitute the locomotory apparatus of the larvae (Jékely et al. 2008)(Jékely, 2011). and in the same time are sensory neurons, as suggested by the fact that they express sensory effector markers, such as the *trpV* channel (fig.31).

It is likely that from such a multifunctional cell (step 1, fig.73A), segregation of functions in different cells was ne of the first steps during nervous system evolution and produced the first simple circuitries (step 2, fig.73B), (Mackie 1970,Arendt 2008, Jékely, 2011).
. The sensory function was retained in the receptor cell, that likely specialized further to respond to different sensory stimuli (such as light or touch) and the contractile function was retained in the muscle cell. These cells remained in contact via specialized protrusions, the axons.

It has been proposed that one of the sensory neurons migrated to integrate different stimuli, becoming and interneuron (step 3, fig.73C). (Parker 1919, Mackie 1970,Arendt 2008, Jékely, 2011).

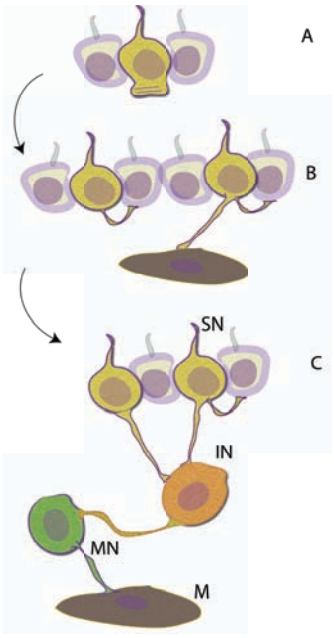


Figure 73. The evolution of the bilaterian sensory-motor circuits. This drawing has been made considering Parker 1919, Macki 1970, Arendt 2008, Jekely 2011.**A:** originally myoepithelial cells and sensory-motile ciliated cells are present.**B:** Segregation of the receptor and effector cells occurs. The receptor cell (sensory) contacts neighbouring cells (motile ciliated cells) and muscle effector cells via specialized protrusions (axons). **C:** Later more cells collect sensory inputs and one of them (the 'protoneuron', Parker 1919) integrates the sensory stimuli, becoming a interneuron. Motoneuron originate and mediate the motor response modifying the initial synapses into neuromuscular junctions. SN: sensory neuron. IN: interneuron.MN: motoneuron. M: muscle

The intermediate steps, where a sensory-motor cell contacts other cells that mediate ciliary locomotion are still present in many protostome larvae. Example of this are the brain serotoninergic sensory cells innervating the ciliary band cells of *Platynereis* (Jékely, 2011).. I have also found that a posterior serotoninergic cell, expressing sensory markers such as *brn3* is in contact with the posterior ciliary band (telotroch, Appendix fig.a1, a2) . Based on the function of sensory-motor serotoninergic cells known in other protostomes, we can speculate that these cells modulate the speed of the ciliary locomotion in *Platynereis*.

III.2 Building the sensory-interneuron-motoneuron basic topography using mediolateral coordinates and canonical Wnt signaling

The '*division of labour*' model (Arendt 2008) likely gave rise to cell types such as the Rohon Beard-like cells and the primary motoneurons at the base of Bilateria, a topography of cell types that still constitutes the primary scaffold of anamniote vertebrates and lampreys (fig.18,74) and mediates escape response in the larvae (Whiting 1948), (Nakao and Ishizawa 1987) (Lamborghini 1980), (Baccaglini and Spitzer 1977).

The generation of such topography of distinct cell types was probably strongly linked to the establishment of mediolateral patterning in Bilateria. This likely creating a specific grid of coordinates that produced from dorsal (lateral) to ventral (medial) sensory, interneurons and motoneurons, compartmentalizing the nervous system in a central and a peripheral component.

Demonstration that the mediolateral patterning shapes the annelid trunk neuroectoderm producing conserved cell types (Denes et al. 2007 and this study) suggests that a primitive form of cell type diversification in functional units predated chordate evolution.
Such a patterning was likely already established in conserved body territories along the dorso-ventral and anterior posterior axis at the dawn of Bilateria (fig.72A, B, C). Nevertheless, in *Platynereis,* intermediate forms of circuits are still present, such as the pygidial serotoninergic neuron of the trunk and the head, and the sensory motor neurons of the ciliary bands. (fig.74A).

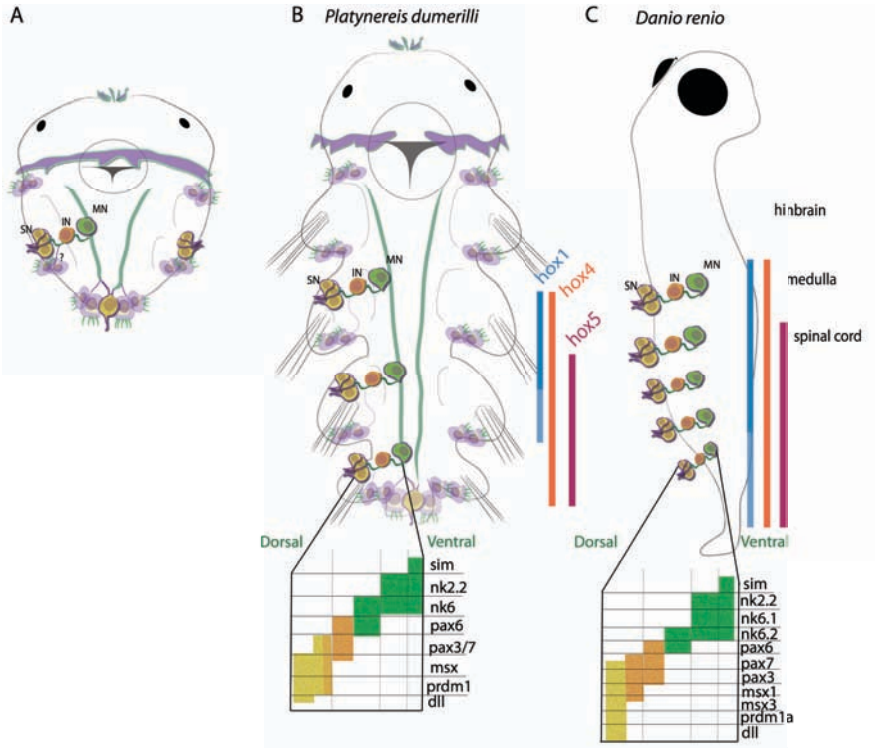


Figure 74. The pygidial serotoninergic neuron and the somato-motor circuit in *Platynereis* and vertebrate trunk along the D-V and A-P axis. A: serotoninergic primary neuron in the pygidium of *Platynereis* at 48h. At this stage it is likely that also sensory-motor circuits are already present along the D-V axis**. B:** The basic sensory-motor circuitry is mapped along the D-V and A-P axis in the juvenile *Platynereis*. Patterning genes expressed in the distinct columns are indicated. The columns are colour coded as the neurons originating from them. Sensory neurons Rohon Beard-like originate at the level of the A-P axis where a conserved *hox code* is present, see paragraph II.1.1. **C:** comparison with vertebrates. This drawing has been done considering Denes et al and this work and fig. 18.

Furthermore this work suggests that signaling pathways already participated in 'shaping' the domain along the medio-lateral axis.

Precisely, in *Platynereis* I found that the dorsal territory is expanded after ectopic ß-catenin activation, similarly to the vertebrates situation. Surprisingly my data suggest that the antagonism between sFRP and Wnt, that instructs the vertebrate medial most precursors (see Introduction, paragraph II.1.2, fig.33), is conserved in *Platynereis*.

From the mRNA expression pattern, it is likely that in the worm Wnt is secreted from the blastopore region as in Cnidaria (Guder et al. 2006), and then extends to the lateral neuroectoderm. Interestingly the Wnt free domain in between is indeed the *pax6/sfrp+* and produce *hb9+* motoneurons. This study shows that also in *Platynereis pax6* and *sfrp* are expressed in the same territory, furthermore it shows that ectopic ß-catenin activation suppresses *pax6* and *sfrp* expression specifically in the trunk, and *nk2.2* + progenitors are ectopically produced in this domain, similarly to vertebrates (Kim et al. 2001), (Lei et al. 2006). It is likely to think that already in *Platynereis* the mediolateral patterning is maintained by Wnt, through the positive activity on the dorsal most territory (see above) and the opposition of Wnt/sFRP in the ventral domain (fig. 75).

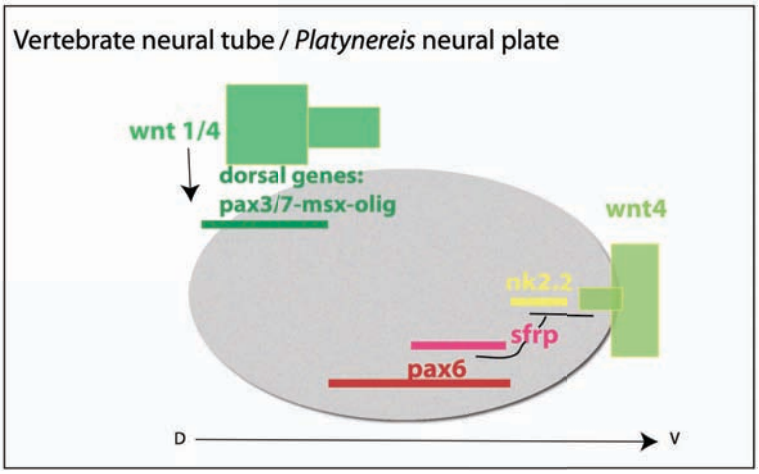


Figure 75. Sfrp/ Wnt antagonism influences the mediolateral patterning.
In vertebrates as in *Platynereis* wnt1/4 are expressed in the dorsal side of the neuroectoderm and wnt4 in the ventral side. Canonical Wnt signaling influences positively the expression of dorsal patterning genes, such as *pax3/7* and *olig*. Ventrally the boundary between *pax6-nk2.2* is kept via the Wnt inhibitor sFRP, that inhibits the dorsal most *nk2.2* domain.

We can envisage that at the transition between non bilaterian and bilaterian nervous system, the Wnt-Sfrp antagonism was 'intercalated' in the ancestral Cnidaria-like *Wnt +* domain at the blastopore. This contributed to the emergence of the bilaterian trunk nervous system from this region by the formation of the slit-like blastopore and convergent extension movements (Dr. Steinmetz PhD thesis, 2006, Meinhardt 2006).
Interestingly in the *Platynereis* posterior growth zone, where new segments are added, the *wnt* expression forms a unique homogenous group from medial to lateral, and no *sfrp* or *pax6* is yet expressed. This constitutes a developmental step that might also reflect an evolutionary event, likewise in the Cnidaria blastopore.

3. The presence of canonical neurotrophic signaling is a bilaterian invention and predated the evolution of the neural crest

IV. Expanding and diverging cell types and circuitries in evolution using neurotrophic signaling

Rohon Beard cells, neural crest and the sensory neurons that derive from neural crest depend on neurotrophic signaling for their development. This pathway is important to confer the correct proliferation, survival, death and migration signals to the developing cells. Data from vertebrates show that neurotrophic signaling plays a pivotal role in the 'methabolism' of sensory cells in the dorsal nervous system (Marmigère and Ernfors 2007; Lallemend and Ernfors 2012). Neurotrophic signaling (TrkC-NT3 signaling) mediates the degeneration of Rohon Beard cells in the spinal cord of fishes (Williams et al. 2000); an event that precedes the differentiation of the early neural crest in sensory neurons of the dorsal root ganglia. These neurons will functionally replace the Rohon Beard cells and are also highly dependent on neurotrophic signals for their expansion, survival and target recognition.
Therefore, to understand how these new cell types (such as *bona fide* neural crest itself and the plethora of sensory neurons) and new neural circuits evolved it is also necessary to ask how trophic signaling and axon path-finding mechanisms evolved.
When did the ancestral neural crest precursors acquired responsiveness to the neurotrophins?

We can imagine that in the early steps of complex nervous system evolution changes in neuronal numbers (increases and decreases), survival and pattern of connectivity might have generated different developmental options to shape the original circuits (von Bartheld and Fritzsch 2006, fig.76). Elaborating signaling systems acting in generating these possibilities might have driven the evolution of complex neural circuits and cell type variety in the nervous system.

This study shows that one of the most important trophic system for the vertebrate nervous system, the neurotrophic signaling, is present also in annelids. It comprises fully conserved molecules: one Trk receptor, one ligand (neurotrophin) and one co-receptor p75, that are expressed in the nervous system and target tissues such as the muscles (fig.49, 55, 56,57,58). Hence, it is likely that this signaling was already available to shape nervous systems of early bilaterians (fig.77).
The present study also suggests that the neurotrophic signaling as such was a bilaterian invention. Except for p75, no clear homologs of the receptors or ligands are found in basal Eumetazoans such as Cnidarians (fig.66,67,68).

Although the function of *Platynereis* neurotrophic signaling has to be determined, we can imagine that at the transition between non-Bilateria and Bilateria, a primitive form of the neurotrophic signaling was used to shape existing connections. This might have occurred by varying the time and the level of expression of both ligands and receptors (von Bartheld and Fritzsch 2006, fig. 76C) and re-directing certain neuron to specific targets

within an initial homogenous group, therefore 'segregating' the neuronal identities as well.

I found that *Platynereis* possesses only one ortholog per each components of the neurotrophic system. Both Trk and NT genes duplicated during vertebrate evolution, generating three Trk receptors and 4 neurotrophin ligands (fig.77). We can envisage that the duplication/elaboration of the neurotrophic signaling facilitated the formation of more precise and segregated circuitries, via a process of '*parcellation*' (von Bartheld and Fritzsch 2006) (Ebbesson 1984).

of circuitries and identities (fig.76C). If this is repeated multiple time during development, in different neuronal precursors and using different receptor/ligand combinations is easy to imagine how this contributed to the complexity of the nervous system seen in vertebrates.

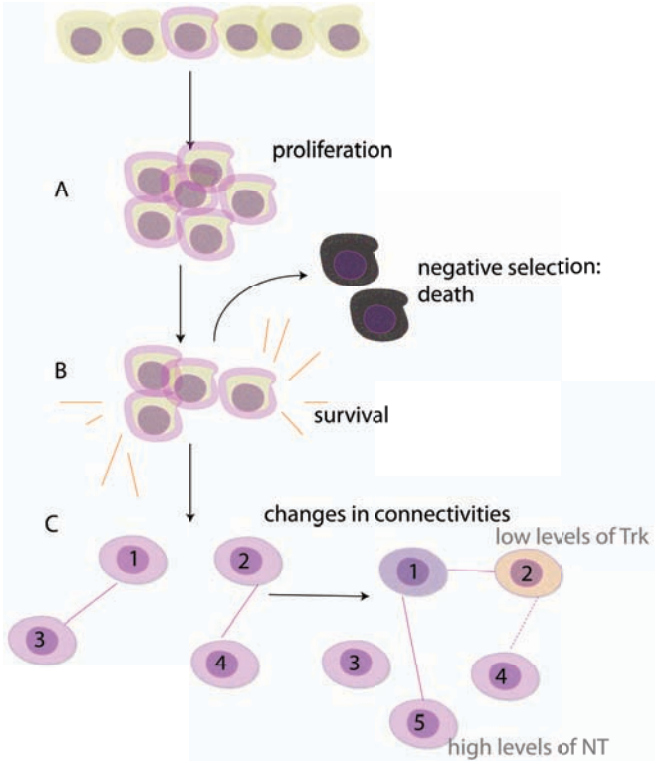


Figure 76. Hypothetical expansion of cell types and shaping of neural circuits mediated by Trk-neurotrophin-like molecules during evolution. See text for details. In **C** changes in connectivity give rise to different cell types (indicated with different colours). Dashed line indicates a degenerating axon. This drawing was based on fig.22.

In terms of expansion of cell types it has been proposed that the emergence of the neural crest correlate with the emergence of neurotrophic ligands (Wittbrodt 2007). The expansion of the neurotrophin ligands was unlikely implicated in the emergence of the neural crest in the first place, because lamprey has neural crest, but it possesses only two lamprey specific Trk paralogs (Hallböök, Lundin, and Kullander 1998) that originated from the ancestral one and one apparent neurotrophin molecule. Different types of sensory neurons are generated from the neural crest (mechanoreceptors, nociceptors,

proprioceptors) and they express different Trk receptors, and therefore generate distinct pattern of connectivity(Lallemend and Ernfors 2012).

It is possible that the duplication of the Trk receptor was implicated in the diversification of these cell types from few original sensory neurons.
It is notable that sympathetic neurons depend on NGF-TrkA for their survival, and that nor NGF or TrkA are present in lamprey, which also lack neural crest derived sympathetic neurons (Butler and Hodos 2005a), (Häming et al. 2011). Hence, it is plausible that this novel population of neural crest derived neurons co-evolved with the multiplication of the neurotrophic signaling only in gnathostomes.

Future research will determine the role of these simple forms of neurotrophic signaling and further test the scenario put forward here: that the evolution of a sophisticated neurotrophic signaling was involved in generating the complexity of cell types and connectivity observed in vertebrates.

IV.1 How did the bilaterian neurotrophic signaling evolve?

Obvious questions are: what was the function of the neurotrophic signalling? When did it evolve? And how did it evolve? These are still open questions but the work presented so far can gives some glimpses and put forward new hypotheses.

Trk receptors belong to a family of tyrosine kinase receptors (including MuSK,Ror, Drd, Trk)(Sossin 2006) with similar tyrosine kinase domains but different extracellular domains. The latter determine the affinity for different ligands and therefore differentiate the function of the receptors.

This study shows that *Nematostella vectensis*, a basal Eumetazoa, possesses a conserved p75 co-receptor, several different tyrosine kinase receptors but no neurotrophin (paragraph VII, Results). A phylogenetic analysis of these receptors is quite difficult due to the great divergence of the extracellular domains, but it suggests that the *Nematostella* receptors are MuSK-like (MuSK-l) receptors with kringle/frizzled like domain in the extracellular portion, belonging to the same RTK (receptor tyrosinase kinase superfamily) as Trk (fig.66).

A similar receptor have been found also in sponges (Sossin 2006), and it has been proposed that the Trk receptors evolved by exon shuffling at the level of the extracellular domain from an ancestral tyrosinase receptor present at the base of Eumetazoa(Sossin 2006). This receptor had extracellular kringle/frizzle like domain, and likely bound Wnt-like molecules. The presence of *Nematostella* Ror and MuSK-l shows that the split between Ror/MuSK occurred already at the base of Eumetazoa, but the lack of a canonical cnidarian Trk receptor indicates that the evolution of Trk occurred only in Bilateria (fig.77). This occurred in Bilaterian lineage likely through *de novo* modular assembly of the extracellular portions of the receptors, a mechanism that has also been used to explain evolution of other important molecules in the nervous systems of vertebrates.

One of the *Nematostella* MuSK-l receptors is expressed in the ectoderm and together with *Nematostella* p75 in the directive mesenteries (fig.67,68), which express many neuronal markers (such as *lhx2/9*)(Srivastava et al. 2010) .This receptor is also quite divergent from the classical MuSK receptor that binds Agrin ligand in vertebrates and is needed to form Ach receptor clusters in the neuromuscular junctions (Glass et al. 1997). Nevertheless, it is interesting that zebrafish MuSK receptors can also coordinate axon guidance of motorneurons towards the muscle central zone via Wnt signalling (Banerjee et al. 2011), likely linked to the function of ancestral MuSK receptors.
Hence, it is tempting to think, as suggested in Sossin 2006, that an ancestral role in axon guidance was the first function of the original eumetazoan signaling (fig.77, ancestral Ror/MuSK/Trk receptor) probably acting via Wnt ligands.

Starting from this hypothesis it is hard to reconstruct all the steps of the evolution of the signaling. First of all only now we start to uncover possible homologs in protostomes and in basal Eumetazoa. Moreover, we should take into account that understanding the emergence of the ligands is also important to understand the evolution of the canonical signaling, but this extremely difficult to trace, because these ligands are notoriously fast evolving; unless we find an intermediate forms of the receptors and of the ligands.

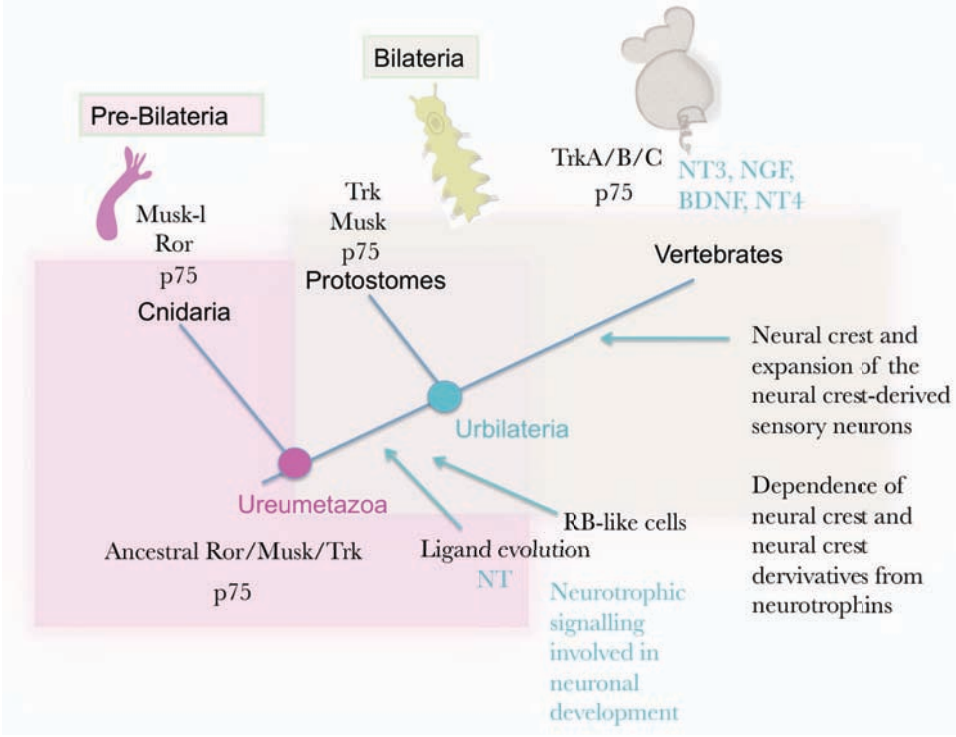


Figure 77. Evolution of the neurotrophic signaling at the base of Bilateria and emergence of the neural crest in vertebrates. The neurotrophic signaling was assembled only at the based of Bilateria, with the evolution of the ligand (neurotrophin, NT) and of the canonical Trk from an eumentazoan MuSK/Trk receptor. A centralized nervous system was already in place, RB-like cells were already populating the lateral neuroectoderm and predated the emergence of neural crest. Likely the ancestral neurotrophic signaling was critical for neuronal development. Gene duplication in the vertebrate lineage led to the moltiplication of the neurotrophic signaling components and to the diversification of their functions. Neural crest evolved and expansion of the derived sensory cells occurred, likely due to survival and trophic function of the neurotrophic signaling. Ureumetazoa and Urbilateria are the common ancestors of Eumetazoa and Bilateria.

V. Concluding remarks and outlook

This work tried to address the evolution of the neural crest from a protostome perspective.
Furthermore, it also aimed at shedding light on the evolution of one of the most important signaling pathways acting in the vertebrate nervous system: the neurotrophic signaling.
Evolutionary precursors for neural crest cells and neurotrophic signaling can be inferred taking into consideration the literature and integrating new data from this work, but many open questions remain and new questions arise:
-If Rohon Beard-like cells were the bilaterian precursors of neural crest, how and when did they acquire migratory behaviour?
-Which molecular events occurring in this ancestral population were responsible for the acquisition of such a great developmental potential?
-Which kinds of stimuli do the trunk sensory neurons perceive in annelids?
-What did the first trunk neuronal circuit involving Rohon Beard-like cells look like?
-How did it evolve?
-What was the function of the first neurotrophic signaling more then 500 MYA?
-And how did the expansion of the repertoire of neurotrophic signaling molecules contribute to the emergence of the neural crest complexity?

We can now argue that a prototype of neural crest precursor cell was already present at the base of Bilateria and Rohon Beard like-cells and supporting cells were likely produced from this cell type.
A canonical neurotrophic signaling was also already part of the ancestral bilaterian molecular inventory.

Next, we need to understand the functional significance of the ancestral most canonical neurotrophic signaling (such as the one I discovered in *Platynereis)* on one side, and reveal the molecular steps that led to the appearance of the neural crest. The latter is possible only when comparison of the neural plate-like precursors in different invertebrates is achieved at the molecular levels, as well as in terms of their development.
I am convinced that with the optimization of the transgenic techniques, live imaging and functional interference this will be soon possible in *Platynereis,* as in other non classical model systems which occupy a crucial phylogenetic positions, to answer these questions (such as amphioxus).
A detailed study of the trunk circuitry of *Platynereis* will test the hypothesis put forward in this study: that RB-like cells are already used in annelids to rely sensory stimuli in a reflex-like circuit, similar to early anamniote larvae.
To understand how the neural crest evolved from the same precursor of these annelid RB-like cells at the lateral neuroectoderm (with partial genetic code already established), gene regulatory network analysis is required on a large scale.

From an evolutionary prospective indeed the neural crest challenges the concept of cell type conservation, as it is not even definable as cell type, because of its plasticity and developmental potential.

In this study the conservation of cellular and molecular features during the evolutionary history of the '*lateral neuroectoderm cells'* has been useful. It led us to determine cell types (sensory cells) that always derives from lateral neuroectodermal cells in all the phyla examined, therefore more likely one of the ancestral most derivatives of the neural crest (fig.70.71).

In the future, detailed studies of the development and of the molecular fingerprint of the precursors of the putative Rohon Beard-like cells in more invertebrate species is needed to validate the scenario that I proposed (paragraph II.1.1, fig. 70.71). This approach will test the hypothesis that primary sensory neurons were among the first cell types originating from the neural crest precursors. Nevertheless, it will not teach us anything about how evolution worked to generate the neural crest as such, with such a great developmental potential and migratory behaviour.

To do this, it is necessary to perform a functional analysis of the crucial neural crest genes already expressed in the neural plate border of *Platynereis*, as well as generate a detailed map of the gene regulatory interactions occurring at the neural plate border-like territory in all the phyla. Indeed, such a study, only if performed in a comparative manner, will give us an idea on which gene co-option occurred and approximately when it occurred during the evolution of the neural crest.

MATERIALS AND METHODS

I. Materials

I.1 *Platynereis dumerilii* culture

Platynereis dumerilii larvae were obtained from an established breeding culture at EMBL, HeideLB (Luria-Bertani) brotherg, following Dorresteijn et al. (1993). After fertilization, *Platynereis* embryos were raised in Zentis cups, in natural sea water (NSW), in an incubator at the temperature of 18 °C, with a 16L:8D light cycle (Type KB53, Binder, Tuttlingen, Germany).

I.2 Equipment

Centrifuges: Eppendorf centrifuges 5417C and 5430, Thermo scientific centrifuge "Multifuge" 3SR+, Thermo scientific Sorvall RC6T
(Beckmann UC rotor Ti70)

PCR machines: Peltier Thermal Cycler PTC-200 (BioRad). ABI7500 Light cycler (Applied Biosystems)
with 96-well blocks for qPCR (EMBL GeneCore).

Microscopes: Zeiss Stemi2000 stereomicroscope for observation of the animals and mounting.
Zeiss Axiovert 40C for injection of *Platynereis* zygotes, equipped with a UMMJ-3FC micromanipulator (Narishige) and an Eppendorf FemtoJet express.
Leica SPE with a 40x and 63x oil-immersion objective for confocal microscopy.
SEM LEO_1530 (at the Core Facility of Electron microscopy, Bioquant INF 267 69120 HeideLB (Luria-Bertani) brotherg)

Others:
Eppendorf Thermomixer compact
Nanodrop ND-1000 (Thermo scientific)
GenePulser Xcell (BIO RAD) for electroporation of bacterial cells
Sonifier 250 Branson
LEICA VT 1200S

I.3 Frequently used chemicals and reagents

Chemicals were purchased from Sigma-Aldrich and Merck, unless indicated otherwise.
Routinely used solutions were prepared after Sambrook and Russell (2001).
-Oligonucleotides for PCR were purchased from Sigma and Invitrogen.
-Agarose for routine nuclei acids separation, and for the preparation of the injection stage : Sigma, cat. n. A9539.
-Low melting agarose used to perform vibrotome sections of the adult *Platynereis*: Ultrapure LMP-Agarose (Gibco).
-'eral Oil : Sigma, cat. n. M5904
-polyDlysin :Sigma, cat.n. p6407
- NTPs: : 15.4mM ATP, 15.4mM CTP, 15.4 GTP, 10mM UTP (Boehringer Ingelheim)
-10x DNA agarose gel loading buffer 50% Glycerol, 100mM EDTA pH 7.5, 1.5mM BromophenoLB (Luria-Bertani) brothlue, 1.9mM Xylenecyanol
-10xTBE: 890 mM Tris, 890 mM boric acid, 20 mM EDTA pH 8.0. Dilute to 1x for running and preparing agarose gels.
-Fixative : 16% PFA Paraformaldehyde, for fixation (Electron Microscopy Sciences, 15710).
1 M NaCl : for 500ml 29.22g NaCl in 500ml H20
1M Tris pH 9.5 : for 500ml 60.57g Tris in 500ml H20

20% Tween: 8ml of Tween-20 in 32ml of H20
-10x PBS : 1L: 70g NaCl; 62,4g Na2HPO4.2H2O; 3,4g KH2PO4, pH 7.4.
10xPBS for *Nematostella* WMISH: 18.6 mM NaH2PO4.H20 (2.56g/Liter) 84.1 mM Na2HPO4.2H2O (14.97g/Liter), 750 mM NaCl (102.2g/Liter)
-PTW 1x : PBS pH 7.4 + 0.1% Tween-20, sterile filtered.

I.4 Commercial antibodies
Primary antibodies:
Mouse anti-acetylated tubulin: Sigma T6793 1:200
Mouse anti-tyrosinated tubulin: Sigma T9028 1:250
Rabbit anti-serotonin: ImmunoStar 20080 1:300
Mouse anti-synapsin: Dev. studies hybridoma bank, 3C11 1:250
AntiRabbit

Secondary antibodies:
DyLight 488 anti-mouse: Jackson Laboratories, 1:500
DyLight 549 anti-rabbit : Jackson Laboratories, 1:500
Anti-Rabbit IgG (H+L), HRP Conjugate: Promega, 1: 3000

I.5 Softwares
-DNA sequences were analyzed using *ApE* :
(http://biologylabs.utah.edu/jorgensen/wayned/ape/).
-Multiple sequence alignments were generated with *MUSCLE*
(http://www.ebi.ac.uk/Tools/msa/) (Edgar, 2004)and with *ClustalW*
(http://www.ebi.ac.uk/Tools/msa/clustalw2/).(Jeanmougin, Thompson, Gouy, Higgins, & Gibson, 1998)
-The software *Excel* was used for statistics.
-The protein aligments were visualized with *Geneious 5.6.4*
(http://www.macupdate.com/app/mac/21135/geneious).
-Phylogenetic trees were performed using *PhyML*(Guindon, Lethiec, Duroux, & Gascuel, 2005)
(http://www.atgc-montpellier.fr/phyml/).
-Protein analysis was performed using these tools from *Expasy*:
Translate (http://web.expasy.org/translate/),
*Prosite (*http://prosite.expasy.org/), (Sigrist et al., 2010)
SignalP 4.0 (Server from Cbs, http://www.cbs.dtu.dk/services/SignalP/)
-Protein 3D models were predicted using:
M4T (http://manaslu.aecom.yu.edu/M4T/), (Fernandez-Fuentes, Rai, Madrid-Aliste, Fajardo, & Fiser, 2007; Rykunov, Steinberger, Madrid-Aliste, & Fiser, 2009).
-The predicted structures were visualized with *Chimera*
(http://www.cgl.ucsf.edu/chimera/).(Pettersen et al., 2004).
-Image processing was performed using *Image J* and *Imaris* (Bitplane).
Brightness and contrast were adjusted equally. Figures and artwork were created using Adobe Photoshop and Adobe Illustrator.

II. Cloning

II.1 Cloning of the *Platynereis* genes of interest to generate RNA probes for ISH

II.1.1 Extraction of RNA from larvae

Around 2 batches (>500 animals each) were mixed for these stages: 24h, 48h. 1 batch was used for this stage: 72h, 5days, and 14days.

I followed this protocol:

1) Collect *Platynereis* larvae in an Eppendorf tube

2) Spin for 30''at 4000 rpm

3) Remove the NSW (natural seawater)

4) Quickly freeze in liquid nitrogen

5) Add 1ml of Trizol (TriFast, Peqlab) and lyse by pipetting up and down for 3'. (For small amounts of material, use only 500 ul of Trizol)

Incubate 5' at r.t.

6) Spin 10' at 12000 rpm at 4 °C

7) Transfer the supernatant in a fresh tube

8) Add 200 ml of chloroform per 1 ml of Trizol

9) Shake vigorously by hand for 15''

10) Incubate 5' at r.t. , spin 15' at 12000 rpm at 4 °C

11) Transfer the supernatant in another tube. Add one volume of phenol:

chloroform:isoamylalcohol 25:24:1

12) Vortex 30'

13) Spin at 14000 rpm, 5' at r.t.

14) Transfer the acqueous phase in another tube. Extract by adding the same volume of chloroform.

15) Vortex 30''

16) Spin at 14000 rpm, 5' at r.t.

17) For small amounts of starting material: transfer the acqueous phase in a fresh tube. Add 0.5ml of Linear Acrylamide (Ambion, AM9520) for 500ml of starting Trizol. Mix

18) Add 500ml of ice-cold isopropanol for 1 ml of starting Trizol

19) Vortex and incubate at -20°C for 1 hr. For small amounts of starting material, incubate overnight

20) Centrifuge at 4°C for 15' at maximum speed (>14000 rpm). For small amounts of starting material, centrifuge for 1 hr

21) Remove the supernatant

22) Add 1 ml of cold 70% EtOH, invert the tube and centrifuge at 4°C for 5'

at the maximum speed (>14000 rpm)

23) Remove the supernatant, and repeat the EtOH wash as above

24) Remove the last supernatant without disturbing the pellet

25) Dry the pellet for about 10 '

Add 10-25 ul of nuclease-free water. To resuspend, agitate at at 30 °C for 15'

26) Measure the RNA concentration with the nanodrop. Store the RNA at -80C

II.1.2 cDNA libraries preparation

cDNA libraries were prepared from RNA of mixed larval stages using the Superscript III Reverse Transcription kit from Invitrogen, according to manufacturer's instructions. RACE

libraries were prepared from RNA of mixed larval stages using the GeneRacer Advanced RACE Kit (Invitrogen, cat. n. L1502-01), according to manifacturer's instructions.

II.1.3 Designing of primers, regular and RACE PCRs

Primers for the gene of interest were designed with a TM of around 70°C for Race primers and 60-63°C for standard primers. The quality of the primers was assessed with: *OligCalc* (http://www.basic.northwestern.edu/biotools/oligocalc.html).

PCRs were performed using these DNA polymerases according to manufacturer's instructions: Phusion High-Fidelity DNA Polymerase (NEB, cat.n. M0530), ExTaq TAKARA (CHEMICON International, RR001B,), HotStarTaq DNA Polymerase (Qiagen, cat. n. 203205) and Advantage2 PCR kit
(Clontech, PT3281-2).

PCR reaction with Phusion:
Template (cDNA library) 0.3 ul
dNTPs 2.5mM 0.8 ul
FW primer 10 uM 0.5 ul
REVprimer 10 uM 1 ul
5x Phusion Buffer 2 ul
Phusion High-Fidelity DNA Polymerase (NEB) 0.1 ul
water up to 10 ul
Cycling program with Phusion (touchdown, primer Tm _ around 60 °C):
98°C 30''
5x: 98°C 10''- 59°C 40''- 72°C 1' (for amplicons around 1kb), 2' (for amplicons >1kb)
5x: 98°C 10''- 57°C 40''- 72°C 1' (for amplicons around 1kb), 2' (for amplicons >1kb)
15x: 98°C 10''- 55°C 40''- 72°C 1' (for amplicons around 1kb), 2' (for amplicons >1kb)
72°C 10'

The PCR on genomic DNA to investigate SNPs in Trk 5' coding and UTR region were perfomed with Phusion polymerase as well. 50ng/ul of genomic DNA was used
Cycling program :
98°C 30''
28x: 98°C 10''- 62°C 30''- 72°C 1',5''
72°C 10'

PCR reaction with ExTaq:
Template (cDNA or plasmids 50 ng/ul) 1.5 ul
dNTPs 2.5mM 8 ul
FW primer 10 uM 3 ul
REVprimer 10 uM 3 ul
10x ExTaq TAKARA Buffer 10 ul
ExTaq TAKARA 0.5 ul
water up to 50 ul
Cycling program with Phusion (touchdown, primer Tm _ around 60 °C):
94°C 1'

5x: 94°C 30''- 58°C 45''- 72°C 1′ (for amplicons around 1kb), 2′ (for amplicons >1kb)
5x: 94°C 30''- 55°C 45''- 72°C 1′ (for amplicons around 1kb), 2′ (for amplicons >1kb)
20x:94C 30''- 53°C 45''- 72°C 1′ (for amplicons around 1kb), 2′ (for amplicons >1kb)
72°C 10′

RACE PCR reaction for *Platynereis* (using Hot start polymerase)
Template (RACE library) 0.4 ul
dNTPs 2.5mM 0.8 ul
Gene Specific Primer 10 uM 0.9 ul
GeneRacer Oligo 10 *m*M 1.8 ul
10x HotStarTaq Buffer 2 u*l*
HotStarTaq DNA Polymerase (Qiagen) 0.2 ul
water to 20 u*l*
Nested RACE PCR reaction for *Platynereis :*
Template (RACE PCR product) 1 ul
dNTPs 2.5mM 4 ul
Gene Specific Primer 10 uM 1 ul
GeneRacer Nested Oligo 10 uM 1ul
10x HotStarTaq Buffer 5 ul
HotStarTaq DNA Polymerase (Qiagen) 0.5 ul
water to 50 ul
Cycling program (touchdown):
98°C 30''
5x: 98°C 10''- 69°C 40''- 72°C 2′
5x: 98°C 10''- 67°C 40''- 72°C 2′
15x:98°C 10''- 65°C 40''- 72°C 2′
72°C 10′

RACE PCR reaction for *Nematostella* (using Advantage2 PCR kit):
(Protocol and cDNA libraries kindly provided by Dr. Heather Marlow)
5′ and 3′ cDNA libraries 1ul
dNTPs 10mM 0.5ul
Gene Specific Primer 10 uM 1ul
Universal primer Mix (from the Kit) 2.5ul
10xReaction buffer 2.5ul
Polymerase mix 0.5ul
Water to 25 ul

Nested RACE PCR reaction for *Nematostella vectensis* and *Capitella teleta :*
1/50 Race pcr 2ul
dNTPs 10mM 1ul
Gene Specific Primer 10 uM 2ul
Nested primer Mix (from the Kit) 2ul
10xReaction buffer 5ul
Polymerase mix 1ul

Water to 50 ul
Cycling program (touchdown):
94°C 5′
5x: 94°C 30′′- 68°C 45′′- 72°C 2′
5x: 94°C 30′′- 65°C 45′′- 72°C 2′
20x: 94°C 30′′- 63°C 45′′- 72°C 2′
72°C 10′

II.1.4 Cloning of the PCR products

I followed this protocol:

1) Analyze PCR products obtained with gel electrophoresis, according to standard protocols (Sambrook and Russell, 2001).

2) Cut the DNA fragments from the gel and purify them using the QIAquick Gel Extraction Kit (Qiagen, cat. n. 28704)

3) Clone the products in the pCRII-TOPO vector with the TOPO TA Cloning Kit Dual Promoter (Invitrogen, K4660-01), following manufacturer's instructions.

4) Transform the DNA into Electrocompetent or chemocompetent, according to standard protocols (Sambrook and Russell, 2001), and plated on LB (LURIA-BERTANI) BROTH agar plates with X-Gal, for blue-white colony screen.

5) Inoculate the white colonies in 5ml of LB (LURIA-BERTANI) BROTH medium.

6) Perform minipreparation of DNA from the bacterial cultures were performed according to Sambrook and Russell (2001).

7) Analyze the minipreps with the restriction endonuclease EcoRI, whichexcises the insert in pCRII-TOPO.

8) Send the positive clones to sequence (GATC Biotech, Konstanz). The pCRII-TOPO vector has T7 and SP6 RNA polymerase binding sites in opposite directions; sequencing is necessary to find out the RNA polymerase to use for antisense transcription.

II.1.5 Sources of the *Platynereis* and *Nematostella* clones used as template for probe synthesis and list of the primers used

During my PhD I could take advantage of the *Platynereis* sequencing project (for both genome and transcriptome) originally started by the Arendt lab and Jekely lab,unpublished), and then continued by Dr. Tomas Larsson and Oleg Simakov, and other *Platynereis* laboratories. To fish the genes of interest, tBlast search was perfomed against the the *Platynereis* resources website (http://4dx.embl.de/platy/). For the *Nematostella vectensis* clones the tBlast was performed against the JGI genome browser (http://genome.jgi-psf.org/Nemve1/Nemve1.home.html).

Sequence fragments with high similarity to the query sequence were then compared and assembled, either manually or using contig assembly algorithms such as '*Codon Conting Aligner*'.

For probe tamplates: if the resulting contings were less then 600-700 bp RACE PCRs were performed. This was done also to clone full-length proteins. Many other probes for WMISH were produced from clones already present in the common database of the lab.

Primers used in the clonings:

All the primers are designed against *Platynereis* DNA sequences, unless indicated.

Race primers

Pdu_p75_5'R AGTAATACCCCTGCCGACATTCGCAGACT
Pdu_p75_5'N GCAGACTGTGTCGTTAGTTATCGTACAGGG
Pdu_p75_3'R GCTTCATGTGTGCAACTACAGAGAGGATAC
Pdu_p75_3'N GATACAGTGTGTATGGAAATGCCCGTCCCAG
ColA_5R CTGCAACGGCATTTCACAGTCGATTTGC
ColA_5R CTGCAACGGCATTTCACAGTCGATTTGC
Eprhin3'R_09011 TGAAAAGTATTCTGCCATCCCCAATG
Eprhin3'N_09011 CACTGAGGGTGGCACTTATTATTAC
Eprhin5'R_09011 GTAATAATAAGTGCCACCCTCAGTG
Eprhin5'N_09011 CATTGGGGATGGCAGAATACTTTTCA
Eprhin3'Race092011 TGAAAAGTATTCTGCCATCCCCAATG
NVTrk_5R_2_03011 CATCAACCGCtGGTCAATGTTCTTGAGACT (for *Nematostella vectensis Musk-l*)
NVTrk_5N_2_03011 GCTTCTGTCGCATCGTCATTGCGTCCT (for *Nematostella vectensis Musk-l*)
5N_NvTrk-l_2011 TCCACTCTCTCCTCGGGCTCTCTCGACCA (for *Nematostella vectensis Musk-l*)
5R_NvTrk-l_2011 GTCCCGCAAACGTTTTTGAATGAGTCCAAA (for *Nematostella vectensis Musk-l*)
Nv_TRK_3R GCCCGAGGAGAGAGTGGACTTTGGACTCATTC (for *Nematostella vectensis Musk-l*)
Nv_TRK_3N CTTTGGACTCATTCAAAAACGTTTGCGGGAC (for *Nematostella vectensis Musk-l*)
C.T.5N_scaffold4 TTCTACGTAGACCTTCTTGCTCAGACATTGT (for *Capitella teleta* NT1)
5′N_FoxD GGCGATCCCGGAATTCCGGACCGGACCAG
5′R_FoxD GCCTGTCATGGTCGTGCAGGGGCGATCCCGG
Sox10_3R_U1 AAGGAGGAGAAGAAACCATTCGTAGAGGAG (Designed by Alexandru Denes)
Sox10_3R_U2: GAGGCAGAGCGATTGAGGGTCCAACAC (Designed by Alexandru Denes)
Sox10_5R_L1: TCTTGTGTTGGACCCTCAATCGCTCTG Designed by Alexandru Denes
Sox10_5R_L2: GCCTCCTCTACGAATGGTTTCTTCTCCTCC Designed by Alexandru Denes
Pdu Trk 5′R_1 GGCACCTTTCCCAGACACAGGGCATCAGGGCC
Pdu Trk 5'R_5_C.W GCAGACTCCATAAAATGTCACAATATTCTCGTG
Pdu Trk 5′R 7 C.W CAGGTAATTCGGATTATCCAACACATGAGG
Pdu Trk 5′N 7 C.W

ACATGAGGTGCATTTAAGGGCATGGTC
Pdu Trk 5′N 8 C.W TCCTAATTCTCGAATAAATCTGATCTGCTT
Pdu Trk 5′N_9 TCTTGGATCAACCTCAATTTCAGGGATTTGACA

Regular primers:

Wnt1_FW ATGGCTCCTCTGCGCCTG
Wnt1_REV TACATTGCCGACCAGTGGTC
EprhinPutative5'Fw TTTGGGCACCAGTGAGGA
Eph_FW GGACACTGACATGGACTGAAGG
Eph_REV AGTGCCACCCTCAGTGAATTCCT
Dcx_fw2 CGGGATGTCACAACGAATGT
Dcx_rev2 TTAGTAGGACTCTTCTTAGGTG
Putative_NVtrkREV_3N1 AAGACCTGAACCACCAGGAACCTT (for *Nematostella vectensis Musk-l*)
C.T.Trk FW ATGTTTTTGAGTGACGTTGCGTGCT (for *Capitella teleta* Trk)
C.T.Trk REV ATCGGCGATGATTTCCAAATATGGTGG (for *Capitella teleta* Trk)
C.T._NT_scaffold4_REV_09011 AGTCAGAGTTGCGGTACAGCA (for *Capitella teleta* NT1)
C.T._NT_scaffold4_FW_0901 ATGCAGCTTGATTGCTGGC (for *Capitella teleta* NT1)
C.t.NT_FWscf_669 ATCGACATGCAGTGGAATCAAAGAAAATCC (for *Capitella teleta* NT2)
C.t.NT_REVscf_669 ATTTTCCATCAACTGAAATCGATCAGAC (for *Capitella teleta* NT2)
C.t.NT_FW_sf_4 ACAATGTCTGAGCAAGAAGGTCTAC (for *Capitella teleta* NT1)
C.t.NT_REVscf_4 TTAGGAAACAGAAGCGTCAGAGTTG (for *Capitella teleta* NT1)
Trk_TK_seqprimerREV_ GGTTCTTTATGAGCACTTTCTGT (to sequence Trk)
PduEGR FW ATGTCCCAAATTAAAATTCACACATTTTAAC
PduEGR REV GACATGGTTACACTGCCGTGGTTACGACCG
PduNeogR_FW CTCAGAGGCGACCCAGGTATTACGTAAG
PduNeogR_REV AATATGTGTGTCCGGGGTAAGTTGGCGGATG
PduNeogR_REV2 CT ATTCTTGGGGAGGGGTCATTTTTGGGTGCC
Pdu_AP-2FW CCGGGATCAGTCAGTGAGGAAGCACGTCT
Pdu_AP-2REV GAGGGCAGCACCCCGACATGACACGGCGT

Nvp75_fw ATGGCTCGAAGTCTCCCTCCGTCGCTAA
Nvp75_rev TCATCTCGGTGTATTTCAAGAAGCAGA
Pdu_p75_FW GCCACCAAGTCACCTCAAAACCCTAACCATGGAT
Pdu_p75_REV ATTCTAAAACACTATACAACTAAAGGTTCGATAGC
Pdu_FoxD3 FW ACAGAGACTGTGTCATTTCCGTCAC
Pdu_FoxD3 REV CTGGGAGGTTTAGAGTCAGAATTGC
PduCLO2A1 FW (I) GGAGAACCAGGACCTACTGGAGTTA
PduCLO2A1 REV (I) CGAATGGAGCTATATCAACAACTGG
PduCLO2A1 FW (II) GTGTACCTGGTCTCCAGGGTATGAA
PduCLO2A1 REV (II) CTGCGCTTGATATCACAGTGAACTT
Pdu_NT_FW GACGGAGGCTGGTCGCAAAAAACATGTCAC
Pdu_NT_REV GGGGGTATCACCGCATATCTTGCAGCAA
Pdu_LillaTRPV FW_2 CGGGATGCTGACATGGAGGTGATGAACCT (for *trpV2*)
Pdu_LillaTRPV REV_2 CAACGCTTTGATTTCTTTCACCAGCCGCT (for *trpV2*)
Pdu_ALEXsubmittedTRPV FW_1 for *trpV1*) TAGACAACGTAGACCAGTTAGTAATGCTC
Pdu_ALEXsubmittedTRPV REV_1 CCAATTGAAGTTTCAGGGACTCTTTCAG (for *trpV1*)
Pdu_TRP FW_2 GGGATCGGGAAGTTCTCCGCGAGGG (for *trpA*)
Pdu_TRP REV_2 TATTCATGGTAGACAGCAGCAGCTATAGA (for *trpA*)
Pdu Brn3 NT 5′ FW GAGGACGCTAGTGACTTTTTAACAC
PduBrn3 CT 3′ REV CTCCGTTTATAAATGTCTTTCTCAATATA
TsumoClone4seqprimerFW ATC ATT GAA GAC CAG AGT G
Runx-fw2 ACGCCTCCGTCACTAACCACAT
Runx-rev ATTTCAATAATGACTAAACCC
Prdm-FW ACACGAGAGGGCTAGTCCAGAAA
Prdm-REV TCATGGCTCTCAAGCAAGCGGTCTCCACC
Trk I FW GCTATCTCTATCTCGACAGTCGCATTAC
Trk I REV AAAATGTCTTGAGGACAGACTCTT (to validate the full-length sequence of Trk)
Trk II FW TCTCCAATCCTCCCAAGATGTTGGGAGTAT (to validate the full-length sequence of Trk)
Trk II REV

ATAAGAGACAGTAATCCGTAATTAAGCAATGA(to validate the full-length sequence of Trk)
Trk 4REV ATAAGAGACAGTAATCCGTAATTAAGCAATGA (to validate the full-length sequence of Trk)
Trk II FW TCTCCAATCCTCCCAAGATGTTGGGAGTAT(to validate the full-length sequence of Trk)
FW_5utr_Trk1_morph TTTAAACCAGGGGGTTGTCATAACAACAC (to perform SNP analysis)
FW_5utr_Trk2_morph GGGGGTTGTCATAACAACACATTTTGGTCA (to perform SNP analysis)
REV_5utr_Trk2_morph GTCTTCAATGATTAGTTCCGTTACATTTCCC (to perform SNP analysis)

II.2 Phylogenetic analysis

The am'oacid sequence data were retrieved from the *JGI genome* portal webserver, from *NCBI* and U*niprot.*

Multiple sequence alignments of protein sequences were generated with *MUSCLE* (http://www.ebi.ac.uk/Tools/msa/muscle/), and *Clustal*W (http://www.ebi.ac.uk/Tools/msa/clustalw2/).

Phylogenetic trees with the maximum likelihood method were computed with *PhyML 3.0* (Guindon et al., 2010), with 1000x bootstrap, (http://www.atgc-montpellier.fr/phyml/). The Phylogenetic trees were visualized with *FigTree.*

II.3 Preparation of constructs for transgenesis

II.3.1 Generation of Minos-LIC-EGFP and pBSMarLIC-EGFP

The generation of the transgenic constructs that I describe in this section was done in collaboration with Dr.Mette Handberg Thorsager and Dr.Maria Antonietta Tosches. Dr. Pavel Vopalensky also contributed to the generation of the final pBSMariLIC-EGFP construct. Dr.Maria Antonietta Tosches and Dr.Mette Handberg Thorsager contributed to the generation of this protocol.

Minos transposon-mediated transient transgenesis was successfully achieved in the lab by Dr.Kristin Tessmar Riable. A construct containing a multiple cloning site, flanked by the inverted repeats recognized by the transposase was available in the lab.

We optimized this original vector to achieve the Ligation Independent Cloning (LIC) for a fast cloning of the regulatory elements upstream the EGFP reporter. The LIC strategy is based on the generation of 12nt-long sticky ends after treatment with the T4 DNA polymerase of the ends of the vector and of the insert (Haun et al 1992). This allows directional cloning of large DNA fragments (i.e. promoters) without the use of restriction enzymes (fig.78)

II.3.1.1 Minos-LIC-EGFP

The original Minos vector, called pMi{3xP3-DsRed} (Pavlopoulos et al 2004, Arendt lab plasmid stock 288) has a 3xP3 synthetic promoter driving expression of DsRed; this expression cassette is flanked by the Minos inverted repeats. This vector was modified by Dr.Florian Raible, with the addition of an ISceI site at the end of the expression cassette and before the right Minos inverted repeat (the new vector was called pMiSce{3xP3-DsRed}, Arendt lab plasmid stock 321).

Since the synthetic 3xP3 promoter does not work in *Platynereis*, and the EGFP is brighter than the DsRed, the 3xP3-DsRed-SV40polyA cassette was replaced with a LIC-MCS-EGFP-SV40polyA cassette with the following procedure:

1) Preparation of the Minos backbone

The *3xP3-DsRed-SV40polyA* cassette was removed from the Minos backbone after digestion with ClaI, and re-ligation. Then, this cloning intermediate was digested with PstI and MluI, and with SpeI and SacI to remove all the other cloning sites from the old multiple cloning site

2) Preparation of the LIC-MCS-EGFP-SV40polyA cassette.

The *MCS-EGFP-SV40polyA* cassette was amplified from the pEGFP-N1 vector (Invitrogen), after some modifications. First, the SmaI cutting site was removed with a KpnI-SmaI double digestion, followed by re-ligation. Then, the LIC sequence was inserted in the BglII site.

The LIC oligos (phosphorilated) were designed with a SmaI restriction site (to open the LIC) and BglII-compatible overhangs:

LIC-F p-GATCCTAGGTTGGTGTCCCGGGAGACCCAGCTTGGCCTGACTGGCCA
LIC-R p-GATCTGGCCAGTCAGGCCAAGCTGGGTCTCCCGGGACACCAACCTAG

-The LIC oligos were mixed to a final concentration of 50ng/ul each, in a final volume of 50ul, and annealed with the following protocol:

94° C 4 min

72 °C 30 min

cool down to 4 °C, with 0.5 C/sec ramping

-The annealed oligos were ligated in the BglII site of the modified pEGFP-N1 vector.

-Finally, the *LIC-MCS-EGFP-SV40polyA* cassette was amplified with primers containing ISceI sites.

-The *ISceI-LIC-MCS-EGFP-SV40polyA-ISceI* cassette was ligated in the Minos backbone using the ISceI sites.

In the meantime, studies in the Raible lab (MPL, Vienna) showed that the Mariner transposon is the most efficient for stable transgenesis (Dr. Florian Raible et al, personal communication). For this reason, we later subcloned the original Minos expression cassette in the Mariner backbone.

II.3.1.2 pBSMarLIC-EGFP

The pBSMarLIC-EGFP vector was generating using a pBlueScript as backbone and cloning the ISceI-LIC-MCS-EGFP-SV40polyA-ISceI cassette flanked by the Mariner inverted repeats.

The Mariner inverted repeats were amplified from the pMos{3xP3-DsRed} construct (courtesy of E. Wimmer, see Horn and Wimmer 2000). This new vector, called pBSMarLIC-EGFP, is the one routinely used for *Platynereis* transgenesis.

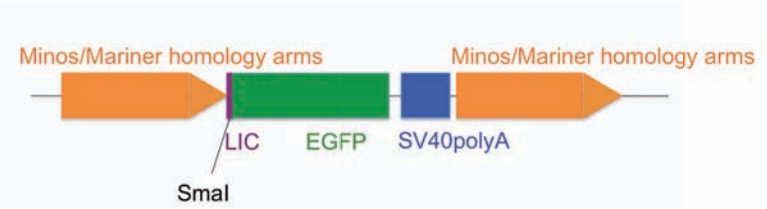


Figure 78. Basic cassette of the Minos-LIC-EGFP and pBSMarLIC-EGFP. Inverted homology arms (Minos for Minos-LIC-EGFP and Mariner for pBSMarLIC-EGFP) are in orange, the EGFP in green, the LIC oligo that allows directional cloning of large DNA fragments is in purple. Polyadenilation signal is in blue.

II.3.2 LIC-based cloning of promoter of interest into pBSMarLic-EGFP

-Genomic DNA extraction

Solutions required:

Lysis Buffer: 10 mM Tris-HCL ph 8.0**,** 0.1M EDTA, 0.5% SDS, 20 ug/ml Dnase.

The genomic DNA was extracted from adult *Platynereis* inbred (line B6.1). The adult worms were quickly dropped in liquid nitrogen, each of them in one 2ml eppendorf tube. I adapted the following protocol from mammalian cell culture preparations.

1) After 10′ blend the sample with mortal and pestle (pre-chilled in liquid nitrogen) until the animal is ground to powder
2) After the liquid nitrogen has evaporated, add 10 volume of the Lysis buffer and incubate 1h at 37C.
3) Add 100 ug/ml of Proteinase K and incubate o/n at 50°C.
4) Cool the solution at r.t. And add an equal volume of Phenol equilibrated with 0.1M of Tris-HCL (ph8.0). Mix the phases and spin at 6500 rpm for 15′ at r.t.
5) Repeat 2x step 4 and mix the aqueous phases obtained.
6) Add 0.2 volume of 10M Ammonium Acetate and 2 volume of EtOH, mix well
7) The genomic DNA precipitates immediately, carefully remove the solution and resuspend in Tris-HCL, ph8.0.

- Cloning

PROMOTER	VECTOR
1) PCR PCR your region of interest from inbred genomic. Use primers with LIC tag overhangs: REV primer: GCTGGGTCTCCCA-specific Sequence (19-26bps) FW primer: GGTTGGTGTCCCA-specific Sequence (19-26bps) Use kapaHiFi polymerase (Peqlab, 07-KK2100-01) *** Prepapre reaction on ice and place the taq at the end. Store immediately the pcr in -20.	1) Preparative digestion with SmaI
2) GEL EXTRACTION Resuspend in 30 ul of sterile water	2) GEL EXTRACTION Resuspend in 30 ul of sterile water
3) T4 DNA POLYMERASE TREATMENT Assemble on ice: T4 DNA polym. Buffer (5x) : 10ul dTTP (10mM) 2.5ul insert all from gel purified (30ul) T4 DNA polym. 1ul (2U) sterile water up to 50ul Incubate in the pcr machine @25 °C 20' Inactivate @75 °C 20'	3) T4 DNA POLYMERASE TREATMENT Assemble on ice: T4 DNA polym. Buffer (5x) : 10ul dATP (10mM) 2.5ul insert all from gel purified (30ul) T4 DNA polym. 1ul (2U) sterile water up to 50ul Incubate in the pcr machine @25 °C 20' Inactivate @75 °C 20'
4) PURIFICATION WITH NRK Resuspend in 30ul of sterile water	4) PURIFICATION WITH NRK Resuspend in 30ul of sterile water
5) T4 PNK TREATMENT T4 PNK buffer (10x) 5ul T4 PNK (10U/ul) 0.7ul dATP(10mM) 5ul DNA all from previous step sterile water up to 50 ul Incubate in the pcr machine @37 °C 30' Inactivate @65 °C 20'	5) SAP PHOSPHATASE TREATMENT SAP BUFFER (10x) 2ul SAP 1ul DNA 1ug sterile water up to 20 ul Incubate in the pcr machine @37 °C 30' Inactivate @65 °C 15'
6) PURIFICATION WITH NRK Resuspend in 30ul of sterile water	6) PURIFICATION WITH NRK Resuspend in 30ul of sterile water

*** To isolate promoter regions I optimized the PCR reaction on genomic DNA with KapaHIFI:

Template (genomic DNA from young larvae) : 0.8 ul

Buffer 5x : 10ul

Primer FW 10 uM: 1.5 ul

Primer REV 10uM: 1.5ul

KAPAHIFI: 1ul

Water to 50ul

Cycling parameters: (primers around 60-63°C)

95°C 5'
5x: 98°C 20''- 57°C 15''- 72°C 2', 10''
20x: 98°C 30''- 55°C 15''- 72°C 2',10''
72°C 5'

Primers used to isolate the regulatory regions of Elav

I performed the PCRs and the cloning of the 3.6 kb regulatory region of *elav* from genomic DNA using primer 1 and 2 (see table below)
PCRs to isolate the 1.6kb *elav* regulatory region from Bac sequence (sequenced by Dr. Peter Hantz) were performed by Dr.Mette Handberg Thorsager using primers 1 and 3 (see table below) during our collaboration.

1)Pdu-elav-R2-LIC GCTGGGTCTCCCATTGTACGAAGGACTAATACAAAGACT
2)Pdu-elav-F2-LIC GGTTGGTGTCCCATGCTTCCGTAGTGCCCATACATACT
3)Pdu-elav-F3-LIC GGTTGGTGTCCCAGAAGGTAAGCACGTGTAGGCTCCTT

7) Perform the ligation indipendent cloning

Vector (PBS_LIC_MARINER:EGFP_SmaI,+dATP,+dephospholilated)	100-150ng
Insert (LIC overhangs_+dTT,+ phosphorilation)	1:3 -1:10 molar ratio
water	up to 20ul
Ligation Buffer (5x)	4ul

Incubate @ RT 1h /@ 16°C o/n.

8) Transform the ligation reaction and screen the colonies
Transform 50% of the ligation in XL1BLUE (not more then 1/10 of the volume)
@37°C 12-14h
-Check the presence of the insert:
digest 100-300 ng of your minipreps with 1ul Isce1 in 10ul for 3h @37°C.
-Sequence the candidate plasmids.
9) Grow for midiprep (max 200ml LB (LURIA-BERTANI) BROTH inoculum).
10) Perform an ENDO-FREE midi/maxiprep.
-The plasmid is ready to inject!

II.3.3 in vitro transcription of the mRNA of the transposase for injection

-Minos

The Minos mRNA was transcribed from the pCS2-Minos vector. This construct was obtained by subcloning the Minos CDS from pBlueSKMimRNA (Pavlopoulos et al 2004) to the pCS2+ vector. pCS2-Minos was linearized with NotI and capped mRNA was synthetized using the SP6 polymerase (mMessage mMachine, Ambion, see protocol below).

-Mariner

The Mariner mRNA was transcribed from the pCS2-Mariner vector. This construct was obtained by subcloning the Mariner CDS from pKhsp82-MOS (Horn and Wimmer 2000) in the pCS2+ vector.

pCS2-Mariner was linearized with NotI and capped mRNA was synthetized using the SP6 polymerase (mMessage mMachine, Ambion, see protocol below).

II.4 Preparation of constructs for mammalian expression of pduTrk, pduNT and pdup75

Primers used:

NheI_SP2xHA_rTrkA_FW TCATCTGCTAGCATGCTGCGAGGCCAGCGGCTCGG
XbaI_REVTrk TCATCTTCTAGATTAAGCAATGATGTCAAGATAA
XhoI_pduTrk-P_FW TCATCTCTCGAGCTAAGCATCCGATGTGCTGTA
XhoI_SP2xHA_rTrkA_REV TCATCTCTCGAGTGCATAGTCCGGGACGTCATAGG
NotI_pduP75-SP_FW TCATCTGCGGCCGCATGCGGCCGTGTGAACCAGGAA
XbaI_pduP75_REV TCATCTTCTAGACTATACAACTAAAGGTTCGATA
NotI_mNT_FW TCATCTGCGGCCGCATCTTTAGACTTCGTGCATGTG
XbaI_mNT_REV TCATCTtctagaTCACCGCATATCTTGCAGCAAGTCC

To generate the vectors I used this protocol:

1) Linearize the vector with the appropriate combination of restriction enzymes o/n
2) Inactivate of the enzymes and purify the vector via gel extraction
3) Dephosporilate of the ends using SAP (shrimp alkaline phosphatase,Thermo scientific, EF0511)
4) Purify of the vector with Nucleotide Removal Kit
5) Perform the PCR amplification of the insert from cDNA with high fidelity polymerase (see protocol above)
6) Purify of the insert via gel extraction
7) Digest the insert with the appropriate enzymes o/n
8) Inactivate of the enzymes and purify of the insert via Nucleotide Removal Kit
9) Ligate o/n 16 C with a ration of concentrations of 1/3 (vector/ insert)
10) Transform of the ligation in chemical competent cells according to manufacturer's instructions
11) Isolate the colonies and preparare bacteria culture with LB (LURIA-BERTANI) BROTH (LURIA-BERTANI) broth medium, grown at 37 o/n
12) Perform minipreparations of DNA to test the presence of the desidered insert

Details of the clonings of the expression vectors:

--To generate *CmvHAHhTrk* I first cloned the coding sequence for rTrkA SP-2xHA-1xHH (kindly provided from Dr. Elia Benito Gutierrez) into the pCDNA 3.1+ using *NheI* and *XhoI* sites.

I then isolated the correct clone and I digested it with *XhoI* and *XbaI.*
I then subcloned the full length of pdu*Trk* without SP (signal peptide) using these restriction sites.
-- To generate *Cmv3xFLAGp75* I cloned the full length of pdup75 without SP downstream the Flag coding sequence in the vector p3XFLAG°CMV-9 *NotI* and *XbaI.*
-- To generate *Cmv3xFLAGmNT* I cloned the mNT sequence without SP downstream the Flag coding sequence in the vector p3XFLAG°CMV-9 using
NotI and *XbaI.*

II.5 Preparation of constructs for the in vitro transcription of neurotrophin full length using the GATEWAY system (protocol courtesy of Dr. Heather Marlow)

The full length of Neurotrophin was amplified with Phusion Taq using the following primers:

preNt+SP_pENTR_TOPO_FW CACCATGTCACAGATCTCA
preNtREVSTOPpENTR_TOPO_FW TCACCGCATATCTTGCAGCAA

4 base pair sequences (CACC) were included at the 5′ end of the primer. This was necessary for directional cloning into pENTR vector.
A stop codon was placed at the end of the full length to avoid the tranlation of a fusion protein between the protein of interest and Venus (present in the final vector). This to avoid misfolding of the proteins, in this case ligands, that would interfere with the receptor recognition.

-General cloning procedure was done as in II.4.
-The PCR product was checked on the gel and purified from gel (see above)
-Cloning in pENTR™/D-TOPO® Cloning Kit (InvitrogenK2400-20) was performed as follow:
1) Assemble this reaction at r.t:
Fresh PCR product 0.5 to 4 ul
Salt Solution1 ul
Sterile Waterto a final volume of 5 ul
TOPO® Vector 1 ul
2) Incubate 5′ at r.t.
3) Transform as in II.4 and isolate positive clones.

- The insert of the pENTR construct was then transferred into the pDEST-Venus (containing a pRN3 backbone (Marlow, Roettinger, Boekhout, & Martindale, 2012; Roure et al., 2007) using the LR GATEWAY reaction protocol:
1) Assemble on ice this recation:
Entry clone (50-150 ng) 1-7 ul
Destination vector (150 ng/ul) 1 ul
TE buffer, pH 8.0 to 8 ul
2) Thaw on ice the LR Clonase ™ II enzyme mix (Invitrogen11791-100) for about 2′.
3) Vortex it briefly 2x2′′
4) Add 2 ul of LR Clonase to the reaction and mix well by vortexing briefly 2x
25°C for 1 h.

5) Add 1 ul of the Proteinase K solution to each sample to ter'ate the reaction. Vortex briefly. Incubate samples at 37°C for 10'.
-Positive clones where then confirmed via sequencing.

III. In vitro transcription of mRNAs

-Capped mRNAs was transcribed in vitro using mMESSAGE mMACHINE® T3 Kit (Ambion, AM1348) as for H2A-RFP mRNA The H2A-RFP mRNA (kindly provided by S. Rohr and Dr. M. Handberg-Thorsager).
1) Assemble the in vitro transcription at r.t. as follow:
nuclease free water up to 20 ul
2x NTP/CAP 10 ul
10x reaction buffer 2 ul
linear DNA template 1 ug
enzyme mix 2 ul
2) Incubate the reaction at 37°C for 2-4 h.
3) Add 1 ul of TURBO Dnase, and incubate for additional 15' at 37°C.
4) Stop the reaction by adding 30 *m*l of nuclease-free water and 30 ul of LiCl precipitation solution.
5) Mix and precipitate for a 'imum of 30', better overnight, at -20°C.
6) Centrifuge at 4°C for 15' at maximum speed.
7) Remove the supernatant. Wash the pellet with 70% ice-cold EtOH, centrifuge again at 4°C for 5' at maximum speed.
8) Remove the supernatant, dry the pellet, and resuspend in 30 ul of 0.22 um filtered nuclease-free water.
9) Measure the concentration with the nanodrop, and adjust the final concentration to the desired value. Analyze the mRNA on gel. Store the mRNA in single use aliquots at -80°C.

IV. Whole mount ISH

IV.1 Whole mount ISH for *Platynereis dumerilii*

IV.1.1 Fixation

For WMISH of 48hpf-5dpf larvae, 4% PFA in 1xPTW was used for 2h
Embryos were then washed in 100% MetOH 3x for 20'

IV.1.2 DIG-labeled probes synthesis

DIG-labeled probes for WMISH were synthesized with in vitro transcription, using linearized DNA or PCR products as templates. The template contained a phage polymerase (T7 or SP6) for the transcription of antisense RNA probes.
Plasmid templates were linearized with restriction digestion using a 5'-overhang restriction enzyme. Usually, 10 ug of plasmid DNA were digested overnight.
The linearized plasmid was purified with the QIAquick Nucleotide Removal kit, following manifacturer's instructions, and eluted in water.

I used the following protocol:
1) Assemble the in vitro transcription as follows:
Linearized DNA template 1 ug
DTT 100mM 2 ul
NTPs 1.3 ul

DIG-UTP 10mM 0.7 ul
Transcription buffer 10x 2 ul
RiboGuard RNAse Inhibitor (Epicentre) 0.5 ul
RNA polymerase (Roche) 1 ul
Nuclease free water up to 20 ul
2) Incubate the reaction for 2-4 hrs at 37 °C.
3) Add 1 *m*l of DNAseI and incubate 15-30 ' at 37°C.
4) Purify the RNA probe with the RNeasy 'i kit (Qiagen, 74104), and elute in 50 ul of nuclease-free water.
5) Check the concentration and the quality of the RNA probe with gel electrophoresis.
6) Dilute the probe to a final concentration of 50 ng/ul with **Hyb-mix** (50% formamide (Fluka, ultra pure), 5xSSC, 50 ug/ml heparin, 0.1%Tween20, 5 mg/ml torula yeast RNA (Sigma), store at -20 °C.

IV.1.3 Standard protocol of WMISH

Required solutions:

20x SSC For WMISH: 3M NaCl (175.32g/l) and 0.3M Dinatrium citrate (88.23g/l), autoclave sterilize

SSCT For WMISH: SSC + 0.1% Tween-20

Staining Buffer pH 7.5 without MgCl2 For WMISH: 100 mM TrisCl pH 7.5, 100 mM NaCl, and 0,1%Tween20

Staining Buffer pH 7.5 with MgCl2 For WMISH: 100 mM TrisCl pH 7.5, 50 mM, MgCl2, 100 mM NaCl, 0,1%Tween20

Staining Buffer pH 9.5 For WMISH: 100 mM TrisCl pH 9.5 (precise), 50 mM MgCl2, 100 mM NaCl, 0,1%Tween20

Rehydratation

1) Incubate the fixed larvae 5' in 75% MetOH-25%PTW
2) 5' in 50% MetOH-50%PTW
3) 5' in 25% MetOH-75%PTW
4) Rinse them 2x5' in 1xPTW

Proteinase K digestion and post-fixation

1) Digest with Proteinase K (100 ug/ml in PTW) without shaking. The length
Of digestion depends on the developmental stage:

<24hpf-24h	30'
>24h-48h	1'
>48h-72h	2'
>72h	5'

2) Stop the digestion with 2x2' washes in 2 mg/ml glycine/1xPTW.
3) Rinse 2x2' in 1xPTW
4) Fix for 20' in 4% PFA/1xPTW

5) Rinse 5x5' in 1xPTW. Transfer the larvae into Eppendorf tubes.

Probe hybridization

1) Prehybridization: remove all the PTW from the tubes, and add 500 ul of Hybmix

2) Incubate at 65°C for 1-2hrs

3) At the end of prehybridization, start preparing the probes

Dilute the probe stocks to a final concentration of 1-2ng/ul, in a final volume of 100ul of Hyb-mix for each tube

4) Denature the probes at 90°C C for 10 '. Transfer immediately on ice

5) Remove the pre-hybridization solution and add quickly the probes

6) Hybridize at 65°C o/n

Washes

1) Warm up the solutions that will be used for the 65°C washes

All the washing steps are perfomed at 65°C

2) Remove the probes, and store at -20°C . Probes can be reused up to 5 times

3) Wash the embryos 15 ' in Hyb-mix

4) Wash the embryos 2x40 ' in 50% formamide-50% 2x SSCT

5) Wash the embryos 20 ' in 2x SSCT

6) Wash the embryos 2x40 ' in 0.2x SSCT

7) Remove the 0.2x SSCT solution and add 1xPTW

Blocking and primary antibody incubation

1) Block in 2.5% sheep serum/1xPTW, for 1hr at r.t. while shaking on a thermomixer at 450rpm

2) Remove the blocking solution, and add 50-100ul of primary antibody solution, prepared in 5% sheep serum/1xPTW

Use the anti-DIG Fab fragments with a 1:5000 dilution, and the anti-acetylated tubulin antibody with a 1:500 dilution

Incubate for one night at 4 C, while shaking on a thermomixer at 450rpm

NBT/BCIP staining

1) Remove the antibody solution, and was 6x5' in PTW, at r.t., shaking on a thermomixer at 450rpm. For a better signal-to-noise ratio, the last PTW wash

can be done o/n at 4°C.

2) Equilibrate the larvae 5' in staining buffer, pH 7.5, without $MgCl_2$, while shaking.

3) Equilibrate the larvae 5' in staining buffer, pH 7.5, with MgCl2, while

shaking.

4) Equilibrate the larvae 2x5 ' in staining buffer, pH 9.5 (precise), with MgCl2,

while shaking. Transfer the larvae in 24-well plates.

5) Prepare the staining solution using 4.5 ul NBT (Roche, final concentration 337.5 ug/ml) and 3.5 ul BCIP (Roche, final concentration 175 ug/ml) per ml of staining buffer pH 9.5. Develop the staining in the darkness.

5) Change the staining solution every day. Staining can take up to 4-5 days.

6) Stop the staining by washing 5 ' in staining buffer pH 7.5. Transfer the

larvae again in Eppendorf tubes.

Secondary antibody, mounting and imaging

1) Wash 2x5' in 1xPTW, at r.t., shaking on a thermomixer at 450rpm.

2) Remove the PTW and block in 2.5% sheep serum/1xPTW, for 30'-1hr.

3) Remove the blocking solution, and add 50-100ul an anti-mouse ''secondary antibody, diluted to a final concentration of 1:500, in 2.5% sheep serum/1xPTW.

4) Incubate 1-2 nights at 4°C, while shaking on a thermomixer at 450rpm.
5) Remove the antibody solution, and was 6x5' in 1xPTW, at r.t., shaking on a thermomixer at 450 rpm.
6) Transfer the larvae in DABCO/glycerol (2.5 mg/ml DABCO in 87% glycerol), shake for 1 hr at 450 rpm. The larvae can be stored in glycerol at 4 C.
7) For imaging, mount the larvae between a slide and a coverslip, with a spacer made by several layers of tape. Use 2 layers of tape on each side.
8) For reflection microscopy (Jékely and Arendt, 2007), use a confocal microscope and illuminate the sample with a 635 nm laser.
Set up the detection window around the same wavelength, to detect the light reflected by the NBT/BCIP precipitates.

IV.1.4 Modified protocols for adult sections, early larvae and treated specimens
This fixative was used : 2mL 0.5M MOPS pH7.4/800uL 25mM EGTA/10uL 1M MgSO4/2.5mL 16% PFA/50uL 20% Tween20 (protocol kindley provided by Dr. Heather Marlow). The embryos were acetylated and the incubation with the probes was done at 63°C for two o/n. Washes were done for longer time. The rest of the protocol was the standard one

IV.2 Whole mount ISH for *Nematostella victensis* embryos (protocol adapted from the original one provided by Dr. Heather Marlow)

Required solutions:
Hybridization Mix: 20ml Formamide, 10mL 20x SSC pH 4.5, 0.1mL Heparin (20mg/mL), 0.5mL 20% Tween-20, 2mL 20% SDS, 0.2mL Salmon Sperm DNA (10mg/mL), 7.5ml of water
Maleic Acid Buffer: for 500ml 5.8g Maleic Acid (0.1 M Maleic acid), 1.46g NaCl (0.05 M NaCl) 500ml of water, ph 7.5. Autoclave sterilize
(10X stock) Boehringer-Mannheim Blocking Buffer: for 50ml 5g Blocking buffer powder (Roche 1096976, 10% w/v), 50ml of Maleic Acid Buffer. Heat and shake to dissolve. Autoclave sterilize

IV.2.1 Fixation
Fixative 1:
1.5 mL 1/3x FSW/0.5 mL 16% PFA/16 uL 25% Glutaraldehyde
Fixative 2:
1.5 mL 1/3x FSW/0.5 mL 16% PFA

1) Prepare fixative in advance and chill on ice.
2) Add Fixative1 and incubate 90''and remove
3) Add Fixative 2 for 1hr at 4C (2hrs for very early stages)
4) Wash 1x in DEPC treated water
5) Wash 2x in ice cold 100% MetOH (pre-chilled on ice)
6) Store at -20°C

IV.2.2 Standard protocol
Rehydratation
Transfer embryos to a 24 well dish and use 500µl for each wash.

(Rehydrate through: 60% MetOH/40% 1xPTw; 30% MetOH/70% 1xPTw; 4 x 100%1xPTw)

Proteinase K digestion and post-fixation

1) Digest with Proteinase-K (0.01mg/ml) without rocking:

The length of digestion depends on the developmental stage:

0-30hpf	5′
30-96hpf	8′
Juvenile Polyps	12′

2) Wash 2x for 3′ in 2mg/ml glycine/1xPTw

3) Wash with 1% triethanolamine in PTw until embryos settle.

4) Add 3 uL/ml acetic anhydride to an aliquot of 1% triethanolamine in 1xPTw and vortex thoroughly. Add to embryos immediately.

5) To the same aliquot of 1% triethanola'e/1xPTw/Acetic anhydride add an additional 3 ul/ml and vortex thoroughly. Add to embryos immediately.

6) Wash 2x in PTw

7) Post-fix the embryos in 4% PFA in 1xPTw, 30′ at r.t.

8) Wash 5x in 1xPTw, removing each wash after the embryos settle.

Probe hybridization

1) Prehybridization: remove all the PTW from the tubes, and add 500 ul of Hybmix.

2) Incubate at 63°C for 1-2hrs.

3) At the end of prehybridization, start preparing the probes.

Dilute the probe stocks to a final concentration of 2ng/ul, in a final volume of 100ul of Hybmix for each tube.

4) Denature the probes at 90°C for 10 '. Transfer immediately on ice.

5) Remove the pre-hybridization solution and add quickly the probes.

6) Hybridize at 63°C for 36h

Washes

Washes are done as for the standard *Platynereis* protocol (see IV.1.3), but at 63°C

Blocking and primary antibody incubation

1) Block in Blocking Reagent (diluited in 1x Maleic Acid) , for 30′at r.t. while shaking on a thermomixer at 400 rpm.

2) Continue as in IV.1.3.

NBT/BCIP staining

1) Remove the antibody solution, and was 6x5′ in PTW, at RT, shaking on a thermomixer at 400rpm.

2) Equilibrate the larvae 5′in AP staining buffer, without $MgCl_2$, while shaking.

3) Wash 2x5′ in AP in staining buffer, with MgCl2, while shaking.

5) Prepare the staining solution using 3.5 *m*l NBT (Roche, final concentration 337.5 ug/ml) and 3.5 ul BCIP (Roche, final concentration 337.5 ug/ml) per ml of staining buffer. Develop the staining in the darkness.

5) Change the staining solution every day. Staining can take up to 4-5 days.

6) Stop the staining by washing 5′ in AP buffer without $MgCl_2$ and transfer the larvae again in Eppendorf tubes.

Secondary antibody, mounting and imaging

As in IV.1.3.

V. Edu incorporation and detection of proliferative cells

V.1 Incubation of *Platynereis* larvae with Edu

To assess the proliferative status of the cells I used the EdU-based Click-iT EdU Imaging Kit (Invitrogen, C10340) and I followed this protocol:

1) Incubated the larvae for 2 hrs in 10 uM EdU at 18°C
2) Wash the larvae 2x in NSW
3) Fix the larvae and store at -20°C

V.2 Detection of EdU

Required solutions:

Click-iT reaction cocktail: 1x Click-iT reaction buffer 430 ul/CuSO4 20 ul/Alexa Fluor azide 647 nm 1.2 ul/Reaction buffer additive 50 ul in 500ul (prepare fresh)

EdU treated larvae were used for immunostainings and WMISH.

After the step 5 of the Standard protocol of WMISH (IV.1.3), the EdU detection reaction was then performed as follow:

1) Remove the PTW from the Eppendorf tubes,
2) Incubate the larvae in in the dark for 30' with 25 ul of Click-iT reaction cocktail
3) wash 2x5'in PTW, shaking at 450 rpm.

The larvae were then incubated in DAPI/PTW for 30', and finally washed 4x5 ' in PTW.

V.3 Analysis of EdU + cells in the confocal scans

The Z-scans of animals incubated with Edu and processed for WMISH were taken at the confocal. The scans were then analyzed in Image J.

The colocalization between the channel containing the scan of the expression of the gene of interest and the Edu was done using the "Colocalization Highlighter" plugin of ImageJ. Although mRNA is found in the cytoplasm and Edu binds to DNA, this was possible because the cytoplasm of the cells at this early stages is a tin layer around the nucleus. Nevertheless the colocalization was checked manually per each gene in each Z-level.

VI. Drug treatments

Treatments with 1-Azakenpaullone (Sigma, A3734) and DAPT (Sigma, D5942-5MG)

The drugs were dissolved in DMSO according to manifacturer's instructions.

The larvae were treated with 1uM, 2uM, 5uM, 8uM of 1-Azakenpaullone and with 2uM, 5 uM, 10 uM of DAPT in 5 ml of FNSW (filtered natural sea water). DMSO was dissolved in the sea water of the control larvae.

VII. Analysis of the co-expression profile

VII.1 Generating average expression per each gene of interest

Average expression patterns were generated as described in Tomer et al. (2010).

WMISH of the genes of interest were performed with properly staged

48hpf larvae. Gene expression patterns of the larval trunks were acquired withconfocal microscopy (reflection microscopy, Jékely and Arendt 2007) together with the acetylated (or tyrosinated) tubulin staining. Each confocal stack was then registered on the reference axonal scaffold, using an ImageJ macro developed by Dr. R. Tomer according to Tomer et al. (2010). Afterwards, the registered axonal scaffold was compared to the reference scaffold for a quality

check. Average was made only for the confocal stacks with a good registration of the scaffold. The average was done by R.Tomer and was based on minimum three good registered scaffolds from three different individuals. The average expression images used for the co-expression profile in this study were generated during this work and from these former members of the lab:

Brn3 ISH and imaging experiments done by me and image processing done with Dr.Raju Tomer
Ngn ISH and imaging experiments done by me and image processing by Dr.Raju Tomer
ColA ISH and imaging experiments done by me and image processing by Dr.Raju Tomer
Neog ISH and imaging experiments done by me and image processing done with Dr.Raju Tomer
SoxE ISH and imaging experiments done by me and image processing done with Dr.Raju Tomer
Phox2 ISH and imaging experiments done by me and image processing by Dr.Raju Tomer
FoxD ISH and imaging experiments done by me and image processing by Dr.Raju Tomer
Vchat ISH and imaging experiments done by Dr. Peter Hantz and image processing by Dr.Raju Tomer
Olig ISH and imaging experiments done by Dr. Peter Hantz and image processing by Dr.Raju Tomer
Pax6 ISH and imaging experiments done by Dr. Peter Hantz and image processing by Dr.Raju Tomer
Nk6 ISH and imaging experiments done by Dr. Peter Hantz and image processing by Dr.Raju Tomer
Msx ISH and imaging experiments done by Dr. Peter Hantz and image processing by Dr.Raju Tomer
Islet Dr. Raju Tomer and Dr. Antje Fischer
Zic Dr. Raju Tomer and Dr. Antje Fischer
Pax3/7 ISH and imaging experiments done by Dr. Peter Hantz and image processing by Dr.Raju Tomer

VII.2 *In silico* analysis of the molecular fingerprint of the lateral neuroectoderm

The analysis was performed using the averaged PrImR expression patterns.

-The first step was the identification of the genes expressed in the lateral columns *msx+*.

I performed the co-localization analysis of the PrImR averages for the genes of interest using an ImageJ macro developed from R. Tomer. This macro uses the "*Colocalization Highlighter*" plugin of ImageJ and displays where two genes co-localize at the cellular level. The co-localization is given for each Z level.

-Next, I selected the genes expressed in the *msx t*erritory. To generate multiple co-expression data for a given co-localization, for example between *msx* and gene *X*, first I have eliminated the channel information for the expression of the single genes (for example *msx* : green and *X*: red). In this way only the image of the co-localization between *msx* and gene *X* was stored in 8 bit format.
-Next I assessed new co-localizations between the previous co-localization and a new gene, using the same macro as above. Co-localizations were verified manually looking at the entire stacks.

VIII. Immunoistochemistry

I developed this protocol to obtain high quality immunofluorescence and detection of difficult epitops (such as the ligands) :

1) Wash 2x embryos in F-NSW
2) Fix in cold ZAMBONI fixative (for 20ml: 14.2ml Na2HPO4 0.2M, 4ml of NaH2PO4 0.2M ,Picric acid 1.3%, from 1% to 2% of PFA). The concentration of PFA depends on the protein and on the stage: usually I increase the concentration when I deal with old stages (>5days)
Fixation time at r.t.:

25'	24h-48h
30'-40'	48h -72h
50'	5days
1h, 30'	2weeks

3) Wash 5x 10' in PBS_1X
4) Treatment with protenase K (stock 20mg/ml) in PBS_1x as for the ISH protocol (this step can be performed also before fixation)
Time of proK incubation:

30''	24h-38h
40''	48h
55''	72h-5days
1', 20''	2w

5) Wash fast 3x in Glycine 2mg/ml in PBS_1x
6) Wash 3x5' in PBS_1x/ Triton_ 0.1%
7) Postfix in ZAMBONI fixative at r.t. for 20' (24h-72h) and 40' (72h-2w)
8) Wash 5x10' in PBS_1x/ Triton_ 0.1%
7) Block 40'-1h at r.t. in 0.5x or 1x_Blocking Reagent in PBS_1x/Triton_0.1%
(the concentration of the Blocking Buffer depends on the antibody. Add 1% BSA fresh if the antibody gives background)
Incubate also the I Antobody in the same Blocking Buffer
8) Incubate 2h at r.t. and then o/n at 4°C with the I Antibody
9) Wash 5x10' in PBS_1x/ Triton_ 0.1%
10) Block 40'-1h at r.t. in 1x_Blocking Reagent in PBS_1x/Triton_0.1%
Incubate also the II Antobody and the DAPI in the same Blocking Buffer
11) Incubate 2h at r.t. and then o/n at 4°C with the II Antibody
(Normally I use 1/500 Dylight from Jackson laboratories, see above)

12) Wash 3x5' in PBS_1x/ Triton_ 0.1%/ Wash 3x15' in PBS_1x/ Triton_ 0.1%
13) Postfix briefly (10') at r.t. in ZAMBONI fixative
14) Wash 3x5' in PBS_1x/ Triton_ 0.1%
15) Equilibrate in Glycerol/ PBS_1x in this order, shaking for 5-10' at r.t.:
20% Glycerol/ PBS_1x- 40%- 50%- 70%, store the samples in 80% Glycerol + DABCO

IX. Vibrotome sections of *Platynereis* adults

Cross-sections of adult *Platynereis* were obtained with the Vibrotome.
This is the protocol I have adopted from the amphioxus protocol provided by Dr. Elia Benito Gutierrez and Manuel :
1) Fix the adults in ZAMBONI fixative with 2% of PFA, o/n at 4°C
2) Wash 5x5' with PBS
3) Embed the samples in 3% low melting agarose in PBS using a 24 well plate
4) When the gel is polymerized cut a squared block of agarose containing the specimens and glue it on the vibrotome holder as in fig.79.

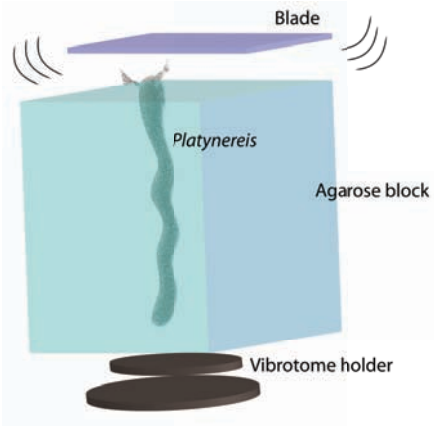


Figure 79. The agarose block containing fix specimen of *Platynereis*

5) Fill the vibrotome chamber with cold PBS
6) Start cutting, parameters used:
Size: 100-300 um for ISH and 300 um for SEM
Amplitude: 1.40 mm
Speed : 50mm/s at the beginning to cut the excess of agarose
26mm/s to cut samples

X. Scanning electron microscopy

I have developed this protocol. Part of this protocol was done at the Core Facility of Electron Microscopy (Bioquant, Heidelberg University) with the help of Dr. Ingrid Haußer-Siller.
1) In my experiments animals at 5days, 2 weeks and adults were fixed in Zamboni fixative (see above). Other fixative where also tried (such as normal 4% PFA with or whithout 3% of Gluteraldheide), but the Zamboni performed the best.
2) Wash 5x 5' in PBS
3) Dehydrate the samples with series of washes from 20% to 100% of Aceton.
4) At the Core Facility the samples (in actetone) were critical point dried in a Leica CPD300.

5) The samples were put on holder for the SEM microscope and using forcepts the skin was manually removed from the speciments.
Different preparations were obatained with less or more removalof the skin
6) The speciments were then spot with Gold (10nm) with the Leica EM MED020
7) The specimens were then analyzed at the electron microscope: at the EM LEO1530, using the SE2 detector

XI. Expression and purification of the pre-neurotrophin and mature neurotrophin full length and polyclonal antibody production

XI.1 Cloning in different expression constructs

The pro (without signal peptide, SP) and mature form of the neurotrophin was cloned in different vectors as indicated in the table below. In addition, the anzymes used are indicated. pTEM 80, 82 and pTEM-28M-SUMO are for *E.coli* expression. PFast Bac_EGT for expression in *baculovirus*.

Expression vectors:	Cloning strategy (amplify the NT sequence with primers containing the sites for the following restriction ezymes)
pTEM 80	NcoI-NotI
pTEM 82	NcoI-NotI
pTEM-28M-SUMO	BamHI-NotI
pFastBac_EGT	NcoI-NotI

To clone I have designed these primers with the overhanging for specific restriction enzymes:

BamHI_pduNT_FW TCATCTGGATCCATGGTGATAGAACCCAACCCTGACA
NotI_pduNT_REV TCATCTGCGGCCGCTCACCGCATATCTTGCAGCAAGTCCA
BamHI_mNT_FW TCATCTGGATCCGTTTGTGACAGTGTCTCAGA

I followed the cloning protocol explained above.

X.2 Expression of the proteins and purification (these experiments were done with the help of Dr. Hüseyin Besir and Ines Racke of the Protein expression and purification core facility at EMBL that provided expertise, reagents and protocols and helped me expressing and purifying the proteins.

Transformation of the construct:
1) Transform 5ul of the constuct in 50ul of the E.coli (BL21 (DE3) CodonPlus RIL)
2) Spread on plate containing kanamaicin and cloranphenicol (50ug/ml) and grow o/n at 37C
3) Preculture one or two colonies in 400ml of LB (LURIA-BERTANI) broth with the right antibiotics, o/n at 37 C
Induction of protein expression:
4) Inoculate the preinoculum in 6L total of LB (LURIA-BERTANI) broth with the antibiotics, 37°C

5) Grow until the OD (optical density) of the solution reaches 0.4-1
6) Incubate the inocula at 28h for 1h
7) Add IPTG 0.5mM to induce the expression of the protein
8) Incubate the inocula at 28h for 3h
9) Spin ar 4000 xg for 20'
10) Resuspend the pellet in PBS 1x

Lysis

11) Lysate the pellets obtained from the previous step for mNT and proNT. I used Standard 1xTris buffer (50mM Tris/HCl pH 8.0, 250mM NaCl, 20mM Imidazole)
12) Add 1ml of protease inhibitor (25X)/ DNAse/ MgCl2/Lysozime, Incubate 30' shaking at r.t.

Sonication and ultracentrifugation

13) Sonicate the sample 5x30'' on ice.
14) Ultracentrifugate the samples at 40000 rpm for 45' at 4C.
15) Wash the pellet 3x in ~25mL of 1xPBS/0.1% Trition X-100

The pellet was then resuspended in 20 ml of 6M Gua/1xPBS.

Protein purification

Ines then filtered the samples and applied to 5mL NiNTA column (AEKTA purifier). The samples were purified using these buffers:

Running buffer	**Elution buffer**
1xPBS	1xPBS
6M Urea	6M Urea
10mM Imidazole	300mM Imidazole
5mM βME	5mM βME

The elute fractions were then tested for the presence of the protein loading on a protein gel (see below)
The gel was then stained with Coomassie Brilliant Blue (Sigma)
The fractions containing the purified proteins were pooled
A buffer exchange was done to obtain a solution at 3M Urea, a concentration that can be injected in the rabbits.

Digestion of the tag SUMO

The Sumo fusion tag was cleaved at 4C o/n with 1: 200 of SenP2 protease. Ines tried different methods to elute only the recombinant mNT and proNT after digestion with no success. Therefore, we kept the solution where there is the recombinant protein of interest and the Sumo.

XI.3 Antibody production protocol

The Laboratory of Animal Resources (LAR) at EMBL did the injections in the rabbits and collection of the bleeds.
250 ul of the protein solution in 3M Urea was injected (proteins concentrated: 500 ug/ml).
The protocol included different steps for the length of around 100 days.
In total 4 boosts with the antigen and adjutants were performed.
Time interval between the boosts and the collection of the bleeds

DAY 14	1 boost
DAY 28	2 boost
DAY 38	I BLEED to test

DAY 56	3 boost
DAY 66	II BLEED to test
DAY 84	4 boost
DAY 94	III BLEED to test

The serum from every bleed was collecting stirring it with a glass rod (done by the facility). The bleeds were then centrifuged for 30' at 5000 g. The surnatant was collected, some was stored at -80C, some was diluited 1/1 with sterile Glycerol, aliquoted, and store at -20°C. The aliquots were tested via western blot and immunofluorescence.

XI.4 SDS-PAGE and Western Blot experiments

Solutions and reagents required:

RIPA buffer: 0.1% SDS, 0.5% DOC (deoxycholate, Sigma, D6750), 1% Nonidet® P40 (AppliChem,A1694), 150mM NaCL, 1mM CaCl2, 50mM Tris-HCL ph7.5 (store at 4°C).

2xSAMPLE BUFFER: 10% Glycerol, 0.01% Bromophenol Blue, 62.5mM Tris-HCL ph 6.8, 3% SDS

1xSDS-PAGE Electrophoresis Running Buffer: 25 mM Tris, 192 mM glycine, 0.1% SDS, and pH8.3

1xTransfer Buffer without SDS: 25 mM Tris, 192 mM glycine, and pH8.3)

20% of MetOH can be add to improve transfer of small proteins

SDS would interfere with the transfer of small proteins

For Coomassie blue gel staining:

Fixing solution: for 50ml: 20ml MetOH, 5 ml Acetic Acid, water.

Coomassie Blue staining solution: 25ml MetOH, 50mg Coomassi Brilliant Blue (BRILLIANT BLUE G, Sigma Prod. No. B5133), 3,5ml Acetic Acid, water.

Destaining solution: for 50ml: 25ml MetOH, 3.5ml Acetic Acid, water.

30% Acrylamide/Bis Solution: (Bio-Rad, 161-0154)

TEMED: (Bio-Rad, 161-0801)

APS: (Bio-Rad, 161-0700)

Non-Fat Dry Milk: (Bio-Rad, 170-6405)

Blocking Buffer: 5% dry milk in 1xPTW

XI.4.1 Preparation of protein extracts from *Platynereis*

- For adult extracts I used adults without gut content (around 5 adults)

-For the larvae I used around 2 full batches (400 larvae). I set up this protocol.

1) Put the animals in 2ml eppendorf tubes, wash 2x in FNSW

2) Incubate the animals on ice for 5'

3) Add cold RIPA buffer (300ul for 5 adults, 100ul of RIPA buffer + 50ul of Sample buffer for 400 larvae)

4) Incubate on ice for 2'

5) Vortex 3x 10''

6) Add proteinase inhibitors (EDTA-free Protease Inhibitor Cocktail

Roche, 04693132001 and Phosphatase Inhibitor Cocktail Tablets, Roche, 04906837001

7) Homogenize on ice with mortar and pestle, vortex 10'' few times while homogenizing the sample

8) Sonicate 3x for 30'' (low duty)

9) Centrifuge at max at 4C for 15'
10) Recover the surnatant and store at -80

XI.4.2 SDS-PAGE Electrophoresis

To assemble the gels I used the glass plates with lanes of 1.5mm and the apparatus from Bio-Rad.

I Prepare a 12% SDS-PAGE following protocol from BioRad:

	Stack	Resolving Gel		
	4%	7.5%	12%	X%
30% Acrylamide/Bis	3.3 ml	25 ml	40 ml	3.3(X%) = (A)* ml
0.5 M Tris-HCl, pH 6.8	6.3 ml	–	–	–
1.5 M Tris-HCl, pH 8.8	–	25 ml	25 ml	25 ml
10% SDS	250 µl	1.0 ml	1.0 ml	1.0 ml
Distilled deionized water	15 ml	48.5 ml	33.5 ml	73.5 - (A)*
TEMED	25 µl	50 µl	50 µl	50 µl
10% APS	125 µl	500 µl	500 µl	500 µl
Total volume	25 ml	100 ml	100 ml	100 ml

Degas before polymerization

* The letter A designates the volume of 30% Acrylamide/Bis Solution required to produce the specified percent gel (X%).

Prepare the samples as follow (do not load more then 20ug per lane):
Dilute the sample in 1x Sample buffer + 5% Beta-mercaptoethanol
2) Boil at 85C for 5'
3) Incubate 2' on ice
4) Centrifuge at max, 5' at r.t.
5) Load your samples on the gel, load also 5ul of SDS-PAGE PageRuler Plus Prestained Protein Ladder. (Termo Scientific, 26619)
5) Run the gel for 2h at 200V (40mA constant)

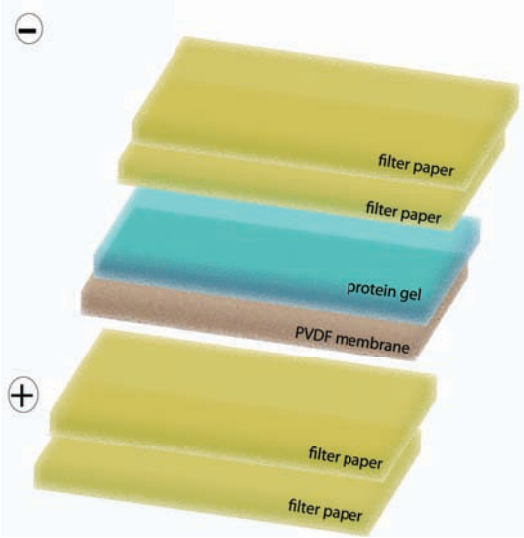
Coomassi Staining (to check the expression of the protein):

For recombinant proteins the gel was visualized with Coomassi Brilliant Blue using this protocol:
Wash the gel 2x in dH2O2
2) Incubate the gel 1h in Fixing solution shaking at r.t.
3) Incubate the gel 1h in Staining solution
4) Wash 2x 20' in Destaining solution at r.t.
5) Wash 2x in dH2O2
6) Add glycerol 2% and it is ready to acquire as picture

XI.4.3 Transfer proteins from the gel to the PVDF membrane:

The tank blotter system was from Bio-Rad
1) Disassemble the gel apparatus and recover the gel, removing the stacking part with a scalpel.
2) Wash 2x in dH202
3) In a box with Transfer buffer assemble the transfer sandwich in the conductive apparatus in

this order:



-2 layers of filter papers

-The gel
-The PVDF membrane (previously activated in MetOH for 30'')

-2 layers of filter papers

4) Place the sandwich in a the tank between the electrodes,
and submerged under the Transfer buffer (Burnette 1981,Gershoni et al. 1985, Towbin et al. 1979).
5) Add the pre-cooled gel block offered by the tank blotter system.
6) Transfer for 1h, 100V.

XI.4.4 Blocking and incubation in I and II Antibody

Disassemble the transfer tank
2) Wash the membrane 1x in dH2O2
3) Pour Red Ponceau (ATX Ponceau S red staining solution, 09276, Fluka**)** to the membrane, shake 10'.
4) Wash 2x in dH2O2. Red Ponceau will bind to all the proteins on the membrane, allowing to check the efficiency of the transfer.
5) Wash 2x in transfer buffer
6) Block shaking 1h at r.t. in Blocking Buffer
7) Incubate also the I Antibodies (in my case the sera from the rabbits) in Blocking Buffer at the desired concentration (I used 1:250).
8) Incubate the membrane with the I Antibody solution prepared in step 7, o/n at 4C shaking.
9) Wash 6x6' at 550 rpm in PTW
10) Repeat from step 6 to 8. This time use a II Antibody (I used the anti -Rabbit conjugated to Horseradish Peroxidase (HRP), 1:5000).

XI.4.5 Signal detection

The membrane was washed 6x6' at 550 rpm in PTW
The detection was done using the ECL system (RPN2132,Amershan Bioscience, Isacsson, U. and Watermark, 1974, Whithead, 1979), according to manufacturer's instructions.
The resulting light was detected on autoradiography film.

XII. Injection of *Platynereis*

XII. 1 General conditions for injection of *Platynereis* zigotes

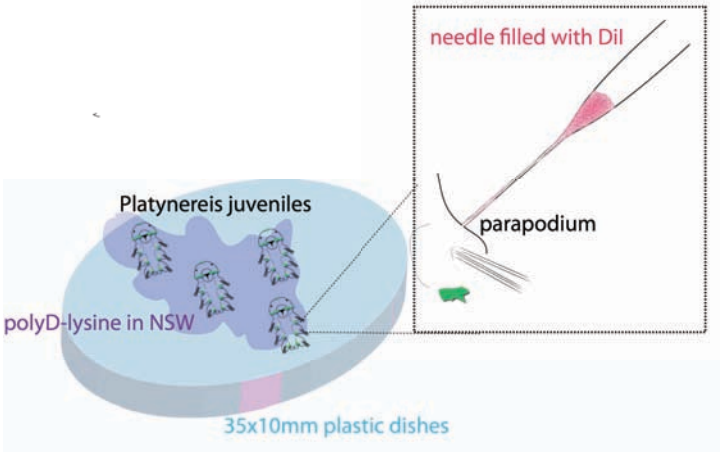
Injections were performed according to the protocol established in the lab (Tosches, unpublished), using a Zeiss Axiovert 40C inverted microscope,
equipped with a micromanipulator and a microinjector (in this case, FemtoJet from Eppendorf).
Needles used were pooled from glass capillaries (1mm diameter,
with filament, Harvard apparatus) using a Sutter needle pooler.
These were the parameters that I used:

PULL	515
HEAT	025
VELOCITY	120
TIME	120

For transgenic constructs the injection solution was:
250ng/ul of the pBSMariner_Elav
200ng/ul of mRNA for H2AmCherry
80 ng of Mos mRNA in a final volume of 5ul
For mRNA:
250ng/ul of H2AmCherry and mYFP were injected in a final volume of 5ul

XII.2 Injection of juveniles with DiI

I developed the protocol to inject DiI in *Platynereis* trochopore larvae and juveniles :
The same microscope as in XI was used.
1) Pour a solution containing 70% of a solution of 50% NSW/50% NSW with 7.5%MgCl2 + 30% of poly-D-Lysine (initial concentration of 0.5 mg/ml) on a nunc plate (35x10mm), creating a bobble of about 100-200ul.
2) Select some animals put them in the bobble.
3) The animals will sink and will adhere to the plastic dish (this take approximately 10' using fresh aliquots of polyD-lysine)
4) With the macromanipulator position the needle above the desired point of injection and exert a small pressure to be able to inject gently underneath the cuticle.



5) Quickly recover the animals in NSW

6) Leave the juvenile in NSW for 1-2h at r.t.
The retro-labeling of axonal tracts was then tested at the confocal mounting the speciments as described below. The imaging of the trochophore larvae was done approximatelt 15-30' after injection.

XIII. DiI in vivo labelling

To label passively the sensory cells and axon tracts in the trochophore larva I used this protocol:
1) Incubate the larvae with a solution of 1mg/ml of CmDiI in NSW and incubate them between 2h-4h at r.t. slowly rotating (I used a total volume of 1.5ml in an eppendorf tube of 2ml).
NB: When labelling older stages, (> 40h) then treat the alive animals with Proteinase K, this will help the penetration of the DiI in the neuroectoderm
2) Wash the embryos transferring them 4x in clean dishes with NSW
3) Mount the alive embryos for imaging as explained below

XIV. Time lapse movies *of Platynereis* larvae

XIV.1 Generation of time lapse movies

For time lapse imaging of developing *Platynereis* embryos, zygotes were injected with H2A-mCherry and mYFP mRNAs at final concentrations of 250 ng/ul. Larvae were raised to the desired stage at 18°C. Larvae with a normal morphology and a strong fluorescence were selected.
If >24h the larvae were left for around 10-20' in 50% NSW/50% NSW with 7.5 % $MgCl_2$. This solution inhibits muscles contraction.

The classical protocol to stop animal movements is to embed it in low melting agarose. This approach was not ideal in my case. I indeed observed that the embedding of *Platynereis* in agarose interferes with the correct formation of the appendages, and leads to misdeveloped animals.
This is most likely due to the mechanimal block that the agarose matrix exerts on the lateral domains of the developing trunk. To avoid this problem I developed the following method. With this approach the animal is efficiently immobilized during the recordings, with no effect on the appendages formation.

This protocol is based on the observation that the presence of polyD-lysine in the water slows down the ciliary beating of the larvae; this occurs because the cilia adhere to the surface coated with polyD-lysine.

1) Spread the polyD-lysine *** (0.5 mg/ml) on the slide and on the coverslip.
2) Incubate it at r.t. for 5'
3) Wash it pouring 2x FNSW (filtered natural sea water)
4) Remove the excess of water
5) Place 30ul of 50%NSW/ 50% of NSW with 7.5% $MgCl_2$ in which the embryo is immersed on the
slide prepared with 2x of tape per each side. Place the coverslip on top

6) Seal the slide with mineral oil (Sigma, M5904) to prevent water evaporation during the recording

***Aliquots of polyD-lysine are stored at -20°C
They can be re-used although a loss of the efficiency is observed over the time

For imaging, a Leica SPE confocal microscope was used, with a 40x oil-immersion objective. The 488 nm and 532 nm lasers were used, respectively, to excite the mYFP and H2AmCherry. Images were acquired with a 512 x 512 pixel resolution. Specific parameters for live imaging are indicated in the main text of this work.

XIV.2 Movies analysis

Images were analyzed with Image J and in Imaris.
Brightness and contrast were adjusted equally on all images. Cells were tracked manually on the original dataset using the MTrackJ plugin (Meijering
et al., 2012).

APPENDIX

A1. The early steps of nervous system differentiation in the trunk of *Platynereis*[10]

A1.1 Embryonic pioneering neurons

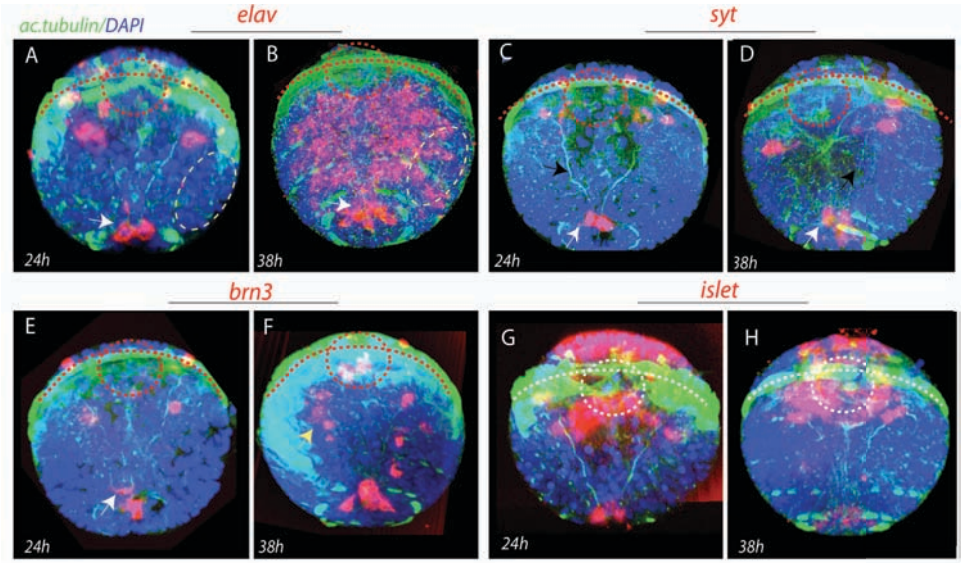
In order to gain a comprehensive view on the trunk neurodevelopment in *Platynereis* and compare with the development of the vertebrate nervous system I started the analysis of the trunk neurogenesis at early stages of embryogenesis.

In vertebrates before the onset of the neural crest in the lateral neuroectoderm, primary neurons, comprising sensory and motoneurons originate in the trunk. Compared to the secondary ones that emerge later, these neurons are few, large and grow axons soon after they arise. They are motoneurons, interneurons and sensory neurons and form early local circuits for escape response (fig.18). (Kimmel, C.B. and Westerfield, M.,1990), (Westerfield, Mcmurray, and Eisen 1986), (Myers, Eisen, and Westerfield 1986), (Tanaka et al. 2011). In *Platynereis* between 20-24h no differentiated neurons are present in the lateral neuroectoderm, that is highly proliferative (fig.a3).

Nevertheless, I found that few neurons are already differentiated in the trunk at this stage. A cluster of 3 cells is present in the posterior part of the trunk (pygidium, in the *cdx+* domain) and few neurons per each side in the more anterior neuroectoderm (fig.a1A,C,E,G,I and scheme in fig.a2D). Those are differentiated neurons and accordingly express *elav* and *synaptotagmin* (white arrows in fig.a1A and C, as reported by a previous PhD in our lab, Denes et al. 2007).

Interestingly I found that some of these neurons express terminal differentation sensory markers as well as, such us the LIM homeodomain *islet* and the POU IV domain *brn3* (fig.a1E and G).

10 The WMISH and the Edu treatments of this session were performed with the help of Dr.Mette Handberg-Thorsager and Franziska Gruhl (see Results, paragraph I.1, footnote 1)



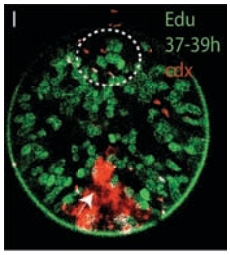


Figure a1. The primitive trunk nervous system of *Platynereis dumerilli* (A-D) : WMISH shows the neural differentiation at 24h (after gastrulation process is over) and at 38h in respect to the developing pioneering axons of the ventral nerve cord. *Elav* (A-B) and *synaptotagmin* (C-D). **(E-H):** WMISH for *brn3* (E, F), *islet* (G, H).White arrows indicate the *elav/syt/brn3/islet* + neurons of the pygidium, the dashed yellow circle in A and B shows the lateral neuroectoderm not differentiated that start to express *elav* at 38h (B). The dashed red line indicates the prototroch; the dashed red circle (white in G-I) indicates the stomodeum field. I: WMISH for cdx after Edu pulse of 2h between 37-39h. White arrow indicates the Edu-, cdx + posterior cells, most likely including the *elav/syt/brn3/islet* + cells. All the WMISH are confocal images obtained by reflection confocal microscopy (Jékely and Arendt, 2007).

A1.1.1 Incubation of the live amrbyos in DiI to label primary neurons and their axons

DiI is is a lypofilic dye commonly used to label cells in other organisms (Kulesa and Fraser 2000),(Hager and David 1997) (Mccauley and Bronner-fraser 2003).

DiI labelling has been extensively used to determine the first neurons and their axons appearing during development, and also follow the formation of the nervous system in later stages (Fritzsch, Fariñas, and Reichardt 1997; Mirnics and Koerber 1995; Wang and Scott 1999).

Hence, In order to label these early neurons and follow their projection patterns I optimized a DiI-retrograde fluorescent labelling method. I incubated the embryos between 20-22h in sea water containing DiI and I was able to label at least one of the neurons in the pygidium (pink arrowhead in fig.a2A). The DiI+ primary neuron protrudes cilia on the surface of the embryo (green arrowhead in fig.a2A), the cilia are likely responsible for the uptake of the DiI from the sorrounding medium.

With this technique I was able to retro-label the axons connecting the pygidial neuron to the anterior most neuroectoderm (labelled passively with DiI, yellow arrowheads in the same figure).

The cells in the telotroch (posterior ciliary band) are multiciliated and uptake the DiI as well.

The DiI + pygidial neuron is serotoinergic (fig.a2C, D). The presence of this posterior serotoninergic neuron has been described in other annelids (McDougall et al. 2006) and the innervation of the ciliary bands by sensory-serotoninergic neurons is quite spread as well in ciliated larvae (B and Hay-schmidt 2000).

A1.1.2 The pygidial neuron and its projetions visualized using transgenesis

Next, in collaboration with Mette Handberg Thorsager we cloned 1.6 upstream regulatory region of elav (isolated from a BAC clone by Peter Hantz, scheme in fig.a2I) into pBSLicMar.

pBSLicMar is a DNA vector generated by collaborative work between Dr. Mette Handberg Thorsager, Dr.Maria Antonietta Tosches, Dr.Pavel Vopalensky and me. I have contributed to the set up the strategy of the clonings starting from the original construct present in the lab and generated by Dr. Kristin Tessmar-Raible and to generate the initial constructs (details in Materials and Methods).

This vector harbours Mariner homology arms for the recognition of the Mariner transposase (Sasakura et al. 2003). It has been shown that it is an efficient transposase system to obtain transgenesis in different invertebrate systems (also in *Platynereis*, communication from Dr. Kristin Tessmar-Raible and Dr. Florian Raible. Details of the cloning and the constructs are in the Material and Methods section).

We injected the pBSMariner containing the regulatory region of elav in the presence of the Mariner mRNA transposase. To be able to label all the nuclei of the embryo we also injected the mRNA for the expression of a fusion protein H2A (Histon2A)-mCherry (mRNA courtesy of Dr. Mette Handberg-Thorsager and Silvia Rohr, original vector courtesy of the Gilmour lab at EMBL). I also established a method to image live animal using confocal microscopy and poly-D-lysine to immobilize the larvae (see Materials and methods).

Using this approach we were able to visualize elav+ neurons, as in the WMISH (fig. a2E-H). We also observed real-time the formation of the growth cones of those pioneering axons; extending from the posterior *elav*+ neurons (white arrowhead in fig.26A) and projecting towards the anterior neuroectoderm (*elav* + axons indicated with yellow arrowheads in the fig. a2E,F). These are the first axons labelled with acetylated tubulin.

These data suggest that a small and simple circuit is already present in the trunk at 20h, and it is composed of primary neurons, among them one is serotoninergic and likely modulates the ciliary beating of the ciliary band as the other serotoninergic cells of *Platynereis* (Gáspár Jékely, unpublished). The pioneer axons of the ventral nerve cords might contribute to establish a primitive scaffold and define routes for coming axogenesis in the larval nervous system, as for vertebrates (Hjorth and Key 2002).

A1.1.3 Putative primary motoneurons at 38h

At 38h I could observe *elav* + neuronal precursors (still *syt*-) spanned along the neural plate (fig. a1B), including cells in the periphery (compared the cells marked with dashed white circle in fig.a1 A and B).

At 38h a chain of additional primary neurons are most likely differentiating along the pioneering axons of the ventral nerve cord; they start to express *brn3* (fig.a1F), *lhx3/4* and *hb9* (information from Mette Handberg-Thorsager) as in the neural tube of fishes (Detrich et al. 2010). This domain corresponds to the one where also secondarly motoneurons will arise later in development (Denes et al. 2007).

Conversely, although *elav* + neurons are present in the lateral neuroectodem (yellow dashed circle in fig.a1B and D and faded brow area in fig.a2J and K) no differentiated neurons are present (for a more detailed analysis of the neurogenesis in the trunk.

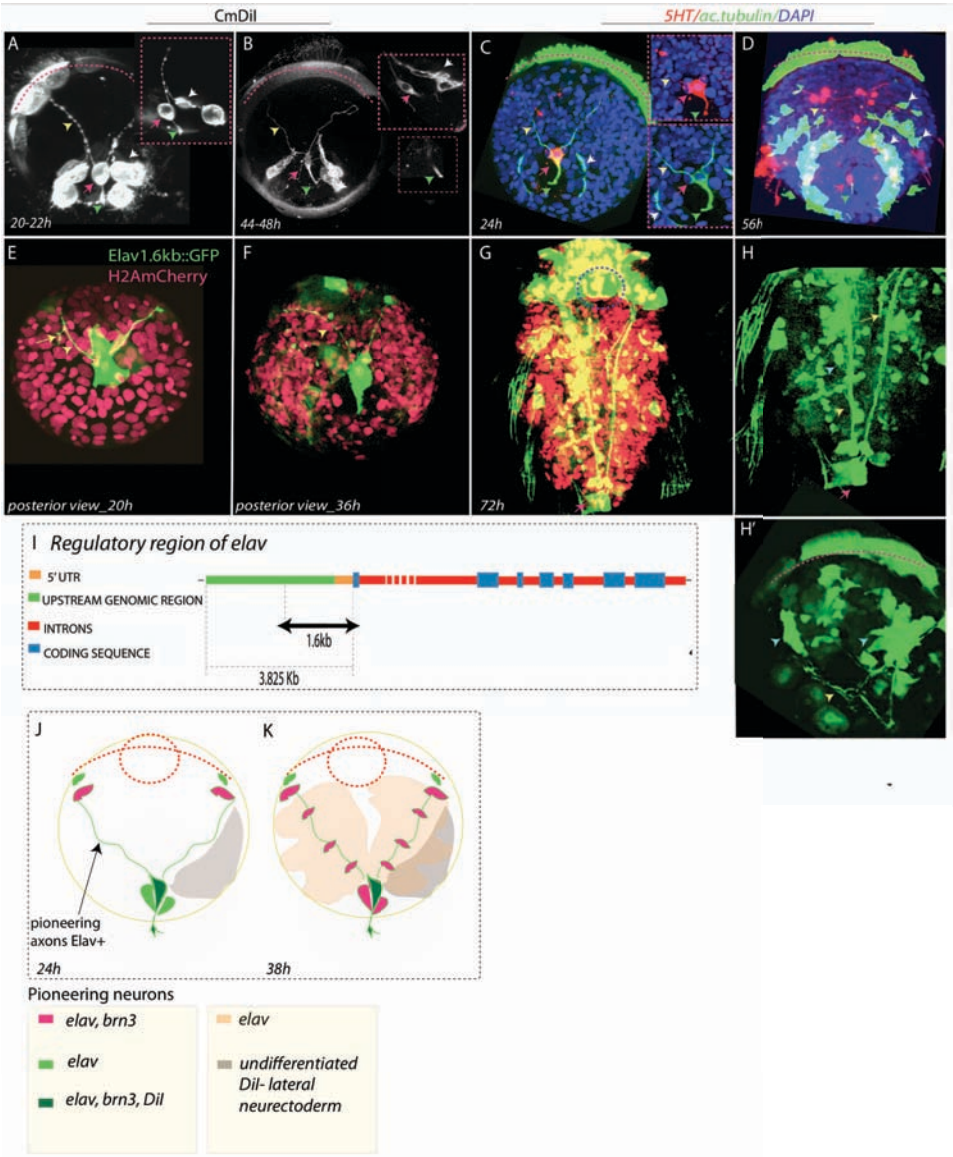


Figure a2. DiI retrograde labeling and transient transgenesis reveals the first trunk circuit in the alive trochophore A-B: : Z-projection of a confocal scan of a live individual after CMDiI retrograde labeling performed between 20-22h (A) and between 44-48h (B). One of the differentiated neuron in the pygidium is labeled (pink arrow), projecting the growing axons anteriorly (yellow arrowhead). CmDiI + telotroch cells are indicated with a white arrow. The inset shows a close up of the *DiI* + neuron, surrounded by the cells of the ciliary bands. This cell protrudes cilia on the surface (green arrow). 70-80% of the labeled animals showed this specific labeling in two independent set of experiments where 20 larvae where used**. C-D:** immmunostaining at 24h (C) and at 56h (D) for sertonin (5HTm, red), acetylated tubulin (green) and dapi (blue). In C the insets show a close up on the neuron in the pygidium, serotonin + (upper inset). **E-F-G:** Z-projection of a confocal scan of a live individual at 20h (E), 36h (F) and 72h (G) injected with a mixture containing a transgenic construct with 1.6k genomic region upstream elav and mRNA of nuclear marker H2AmCherry. **H:** a close up of the trunk of fig.G.light blue arrow indicates elav+ neurons in the trunk nervous system. Yellow arrows in E and H indicate the tip of growing axons.**I:** genomic structure of *elav* indicating exons (blue) and introns (red) and the 1.6kb upstream region used to recapitulate the *elav* expression. **H':** Z-projection of a confocal scan of a live individual injected with Elav 1.6kb::GFP.
J, K : schematic drawings summarizing the results of fig.1 and fig.2. It shows the primitive trunk nervous system of *Platynereis dumerillii* at around 24h and 38h. Dashed pink lines in A-D and red in I, J indicate the prototroch; dashed red circle in J and I indicates the stomodeal field.

Here, I would like make a clarification about the transgenesis : in the transgenic embryos few neurons are labeled. This happens because the integration of the trangene occurs likely in a mosaic fashion (only in some cells). Where the

transgene is not interegrated, then it most likely remains episomal, therefore a dilution of the signal is observed after cell proliferation. These transposase-based transgenic constructs have been used in other invertebrates, such as planarias (González-Estévez et al. 2003), achieving always mosaic patterns as well. Unfortunately we still don't know how to improve the integration efficiency and our laboratory, together with other *Platynereis* laboratories are making efforts to optimize this. The screening of the animals in which the integration occurred in the germline will be also performed, in order to generate a stable line.

Nevertheless, per each injection round, in around 10% of the injected embryos, many neurons *elav* + were still present in the neuroectoderm of older larvae. Between 44h-48h we detected increase number of *elav* + neurons (fig.a2H') and almost all the nervous system was labelled at 4days, as detected by WMISH (fig. a1,a2).

In the injected animal at 4days I could observe that the first neurons in the pygidium do not disappear, they are most likely integrated in the juvenile trunk nervous system. I reported similar results injecting a longer genomic fragment (3.6 Kb) upstream elav. [1]

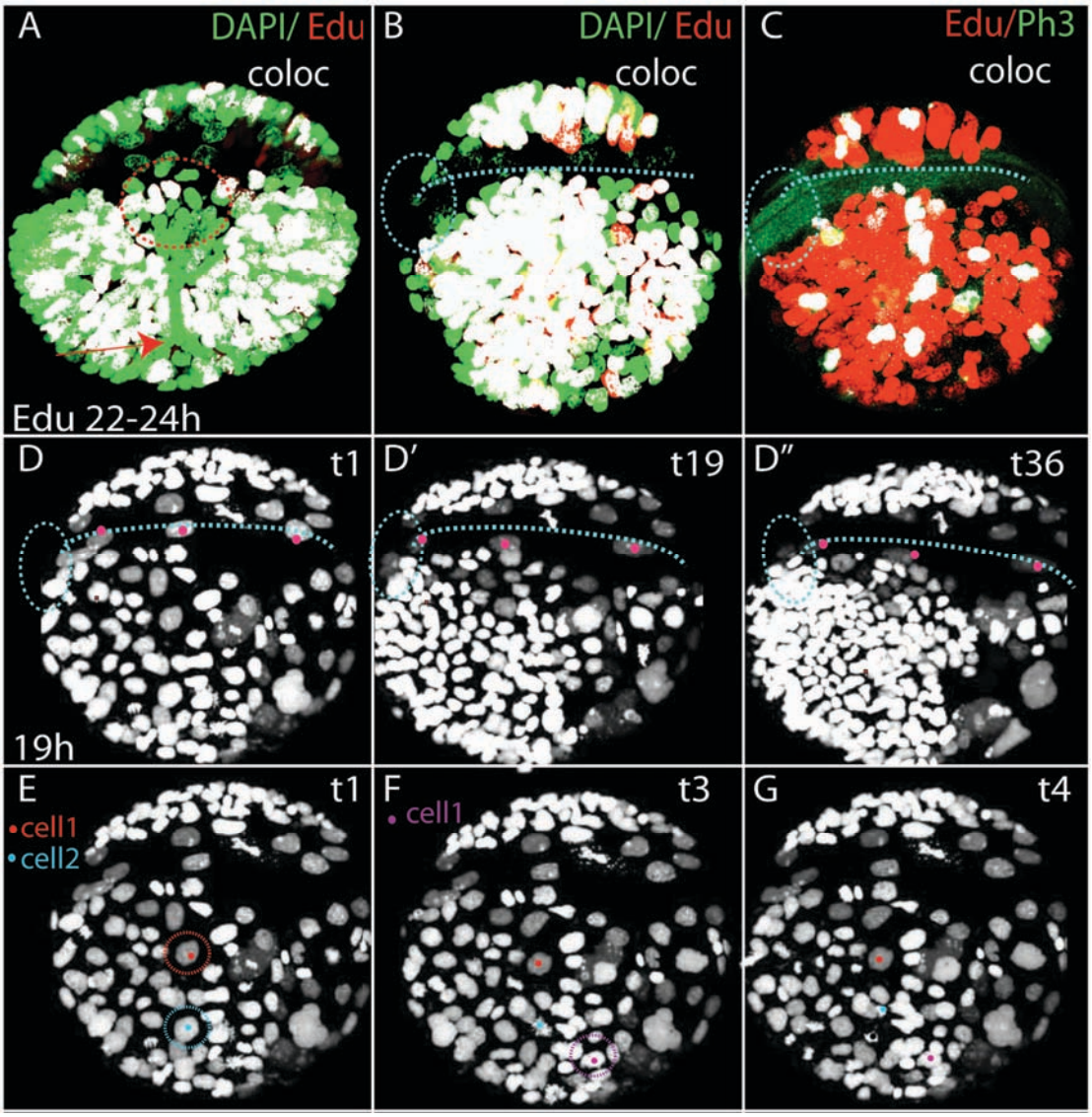
These data show that simple neurons, likely sensory-motor neurons are present in the early nervous system of *Platynereis,* far before the formation of lateral sensory neurons (differantiating only between 38-40h). The sensory-effector (serotoninergic) neuron of the pygidium might represent an ancestral type of neuron, that as the other serotoninergic neurons of the brain innervate the locomotory apparatus of the worm (the ciliary band) and might be implicated in modulating the motor response(Jékely et al. 2008), (Jékely 2011). The cells of the ciliary band are effector cells, they indeed are the swimming apparatus of the larva, but are also sensory neurons (expressing the sensory marker *trpV* channel). These cells, together with the pygidial neuron might represent an ancestral form of circuitry that does not rely jet on the amplification of the signal via interneurons, but already involving different type of neurons (see Discussion).

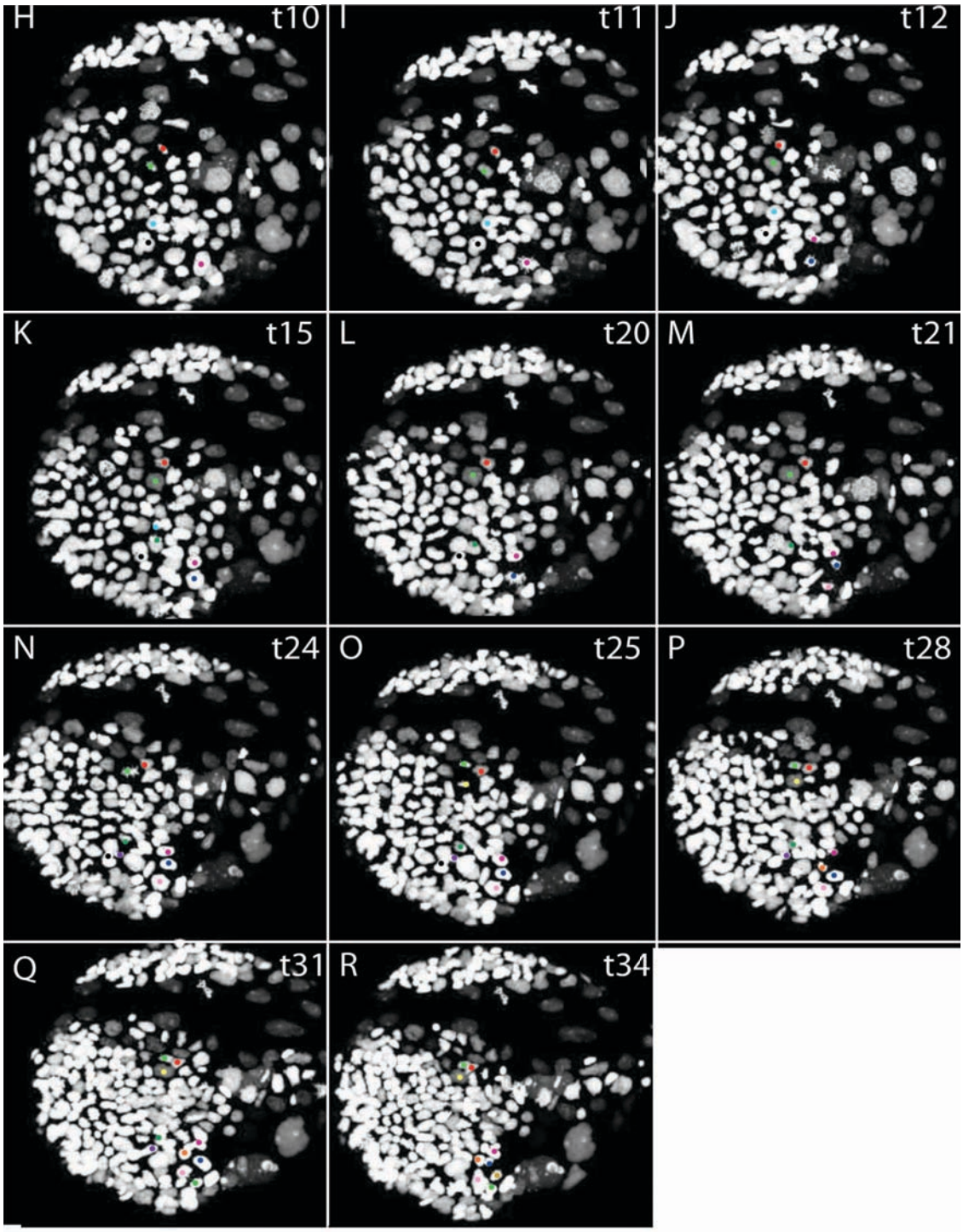
A.2. Time lapse movies reveal intense proliferation of the lateral precursors

In order to investigate the behaviour of the cells of the lateral neuroectoderm at early stages I performed time lapse movies. To achieve this I injected the mRNA for the nuclear marker H2AmCherry (as explained in the main text, Results, paragraph II.1). With this approach I followed the cell behaviour from 19h onwards. This experiment has been repeated at least twice, giving similar outcomes. During this period the trunk neuroectoderm undergoes extensive convergent extension movements (as described in Denes et al. 2007). In addition to this, substantial proliferation occurs (fig.aD-D''). Indeed, starting from few large precursor cells in the monostratified epithelium at around 19h, the neuroectoderm becomes tick and compact, composed of many small cells.

[1] The development of this transgenic tool to label and follow neurons and our pioneer experiments are extremely northwardly, because it the first tool of this kind to be used to study the neurodevelopment of *Platynereis*.

At 24h I have defined the neural plate border-like region as the domain where all the neural plate border genes are expressed (Results, paragraph I.1, fig.26 J). This domain comprises the posterior most portion of the second row and the third row of the ciliary band (prototroch). In vertebrates neural crest derives from the homologous domain, the neural plate border, and migrate extensively. To deepen our understanding on the lateral cells in the *Platynereis* lateral neuroectoderm and compare to the vertebrates, I investigated whether they migrate. This analysis was perfomed at different stages (including later stages, fig.47), and I could not observed cell migration in the lateral neuroectoderm. As previously mentioned, I could observed only the intense proliferation of the precursors. I followed some of the cells and I could appreciate that different types of divisions occurs. Some of the neuroblasts divide asymmetrically, producing one cell that does not divide any more and one small intermediate precursor cell that then divide asymmetrically after long time once or more times (red cell#1 and blue cell #2 in fig.b3). Other precursors divide symmetrically and produce intermediates, some of these stop dividing and other continue for at least 2 more generations (purple cell#3 in fig.b3). This mechanism has been observed also in the brain (Tosches et al, unpublished), therefore it represents a general mechanism for neurogenesis in *Platynereis.* A similar but not identical mechanism has been described in *Drosophila* (Boyan and Reichert 2011).


A
DAPI/ Edu
coloc
Edu 22-24h
B
DAPI/ Edu
coloc
C
Edu/Ph3
coloc
D
t1
19h
D′
t19
D″
t36
E
t1
cell1
cell2
F
t3
cell1
G
t4



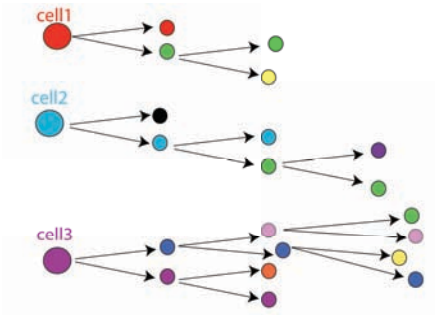


Figure a3. Proliferative cells between 22-24h and analysis of the proliferation behaviour of the precursors in the lateral neuroectoderm between ~19h-26h. A-B: ventral (A) and lateral view (B), co-localization between Edu (red) and DAPI (green). Red arrow in A indicates the cells of the early midline and the pygidial cells Edu-. **C:** co-

localization between Edu (red) and Ph3 (phospho-Histon 3 in green). Ph3 marks the dividing cells. **D-D'':** Lateral view. Confocal z-projection of the live embryo at the beginning of the time lapse (around 19h), after …and at the end of the time lapse. The pink dots label the cells of the prototroch (ciliary band). In A-D'' dashed light blue line indicates the ciliary band, dashed light blue circle indcates the stomodeum **E-R:** Confocal z-projections of the live embryo at different time point (t) of the time lapse. The time interval is 10'. Each cell analyzed is marked with a colored dot, as well as its progeny. The initial progenitor cells are indicated wth a dashed circle as well. The drawing on the bottom left of the figure represents the division patterns observed per each cell.

A.3 Dcx: a new marker to follow the neurogenesis in the trunk

The neurogenesis in the trunk of *Platynereis* occurs mostly through apical-basal divisions, as shown in Denes et al. 2007. The proliferating neuroblasts are found on the surface of the neuroepithelium and the differentiating neurons deeper into the neuroectoderm.

I isolated a new marker to follow the neurogenesis: dcx (doublecourtin). Dcx is a microtuble-associated protein transiently present in dividing neuroblasts and immature neurons (Brown et al. 2003; Horesh et al. 1999; Kim et al. 2003; Couillard-Despres et al. 2005). Accordingly, in *Platynereis* I was able to follow the differentiating neurons, as they express *dcx* and *elav*.
Besides the early differentiated neurons present in the pygidium at 30h (green arrow in fig.a4C), combining these two markers it was possible to observe that the neurogenesis proceedes from an anterior to posterior. For instance, the anterior most neuroectoderm starts to extinguish progenitors before the other segments, as revealed following the wave of *dcx +/Edu –* cells emerging first from the anterior most segments (after Edu pulse between 28h-30h and 37-39h,yellow arrows in fig. a4 B-E). Only later, more posterior cells start to differentiate (Edu pulse between 46-48h and 48h-50h, compare fig.a4B to E, H and K and compare C with F and I, pink arrows indicate proliferating progenitors along the A-P axis).
During development more and more progenitors in the elongating larva start to be committed towards the neuronal fate and accordingly accumulate *dcx* and *elav* mRNA. By 50h then still some proliferating progenitors (*dcx+/Edu +* and *dcx-/Edu +)* are present on the superficial neuroectoderm per each segment, but intermingled with *dcx+ /elav +/Edu* - immature neurons, as differentiated neurons (*syt +*) are deeper in the ventral neuroepithelium (fig.a4 J,K; according to Denes at al., Cell, 2007).

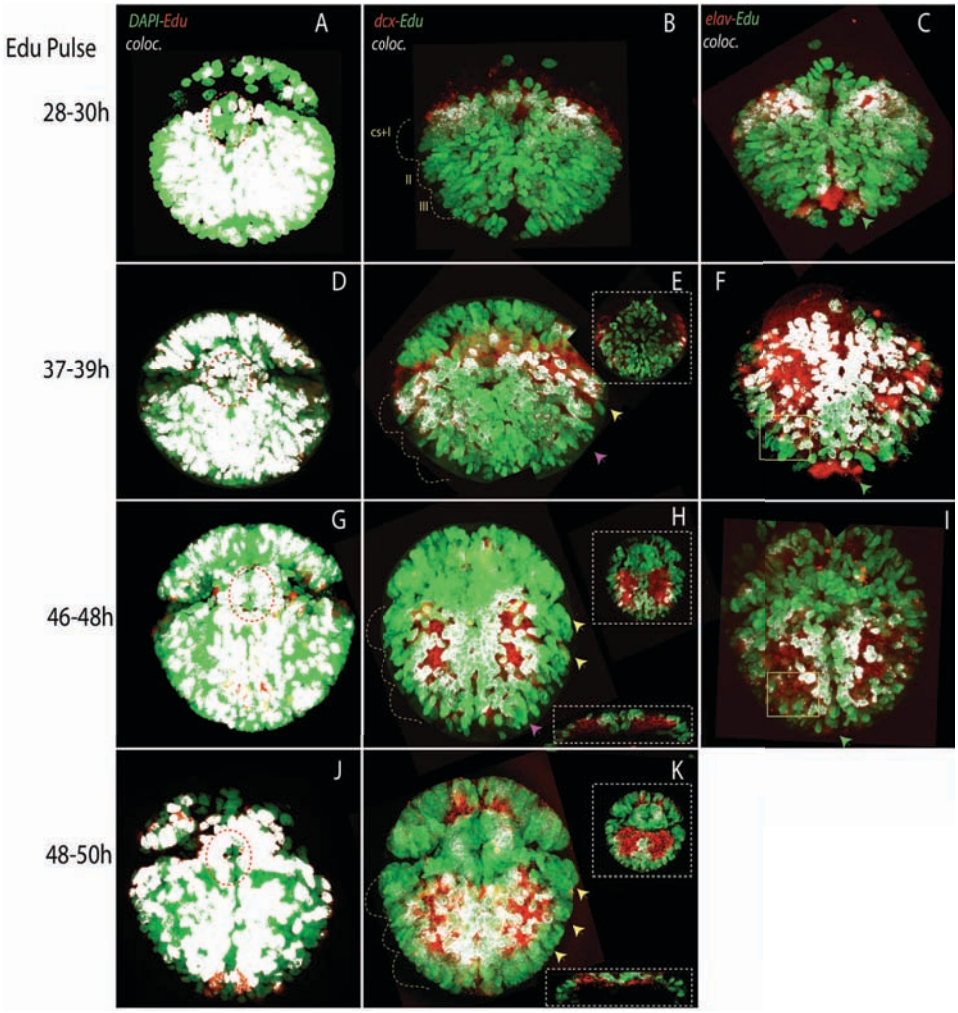


Figure a4: the neurogenesis along the A-P axis in *Platynereis*. WMISH on embryos treated with Edu for 2h, between 28h and 50h. Co-localization is shown in white. **A,D,G, J:** co-localization of Edu (red) and DAPI (green) after the pulses of Edu for the time indicated. **B, E, H, K**: co-localization of Edu (green) with *dcx* mRNA. Per each stage the trunk segments are indicated with yellow dashed lines and roman numbers (I,II, III). *Cs*: criptic segment. In all the pictures yellow arrows indicate the segment where cells that are positive for the *dcx* and /or *elav* and negative for Edu start to arise. The pink arrows indicate the posterior segments where the cells that still Edu +, among which some *dcx* and /or *elav* + start to be visible. The co-localizations are obtained with the plugin of Image J. The insets on the top right in E indicate the lateral domains. Insets in H and K show the deeper layer in the neuroectoderm where all neurons are *dcx*+ and Edu-/. The insets on the bottom right in H and K show a virtual crossed section of the neuroectoderm, where Edu + cells are found in the midline at every level and in the more lateral site less Edu+ cells are found on the surface. C, F, I : co-localization of Edu (green) with *elav* mRNA.

B. Additional data mentioned in the main text

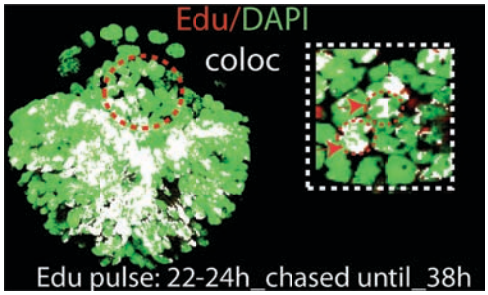


Figure b.1 Typical dilution of the Edu signal after pulse chase experiments. Embryo pulsed with Edu between 22-24h and chased until 38h. Z-projection of confocal scan. Ventral view. The cells undergoing massive proliferation show diluted Edu signal (close up, red circle and arrows). Conversely the cells in the neuronal midline shows homogenous chromatin staining, likely because they do not proliferate extensively (as visible in the live imaging movies).

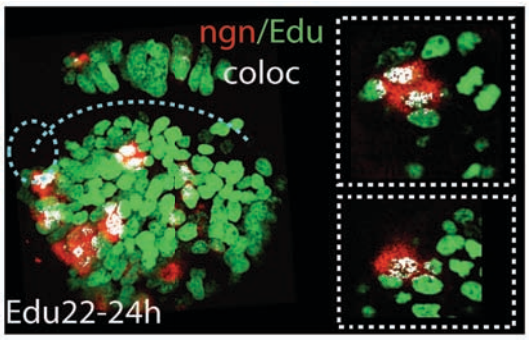


Figure b.2. Ngn/Edu + cells after Edu pulse between 22-24h. Lateral view. In the insets: close up of some of the *ngn* + cells

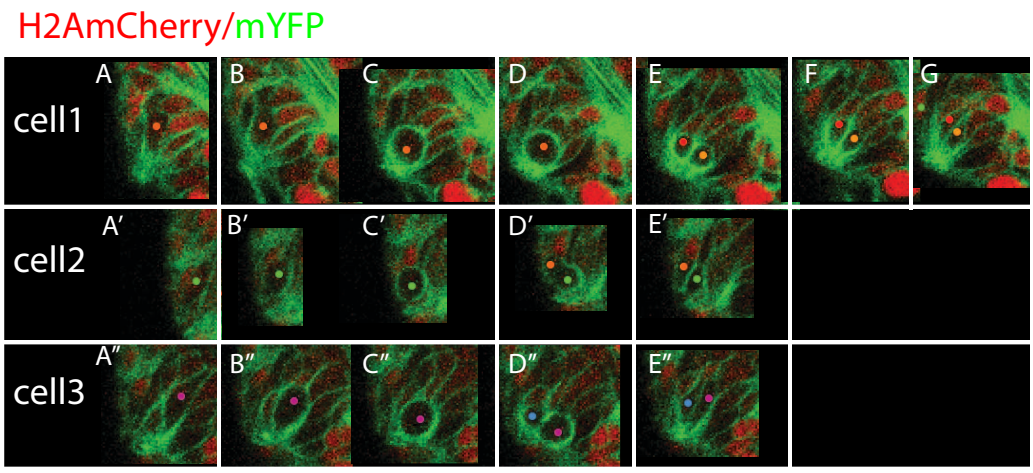


Figure b3. Interkinetic nuclear migration during cell division in the peripheral ganglia at 3 days. The pattern of division of three cells is shown (see main text for details, paragraph II, Results).

pduTRK 834 aa

MLGVLVLVTLTLITRATTFDLCDHACTCKPLSIRCAVGEQLGRIPHVRSKSYMGNVTELIIEDQSALTSIG
PDDLSPYKQLHKLTIRRCGLKGLAQSVFAHNRHLISMDLKDNEIELFPLSLTLHLHLQDLILDGNPLACNC
SHKWLQLLQKKKLGVLGNSLTCVLERQELALADVNITECQIPEIEVDPRRISINASSDIMITCSLLKGSPT
PELTWDVSEVVSNFTVESSISAEGRREETLMLNDVQVEDSGEIYCLATNEAGRTRRPVTITVYGPPKLLSF
KFHANAFYISFTFEVIGAPTPELRWLKNGVPLHVEEKESMNLKIQEQTDYYVRGNLQFTNPPTNTHNGNYT
LIVKNKYGIHNMTTKAIFLENSPVLSSGKPRTGPIRKLKPAPNTEGSSVEGMERDDHVTVMDDTEDLRRSD
RKKTFIIAAAVASGLTLTLGLSLLFACCVRRYKRLKSDVRRQESPFQSMLPLXAMTASPHARQLFTRETMP
LNAPHVLDNPNYLSKQEPFTGGASICHVKRKQIRFIRELGEGAFGRVYLGLCYALHDSEHPTMVAVKTLKE
TRMDDNRRDFDREAELLTTLHHENIVTFYGVCIDGEQMMMIFEYMENGDLNNFLRCHGPDALCLGKVPGVV
VVSAGGDGATESAHKEPHFTLSIPQLLHVCKQIAAGVEYLASQHFVHRDLATRNCLVGDQLTVKIGDFGMS
RDLYSTDYYRVGGHTMLPVRWMPPESILYRKFTVESDVWSFGVVLWEIFTYGKQPWYELSNHEVIQHIQVG
KLLECPRVCPQDIFKIMLGCWQSHPHDRLTMAQIHKQLQHMCSNQPVYLDIIA

Signature

Signal peptide	SP	To guide the protein towards the ER
Leucin reach domain	LRR + flanking cyst domains	Implicated in the binding with the ligand
Immunoglobulin-like domain	IgC-Like domains	The IgC2 (II) is responsible for the binding to the neurotrophin
	Transmembrane domain NPNY	 Juxtamembrane site : binding site for PTB containing adaptors (Src)
Intracellular TK domain	TK domain : LGEGAFGRV…K FVHRDLATRNCLV DLYSTDYYR	 ATP binding sites Conserved Aspartate (D) Active catalytic site Autophosphorylation domain
Docking site	PVYLDIIA	Binding to PLC gamma

pdup75 393 aa

MDYLTVAVSSVLLLLAHGPVSILTEVEMRPCEPGISDHLTSGCCSVCPAGTGVVEACESG
RDTLCSPCDDGSTYSSSQSHEERCKNCRVCDENAKLISPCTITNDTVCECRQGYYFEKYE
KICKRCDSCPEGFGMVKKCTPHHNTKCKRCPEGLFSDRRSHKGCRYCSVCRPDQLTLHVC
NYREDTVCMEMPVPVTSSDLQSSNLDSNNEGSKTSHGDIIPVYCSLLGALVLGLIIYVII
AHRRRQAANKLNRTGADGASPTSKQGSDSGVYVEPDQKQALLAKRFLDLAPSTIRVVEGM
LMTAGNNASDRRHLSTKLGYNTSQTANIEIRSRQEGCSPSHCLLADWGATDGATCGELVK
ALRTIARDDIARILQPGNSDARRNGDAIEPLVV

Signature

Signal peptide	SP	To guide the protein towards the ER
4 Cysteine-rich domains	CRD	NFR/NGFR family cysteine-rich domains with 6 cysteins each
	Transmembrane domain	
Intracellular death domain	DD domain	Involved in apoptosis and inflammation through their activation of caspases and NF-kappaB

pduNT 284 aa

MSQISHTLLVLLSFTTAIQCMVIEPNPDNAIPQFTSTHAHWRRHRRQRHNSNNRRHQRHHK
NSIRHRQLNDLNEVEPPSQVTDMQFTYSPRVVLSPHKPLLPPVALLEPPEDDMDDFALAAV
AISRNSTEESPQGSQDHSQGHTQGHTQGHAQGHAKGRSKRSLDFVHVPVCDSVSEWVERHE
ARNMWGHKVEVLQEIDIGGARVNQYFYETKCREEKSACVGIDTQQFYSVCKNKHVWAYAKI
RTSAGDEGWNLIKIPGSCNCALFKKKVRRVSLLDLLQDMR

Signal peptide	SP	To guide the protein towards the ER
N-glycosilation site	NSTE in the pro-sequence	Putative glycosilation site in the pro-sequence implicated in the secretion and degradation protection
Furine cleavage site	RSKR	Pro-peptide cleavage site to yield to the mature form
Intracellular death domain	Cys Knot	Mature protein containing the core of Cysteins that induce the 3D structure

Figure b4. Sequence analysis of pduTrk, p75 and NT. The domains are indicated

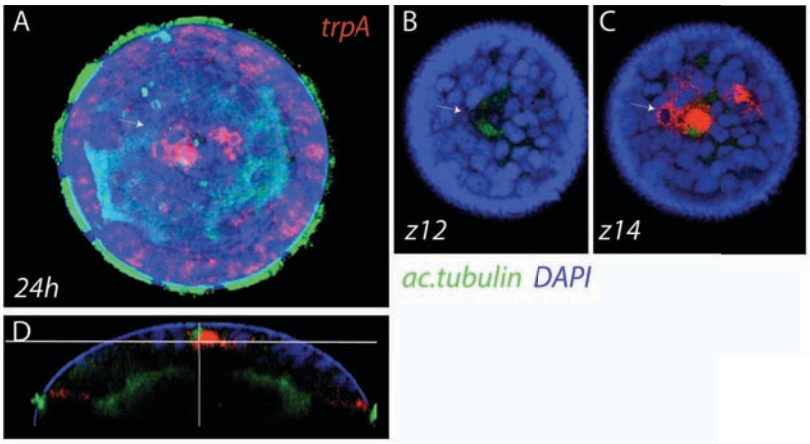


Figure b5. Expression of trpA in the sensory cells of the apical organ and in the ciliary bands. A, B, C are apical view.D is a virtual cross-section showing the expression in the apical organ

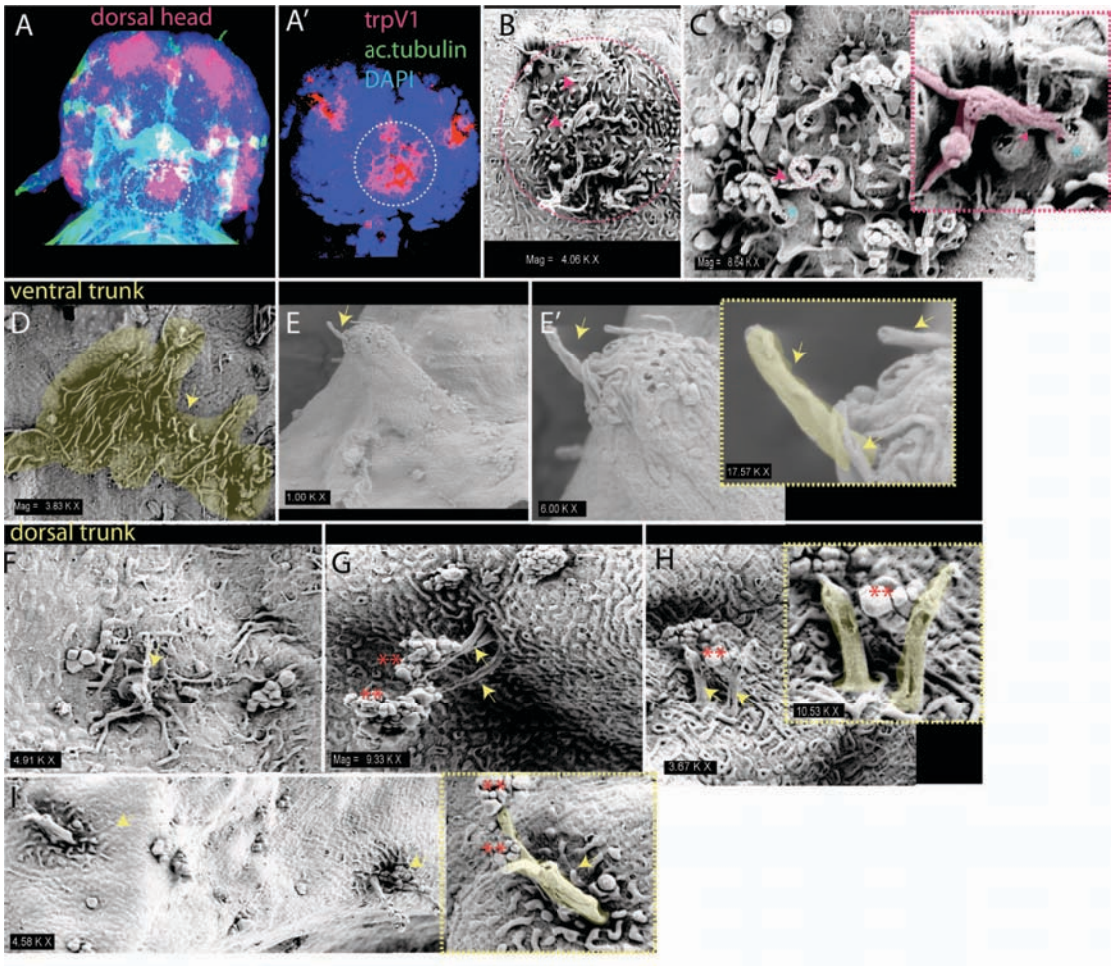


Figure b6. Different type of cilia at 5 days, visualized via scanning electron microscopy. A-C: sensory structures in the head. This cluster of cells in the medial part of the head is *trpV1+* (A, A') and protrudes long cilia deriving from different dendrites on the skin surface. The inset in C shows a close up of scanning electron micrograph, dendrites are pseudo-coloured in pink. Socket[2]-like cells (blue asterisk) are also visible with their typical doughnut-like ring of tissue around the cilia. **D:** cilia of the prototroch. **E-E':** cilia protruding from the ventral cirrus of the peripheral appendage. The inset shows a close up on the cilia, pseudo-colored in yellow. **F-I:** cilia protruding from different positions of the dorsal skin. Insets in H and I show magnifications of the cilia, pseudo-colored in yellow. Red asterisks indicate sea water crystals attached to the cilia.The real maginification used in the experiments is indicated per each panel (down left)

[2] The sockets cells are specialized epithelial cells associated with the sensory cilia of the dendrites. They are found in chemoreceptors and mechanoreceptors in *Drosophila* and *C.elegans*

C . Effect of Delta signaling inhibition on the trunk nervous system of *Platynereis*

Primary motoneurons and sensory neurons such the fish Rohon Beard cells and increase in number after Delta signalling inhibition (Cornell and Eisen 2000),(R. A. Cornell and Eisen). This occurs because the physiological *'lateral inhibition'* mechanism (see Discussion, II.2.2.1) is disrupted. Early during development *delta* expressing Rohon Beard cells are intermingled with the *notch* expressing neural crest cells at the neural plate border. Mutants with a reduced delta signaling have supernumerary Rohon Beard cells and lack neural crest cells (Cornell and Eisen 2000). Recently a similar mechanism has been shown to occur during amphioxus peripheral nervous system development. Indeed *DAPT* treatments in amphioxus (that blocks the intracellular Notch signaling) cause an increase of the ectodermal sensory neruons that form in cluster (Lu, Luo, and Yu 2012).
I asked whether a similar mechanism is present during sensory neurogenesis in the trunk of *Platynereis*. Therefore I treated *Platynereis* larvae with *DAPT* between 48h-4days; when the second wave of neurogenesis in the trunk occurs. These experiments are preliminary, but, similar to the situation in amphioxus and vertebrates, I observed an increase of the neurons, both peripheral (light blue arrows) and central neurons (white arrows) visualized with a WMISH for *islet* (fig.c1) and *elav* (not shown). Compared to the control (fig.c1A), clusters of *islet*+ cells are observed in the trunk (fig.c1B,C). Indeed conversely to the solitary neurons present in the control (fig.c1D), groups of islet + cells are widespread in the trunk after Delta-signaling inhibition (fig.c1E). These are preliminary experiments, and it remains to be understood wheter a special subset of the neurons are increased after *DAPT* treatments. Nevertheless, these experiments suggests that a mechanism involving Delta-Notch signalling was already acting in the trunk nervous system at the base of Bilateria, inducing both peripheral and central fates.

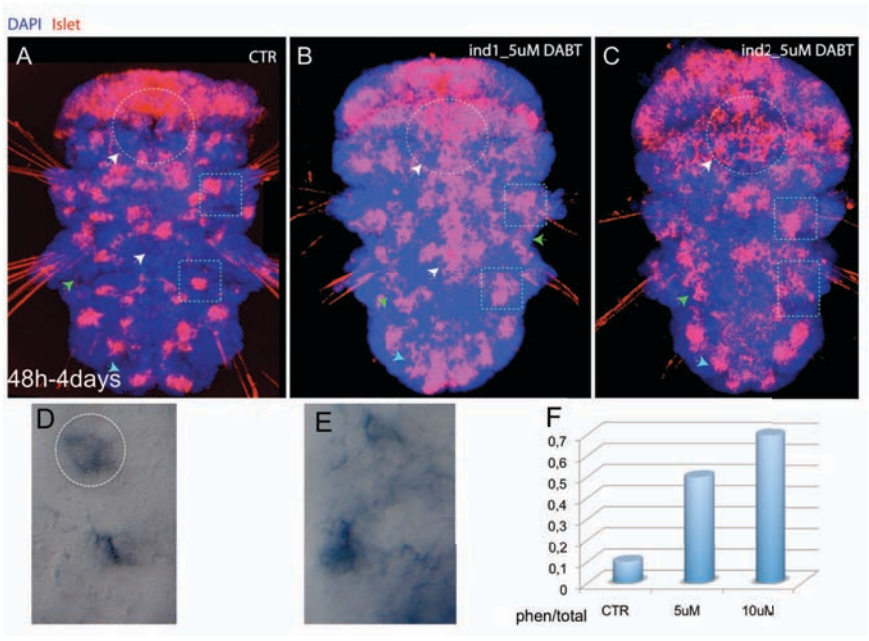


Figure c1. Effect of *DAPT* treatment on the expression of *islet* between 48h-4days. A: Z-projection of confocal scans. Ventral view. Crt animal incubated in DMSO. **B-C:** Z-projection of confocal scans. Ventral view. Two different individuals treated with 5uM of *DAPT*. Yellow arrows indicate the medial neuroectoderm, where more islet+ neurons are found after treatment (compare A-B). Green and white arrows indicate a cluster of *islet*+cells connecting the *islet*+ cells present in each segment, such a cluster is absent in the control. **D-E:** Bright field.Close up on the islet + cells in the trunk in the ctr animal

(D) and in the treated animal (E). **F:** quantification of the number of the animals showing this phenotype on a total of 20 individuals. The experiment has been repeated only once.

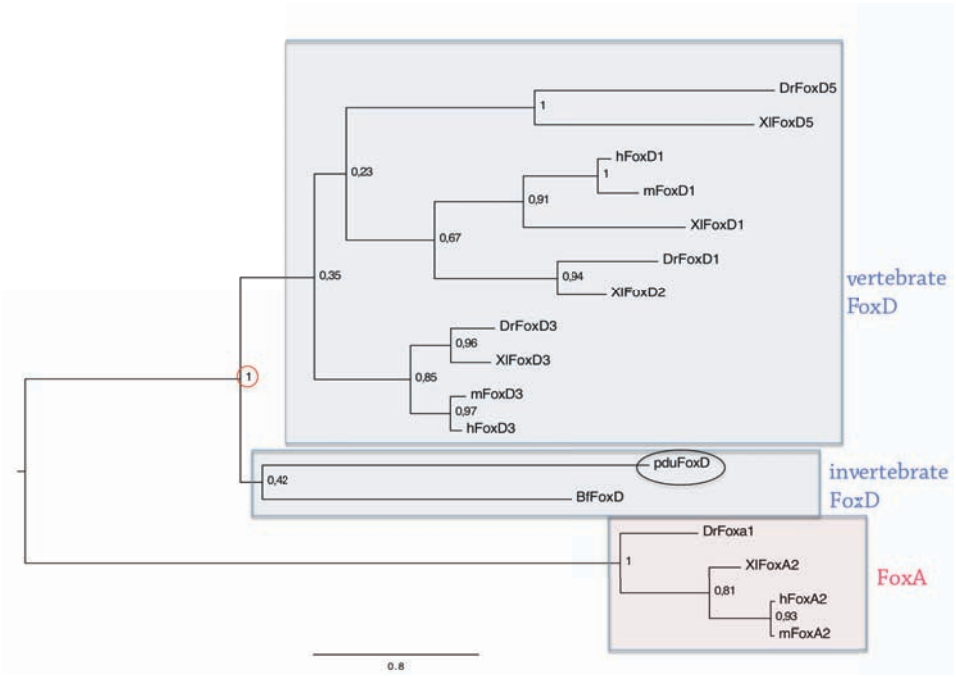
D. Phylogenetic analysis of the newly cloned genes

Protein sequences were downloaded from Uniprot(Consortium 2010; Consortium 2012) and JGI genome server. Alignment were generated with Muscle and maxim um likelihood (ML) trees were generated with PhyML (Guindon et al. 2005), (Guindon et al. 2010). Statistical supports per each node is shown. Outgroups or mid-branching were used to root the trees.

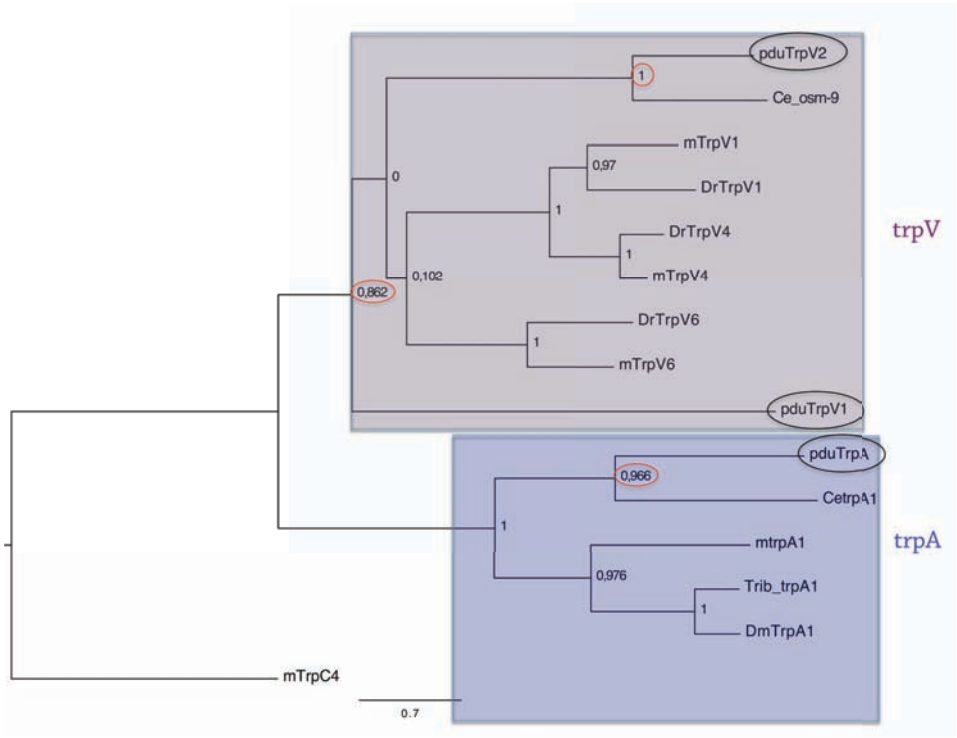
I used this abbreviations: m(*Mus musculus*), Dr (*Danio renio*), h (*homo sapiens*) Xl (*Xenopus laevis*), Bf (*Brachiostoma floridae*), Bf (*Brachiostoma belcheri*), Ce (*Caenorhabditis elegans*), Dpulex (*Daphnia pulex*), Dm (*Drosophila melanogaster*), S.kow (*Saccoglosus Kovalensky*), ArMa (*Arenicola marina*), Riftia P (Riftia *pachyptila*), AlvPomp (*Alvinella pompejana),*Nv *(Nematostella vectensis),* Ct *(Capitella teleta),* Hym *(Hydra magnipapillata*), HALTU (*Haliotis tubercolata),* EM (*Ephydatia muelleri*), Lj (*Lampetra japonica*), Hyv (*Hydra vulgaris*), Shm (*Schmidtea mediterranea*), A.g (*Anopheles gambiae),* Pm *(Petromyzon marinus), Sp (Strongylocentrotus purpuratus),*

Significative supports values to assign the ortology are indicated with red circles. The *Platynereis* sequences are indicated with black circles.

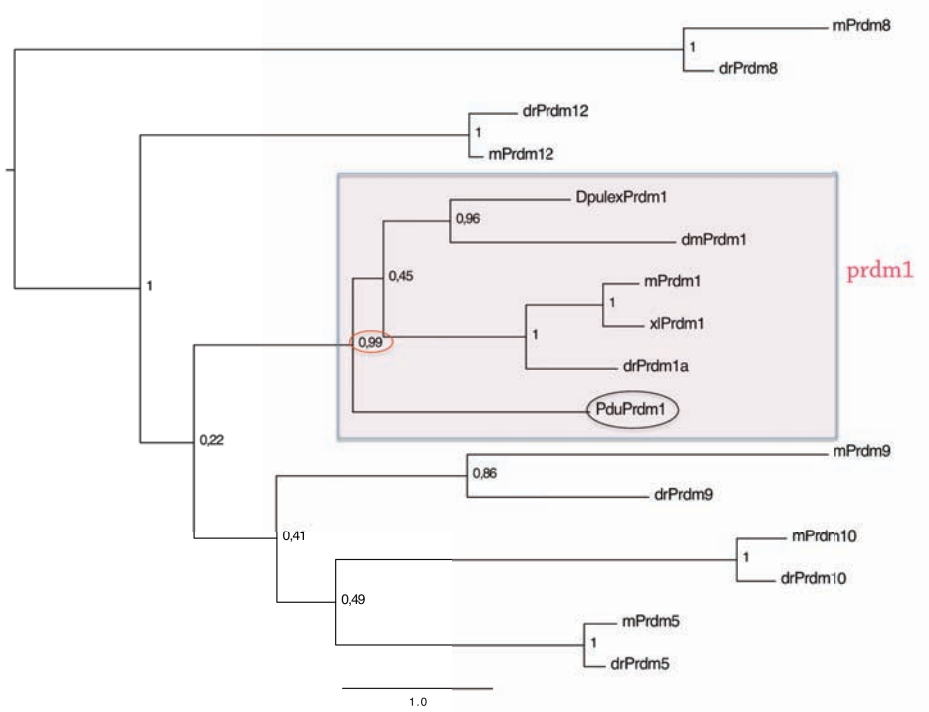
D.1 FoxD phylogenetic tree



D.2 TrpV/A phylogenetic tree

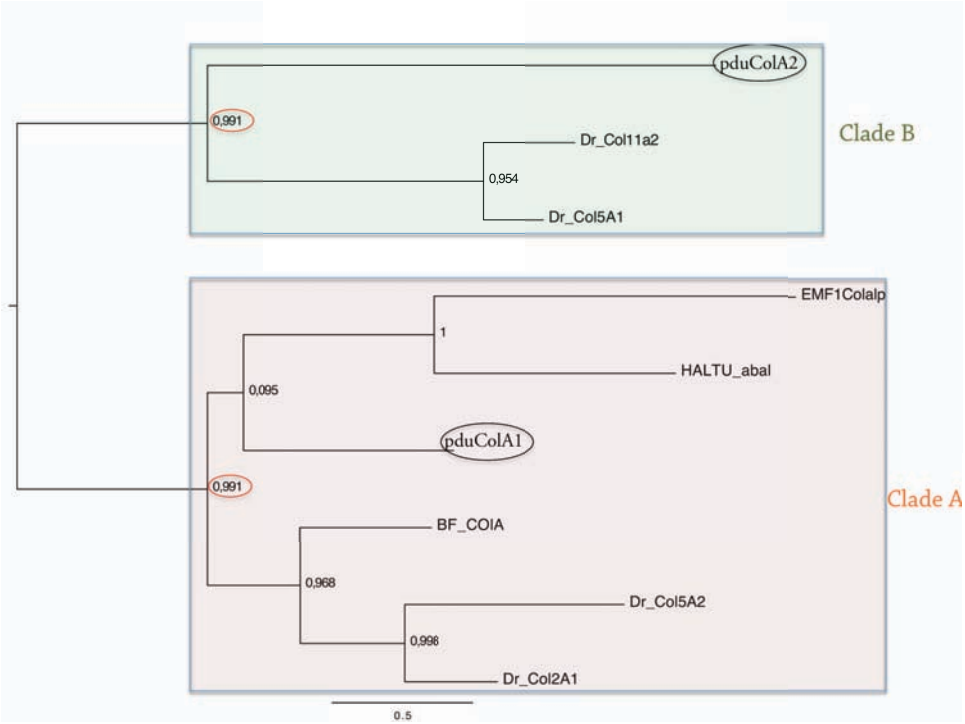


D.3 Prdm phylogenetic tree

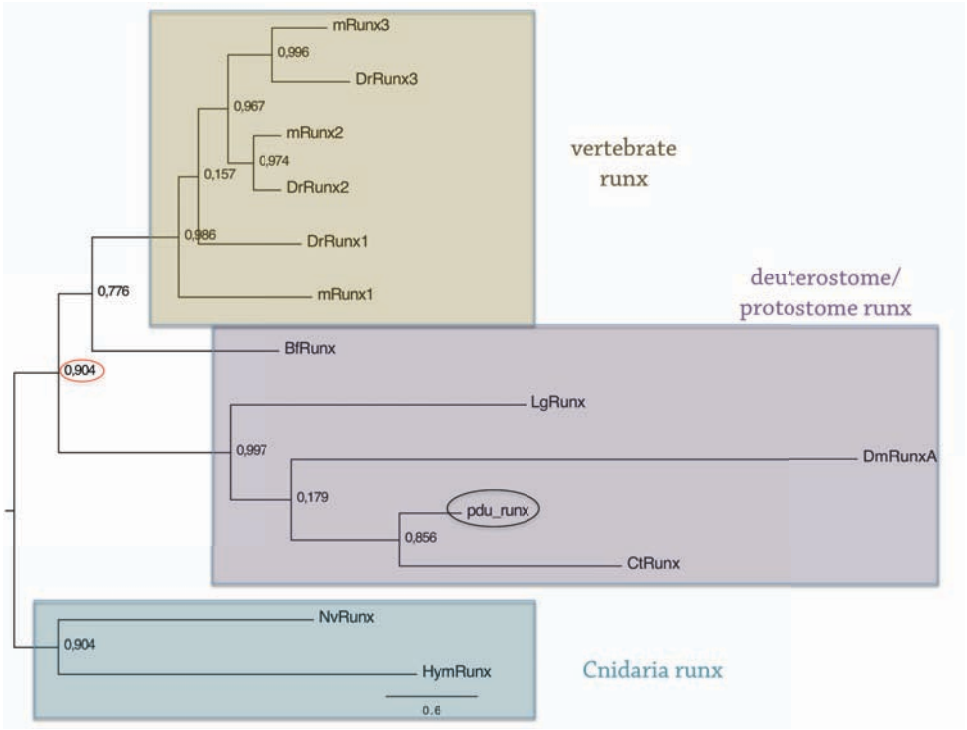


D.4 Fibrillar collagen phylogenetic tree

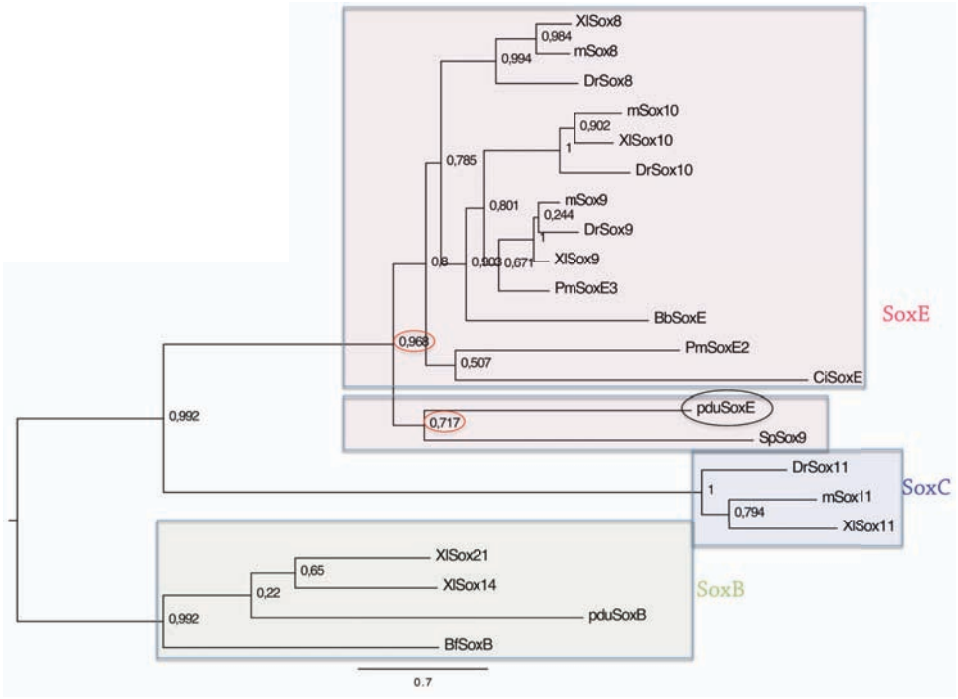
Fibrillar collagen is present in protostomes as well as in sponges, therefore is a eumetoazoan feature. Fibrillar collagen genes are divided into three clades (A-B-C). Recent data show the presence of collagens belonging to clade B (the minor collagens) also in protostomes (Exposito and Garrone 1990; Aouacheria et al. 2004; Boot-Handford et al. 2003; Boot-Handford and Tuckwell 2003), therefore it is likley the split occurred in Bilateria. In *Platynereis* I found two different fibrillar collagen. Only partial sequences for pduColA1 and pduColA2 are available, nevertheless this preliminary phylogenetic analysis supports the presence of a clade A-like ColA and a clade B-like ColA also in *Platynereis*.



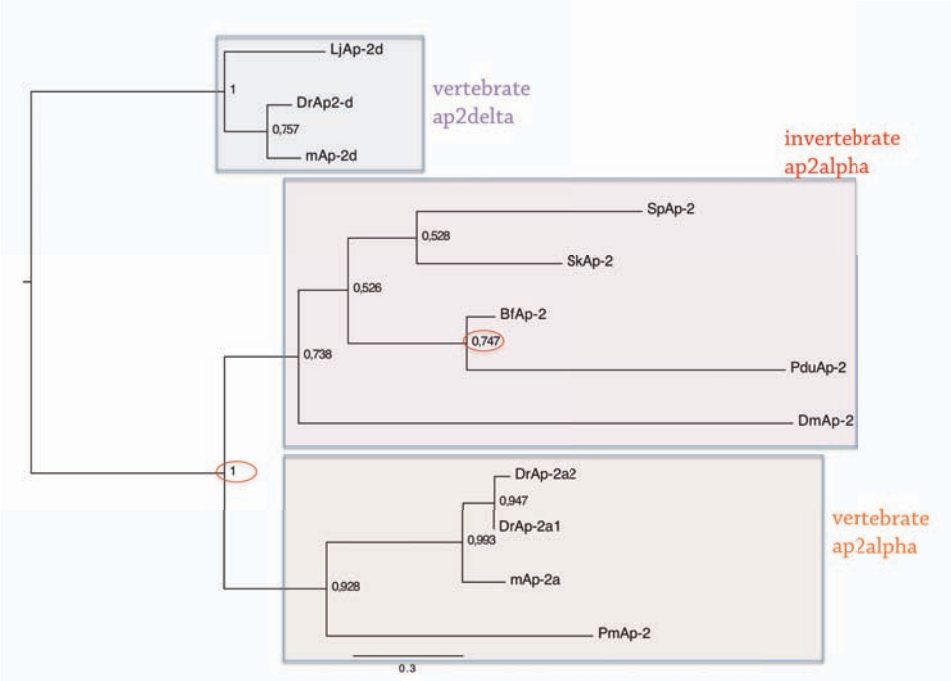
D.5 Runx phylogenetic tree



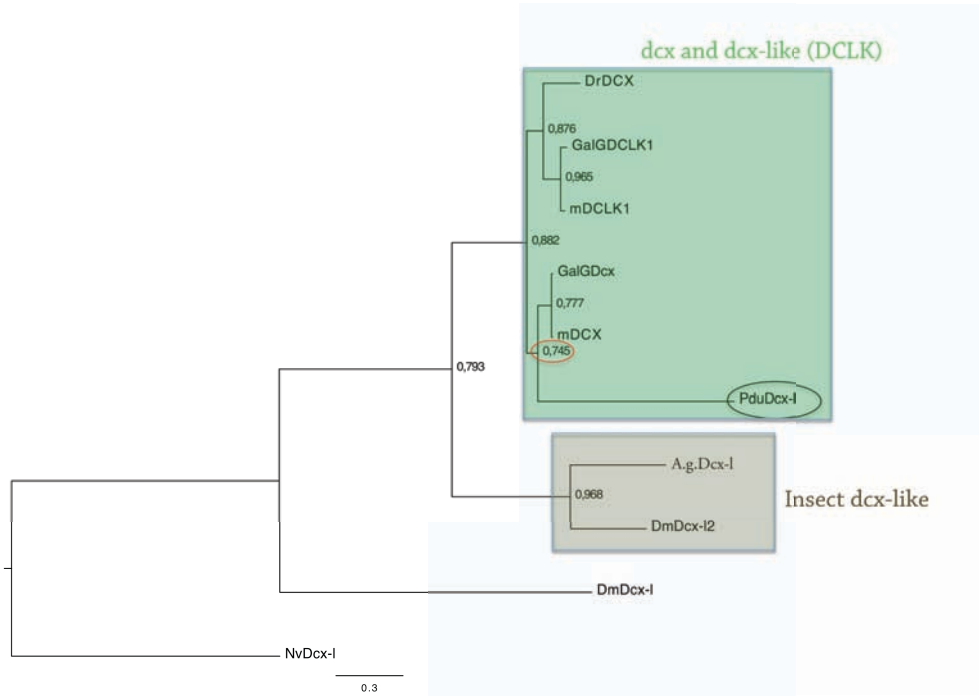
D.6 SoxE phylogenetic tree



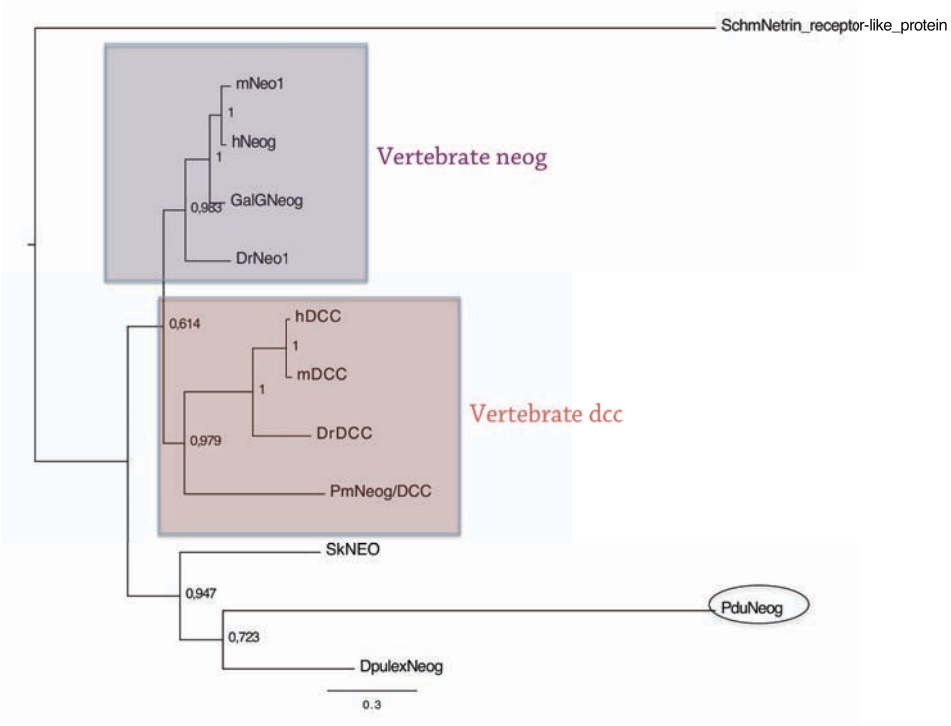
D.7Ap2 phylogenetic tree



D.8 Dcx phylogenetic tree



D.9 Neogenin/Dcc phylogenetic tree



Only a short sequence was available for *Platynereis* putative neogenin. To solve this tree a longer sequence is needed

REFERENCES

Abzhanov, Arhat, Eldad Tzahor, Andrew B Lassar, and Clifford J Tabin. 2003. "Dissimilar Regulation of Cell Differentiation in Mesencephalic (cranial) and Sacral (trunk) Neural Crest Cells in Vitro." *Development (Cambridge, England)* 130 (19) (October): 4567–79. doi:10.1242/dev.00673. http://www.ncbi.nlm.nih.gov/pubmed/12925584.

Agalliu, Dritan, Shinji Takada, Ilir Agalliu, Andrew P McMahon, and Thomas M Jessell. 2009. "Motor Neurons with Axial Muscle Projections Specified by Wnt4/5 Signaling." *Neuron* 61 (5) (March 12): 708–20. doi:10.1016/j.neuron.2008.12.026. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2741579&tool=pmcentrez&rendertype=abstract.

Alcántara, S, J Frisén, J A Del Río, E Soriano, M Barbacid, and I Silos-Santiago. 1997. "TrkB Signaling Is Required for Postnatal Survival of CNS Neurons and Protects Hippocampal and Motor Neurons from Axotomy-induced Cell Death." *Journal of Neuroscience* 17 (10): 3623–3633. http://www.ncbi.nlm.nih.gov/pubmed/9133385.

Alenina, Natalia, Saleh Bashammakh, and Michael Bader. 2006. "Specification and Differentiation of Serotonergic Neurons" 00: 5–10.

Aloe, Luigi. 2004. "Rita Levi-Montalcini: The Discovery of Nerve Growth Factor and Modern Neurobiology." *Trends in Cell Biology* 14 (7) (July): 395–9. doi:10.1016/j.tcb.2004.05.011. http://www.ncbi.nlm.nih.gov/pubmed/15246433.

Altmann, C R, and A H Brivanlou. 2001. "Neural Patterning in the Vertebrate Embryo." *International Review of Cytology* 203 (January): 447–82. http://www.ncbi.nlm.nih.gov/pubmed/11131523.

Antonio, Maurizio D, David Michalovich, Morris Paterson, Anna Droggiti, Ashwin Woodhoo, Rhona Mirsky, and Kristjan R Jessen. 2006. "Gene Profiling and Bioinformatic Analysis of Schwann Cell Embryonic Development and Myelination" 515 (December 2005): 501–515. doi:10.1002/glia.

Aoki, Yoichiro, Natasha Saint-Germain, Michael Gyda, Emily Magner-Fink, Young-Hoon Lee, Christine Credidio, and Jean-Pierre Saint-Jeannet. 2003. "Sox10 Regulates the Development of Neural Crest-derived Melanocytes in Xenopus." *Developmental Biology* 259 (1) (July): 19–33. doi:10.1016/S0012-1606(03)00161-1. http://linkinghub.elsevier.com/retrieve/pii/S0012160603001611.

Aouacheria, Abdel, Caroline Cluzel, Claire Lethias, Manolo Gouy, Robert Garrone, and Jean-Yves Exposito. 2004. "Invertebrate Data Predict an Early Emergence of Vertebrate Fibrillar Collagen Clades and an Anti-incest Model." *The Journal of Biological Chemistry* 279 (46) (November 12): 47711–9. doi:10.1074/jbc.M408950200. http://www.ncbi.nlm.nih.gov/pubmed/15358765.

Appel, B, A Fritz, M Westerfield, D J Grunwald, J S Eisen, and B B Riley. 1999. "Delta-mediated Specification of Midline Cell Fates in Zebrafish Embryos." *Current Biology* 9 (5): 247–256. http://www.ncbi.nlm.nih.gov/pubmed/10074451.

Appel, Bruce, Lee Anne Givan, and Judith S Eisen. 2001. "Delta-Notch Signaling and Lateral Inhibition in Zebrafish Spinal Cord Development." *BMC Developmental Biology* 1 (1): 13. http://www.biomedcentral.com/1471-213X/1/13.

Arduini, Brigitte L, Kevin M Bosse, and Paul D Henion. 2009. "Genetic Ablation of Neural Crest Cell Diversification." *Development Cambridge England* 136 (12): 1987–1994. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2685722&tool=pmcentrez&rendertype=abstract.

Arendt, D, and K Nübler-Jung. 1994. "Inversion of Dorsoventral Axis?" *Nature* 371 (6492) (September 1): 26. doi:10.1038/371026a0. http://www.ncbi.nlm.nih.gov/pubmed/8072524.

———. 1999. "Comparison of Early Nerve Cord Development in Insects and Vertebrates." *Development (Cambridge, England)* 126 (11) (June): 2309–25. http://www.ncbi.nlm.nih.gov/pubmed/10225991.

Arendt, Detlev. 2003. "Evolution of Eyes and Photoreceptor Cell Types." *The International Journal of Developmental Biology* 47 (7-8) (January): 563–71. http://www.ncbi.nlm.nih.gov/pubmed/14756332.

———. 2005. "Genes and Homology in Nervous System Evolution: Comparing Gene Functions, Expression Patterns, and Cell Type Molecular Fingerprints." *Theory in Biosciences = Theorie in Den Biowissenschaften* 124 (2) (November): 185–97. doi:10.1016/j.thbio.2005.08.002. http://www.ncbi.nlm.nih.gov/pubmed/17046355.

———. 2008a. "The Evolution of Cell Types in Animals: Emerging Principles from Molecular Studies." *Nature Reviews. Genetics* 9 (11) (November): 868–82. doi:10.1038/nrg2416. http://www.ncbi.nlm.nih.gov/pubmed/18927580.

———. 2008b. "The Evolution of Cell Types in Animals: Emerging Principles from Molecular Studies." *Nature Reviews. Genetics* 9 (11) (November): 868–82. doi:10.1038/nrg2416. http://www.ncbi.nlm.nih.gov/pubmed/18927580.

Arendt, Detlev, Kristin Tessmar-Raible, Heidi Snyman, Adriaan W Dorresteijn, and Joachim Wittbrodt. 2004. "Ciliary Photoreceptors with a Vertebrate-type Opsin in an Invertebrate Brain." *Science (New York, N.Y.)* 306 (5697) (October 29): 869–71. doi:10.1126/science.1099955. http://www.ncbi.nlm.nih.gov/pubmed/15514158.

Artinger, K B, a B Chitnis, M Mercola, and W Driever. 1999. "Zebrafish Narrowminded Suggests a Genetic Link Between Formation of Neural Crest and Primary Sensory Neurons." *Development (Cambridge, England)* 126 (18) (September): 3969–79. http://www.ncbi.nlm.nih.gov/pubmed/10457007.

B, Proc R Soc Lond, and Anders Hay-schmidt. 2000. "The Evolution of the Serotonergic Nervous System The Evolution of the Serotonergic Nervous System": 1071–1079. doi:10.1098/rspb.2000.1111.

Baccaglini, Paola I, and Nicholas C Spitzer. 1977. "Developmental Changes in the Inward Current of the Action Potential of Rohon-Beard Neurones." *The Journal of Physiology* 271 (1): 93–117.

http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1353609&tool=pmcentrez&rendertype=abstract.
Bacher, Beatrice E. 1973. "The Peripheral Dependency of Rohon-Beard Cells." *Journal of Experimental Zoology* 185 (2) (August): 209–215. doi:10.1002/jez.1401850208. http://doi.wiley.com/10.1002/jez.1401850208.
Baker, C V, and M Bronner-Fraser. 1997. "The Origins of the Neural Crest. Part II: An Evolutionary Perspective." *Mechanisms of Development* 69 (1-2) (December): 13–29. http://www.ncbi.nlm.nih.gov/pubmed/9486528.
Banerjee, Santanu, Laura Gordon, Thomas M Donn, Caterina Berti, Cecilia B Moens, Steven J Burden, and Michael Granato. 2011. "A Novel Role for MuSK and Non-canonical Wnt Signaling During Segmental Neural Crest Cell Migration." *Development (Cambridge, England)* 138 (15) (August): 3287–96. doi:10.1242/dev.067306. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3133918&tool=pmcentrez&rendertype=abstract.
Bargmann, Cornelia I. 2006. "Chemosensation in C. Elegans." *WormBook : the Online Review of C. Elegans Biology* (January): 1–29. doi:10.1895/wormbook.1.123.1. http://www.ncbi.nlm.nih.gov/pubmed/18050433.
von Bartheld, Christopher S, and Bernd Fritzsch. 2006. "Comparative Analysis of Neurotrophin Receptors and Ligands in Vertebrate Neurons: Tools for Evolutionary Stability or Changes in Neural Circuits?" *Brain, Behavior and Evolution* 68 (3) (January): 157–72. doi:10.1159/000094085. http://www.ncbi.nlm.nih.gov/pubmed/16912469.
Bartolomaeus, T., and G. Purschke. 2005. *Morphology, Molecules, Evolution and Phylogeny in Polychaeta and Related Taxa (Google eBook)*. Springer. http://books.google.com/books?id=PGKeXGqqbhsC&pgis=1.
Baskin, Denis G. 1971. "FINE S T R U C T U R E , F U N C T I O N A L ORGANIZATION AND SUPPORTIVE OF N E U R O G L I A IN NERE / S ROLE" 3 (May).
Beck, Gad, David W Munno, Zehava Levy, Helga M Dissel, Naweed I Syed, and Mike Fainzilber. 2003. "Neurotrophic Activities of Trk Receptors Conserved over 600 Million Years of Evolution ABSTRACT : Duced Autophosphorylation Followed by MAP Kinase Ac-": 12–20. doi:10.1002/neu.10329.
Belo, J A, T Bouwmeester, L Leyns, N Kertesz, M Gallo, M Follettie, and E M De Robertis. 1997. "Cerberus-like Is a Secreted Factor with Neutralizing Activity Expressed in the Anterior Primitive Endoderm of the Mouse Gastrula." *Mechanisms of Development* 68 (1-2) (November): 45–57. http://www.ncbi.nlm.nih.gov/pubmed/9431803.
Del Bene, Filippo. 2011. "Interkinetic Nuclear Migration: Cell Cycle on the Move." *The EMBO Journal* 30 (9) (May 4): 1676–7. doi:10.1038/emboj.2011.114. http://dx.doi.org/10.1038/emboj.2011.114.
Benito-Gutiérrez, Elia, Jordi Garcia-Fernàndez, and Joan X Comella. 2006. "Origin and Evolution of the Trk Family of Neurotrophic Receptors." *Molecular and Cellular Neurosciences* 31 (2) (February): 179–92. doi:10.1016/j.mcn.2005.09.007. http://www.ncbi.nlm.nih.gov/pubmed/16253518.
Benito-Gutiérrez, Elia, Christian Nake, Marta Llovera, Joan X Comella, and Jordi Garcia-Fernàndez. 2005. "The Single AmphiTrk Receptor Highlights Increased Complexity of Neurotrophin Signalling in Vertebrates and Suggests an Early Role in Developing Sensory Neuroepidermal Cells." *Development (Cambridge, England)* 132 (9) (May): 2191–202. doi:10.1242/dev.01803. http://www.ncbi.nlm.nih.gov/pubmed/15799999.
Betancur, Paola, Marianne Bronner-Fraser, and Tatjana Sauka-Spengler. 2010. "Assembling Neural Crest Regulatory Circuits into a Gene Regulatory Network." *Annual Review of Cell and Developmental Biology* 26 (November 10): 581–603. doi:10.1146/annurev.cellbio.042308.113245. http://www.ncbi.nlm.nih.gov/pubmed/19575671.
Bibel, M, E Hoppe, and Y A Barde. 1999. "Biochemical and Functional Interactions Between the Neurotrophin Receptors Trk and p75NTR." *The The European Molecular Biology Organization Journal* 18 (3): 616–622. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1171154&tool=pmcentrez&rendertype=abstract.
Bone, Quentin. 1960. "The Central Nervous System in Amphioxus." *The Journal of Comparative Neurology* 115 (1) (August): 27–64. doi:10.1002/cne.901150105. http://doi.wiley.com/10.1002/cne.901150105.
Boot-Handford, Raymond P, and Danny S Tuckwell. 2003. "Fibrillar Collagen: The Key to Vertebrate Evolution? A Tale of Molecular Incest." *BioEssays : News and Reviews in Molecular, Cellular and Developmental Biology* 25 (2) (February): 142–51. doi:10.1002/bies.10230. http://www.ncbi.nlm.nih.gov/pubmed/12539240.
Boot-Handford, Raymond P, Danny S Tuckwell, Darren a Plumb, Claire Farrington Rock, and Richard Poulsom. 2003. "A Novel and Highly Conserved Collagen (pro(alpha)1(XXVII)) with a Unique Expression Pattern and Unusual Molecular Characteristics Establishes a New Clade Within the Vertebrate Fibrillar Collagen Family." *The Journal of Biological Chemistry* 278 (33) (August 15): 31067–77. doi:10.1074/jbc.M212889200. http://www.ncbi.nlm.nih.gov/pubmed/12766169.
Boyan, George S, and Heinrich Reichert. 2011. "Mechanisms for Complexity in the Brain: Generating the Insect Central Complex." *Trends in Neurosciences* 34 (5): 247–257. http://www.ncbi.nlm.nih.gov/pubmed/21397959.

Briscoe, J, and J Ericson. 1999. "The Specification of Neuronal Identity by Graded Sonic Hedgehog Signalling." *Seminars in Cell & Developmental Biology* 10 (3) (June): 353–62. doi:10.1006/scdb.1999.0295. http://www.ncbi.nlm.nih.gov/pubmed/10441550.
Briscoe, J, L Sussel, P Serup, D Hartigan-O'Connor, T M Jessell, J L Rubenstein, and J Ericson. 1999. "Homeobox Gene Nkx2.2 and Specification of Neuronal Identity by Graded Sonic Hedgehog Signalling." *Nature* 398 (6728) (April 15): 622–7. doi:10.1038/19315. http://www.ncbi.nlm.nih.gov/pubmed/10217145.
Bronner-Fraser, Marianne. 1986. "Analysis of the Early Stages of Trunk Neural Crest Migration in Avian Embryos Using Monoclonal Antibody HNK-1." *Developmental Biology* 115 (1) (May): 44–55. doi:10.1016/0012-1606(86)90226-5. http://dx.doi.org/10.1016/0012-1606(86)90226-5.
Brown, Jason P, Sébastien Couillard-Després, Christiana M Cooper-Kuhn, Jürgen Winkler, Ludwig Aigner, and H Georg Kuhn. 2003. "Transient Expression of Doublecortin During Adult Neurogenesis." *Journal of Comparative Neurology* 467 (1): 1–10. http://www.ncbi.nlm.nih.gov/pubmed/14574675.
Buj-Bello, a, L G Pinon, and a M Davies. 1994. "The Survival of NGF-dependent but Not BDNF-dependent Cranial Sensory Neurons Is Promoted by Several Different Neurotrophins Early in Their Development." *Development (Cambridge, England)* 120 (6) (June): 1573–80. http://www.ncbi.nlm.nih.gov/pubmed/8050363.
Butler, Ann B, and William Hodos. 2005a. *Comparative Vertebrate Neuroanatomy: Evolution and Adaptation*. *Comparative Vertebrate Neuroanatomy*. Vol. 2nd Editio. John Wiley and Sons. doi:10.1002/0471733849. http://books.google.com/books?id=6kGARvykJKMC.
Butler, Ann B., and William Hodos. 2005b. *Comparative Vertebrate Neuroanatomy: Evolution and Adaptation (Google eBook)*. John Wiley & Sons. http://books.google.com/books?id=6kGARvykJKMC&pgis=1.
Butte, M J. 2001. "Cellular and Molecular Life Sciences Neurotrophic Factor Structures Reveal Clues to Evolution , Binding , Specificity , and Receptor Activation" 58: 1003–1013.
Candiani, Simona, Roberta Pennati, Diana Oliveri, Annamaria Locascio, Margherita Branno, Patrizio Castagnola, and Mario Pestarino. 2005. "Ci-POU-IV Expression Identifies PNS Neurons in Embryos and Larvae of the Ascidian Ciona Intestinalis": 41–45. doi:10.1007/s00427-004-0444-y.
Cattell, Maria, Su Lai, Robert Cerny, and Daniel Meulemans Medeiros. 2011. "A New Mechanistic Scenario for the Origin and Evolution of Vertebrate Cartilage." *PloS One* 6 (7) (January): e22474. doi:10.1371/journal.pone.0022474. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3142159&tool=pmcentrez&rendertype=abstract.
Chen, Chih-Li, Daniel C Broom, Yang Liu, Joriene C De Nooij, Zhe Li, Chuan Cen, Omar Abdel Samad, Thomas M Jessell, Clifford J Woolf, and Qiufu Ma. 2006. "Runx1 Determines Nociceptive Sensory Neuron Phenotype and Is Required for Thermal and Neuropathic Pain." *Neuron* 49 (3): 365–377. http://www.ncbi.nlm.nih.gov/pubmed/16446141.
Christodoulou, Foteini, Florian Raible, Raju Tomer, Oleg Simakov, Kalliopi Trachana, Sebastian Klaus, Heidi Snyman, Gregory J Hannon, Peer Bork, and Detlev Arendt. 2010. "Ancient Animal microRNAs and the Evolution of Tissue Identity." *Nature* 463 (7284): 1084–1088. http://www.ncbi.nlm.nih.gov/pubmed/20118916.
Clouthier, David E, and Thomas F Schilling. 2004. "Understanding Endothelin-1 Function During Craniofacial Development in the Mouse and Zebrafish." *Birth Defects Research. Part C, Embryo Today : Reviews* 72 (2) (June): 190–9. doi:10.1002/bdrc.20007. http://www.ncbi.nlm.nih.gov/pubmed/15269892.
Colbert, H a, T L Smith, and C I Bargmann. 1997. "OSM-9, a Novel Protein with Structural Similarity to Channels, Is Required for Olfaction, Mechanosensation, and Olfactory Adaptation in Caenorhabditis Elegans." *The Journal of Neuroscience : the Official Journal of the Society for Neuroscience* 17 (21) (November 1): 8259–69. http://www.ncbi.nlm.nih.gov/pubmed/9334401.
Consortium, The Uniprot. 2010. "The Universal Protein Resource (UniProt) in 2010." *Nucleic Acids Research* 38 (Database issue): D142–D148. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2808944&tool=pmcentrez&rendertype=abstract.
———. 2012. "Reorganizing the Protein Space at the Universal Protein Resource (UniProt)." *Nucleic Acids Research* 40 (Database issue): D71–5. doi:10.1093/nar/gkr981. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3245120&tool=pmcentrez&rendertype=abstract.
Cornell, R a, and J S Eisen. 2000. "Delta Signaling Mediates Segregation of Neural Crest and Spinal Sensory Neurons from Zebrafish Lateral Neural Plate." *Development (Cambridge, England)* 127 (13) (July): 2873–82. http://www.ncbi.nlm.nih.gov/pubmed/10851132.
Cornell, Robert A, and Judith S Eisen. "Delta / Notch Signaling Promotes Formation of Zebrafish Neural Crest by Repressing Neurogenin 1 Function Delta / Notch Signaling Promotes Formation of Zebrafish Neural Crest by Repressing Neurogenin 1 Function": 2639–2648.
Couillard-Despres, Sebastien, Beate Winner, Susanne Schaubeck, Robert Aigner, Maurice Vroemen, Norbert Weidner, Ulrich Bogdahn, Jürgen Winkler, Hans-Georg Kuhn, and Ludwig Aigner. 2005. "Doublecortin Expression Levels in Adult Brain Reflect Neurogenesis." *European Journal of Neuroscience* 21 (1): 1–14. http://www.ncbi.nlm.nih.gov/pubmed/15654838.
Creuzet, Sophie, Gérard Couly, and Nicole M Le Douarin. 2005. "Patterning the Neural Crest Derivatives During Development of the Vertebrate Head: Insights from Avian Studies." *Journal of*

Anatomy 207 (5) (November): 447–59. doi:10.1111/j.1469-7580.2005.00485.x. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1571568&tool=pmcentrez&rendertype=abstract.

de Crozé, Noémie, Frédérique Maczkowiak, and Anne H Monsoro-Burq. 2011. "Reiterative AP2a Activity Controls Sequential Steps in the Neural Crest Gene Regulatory Network." *Proceedings of the National Academy of Sciences of the United States of America* 108 (1) (January 4): 155–60. doi:10.1073/pnas.1010740107. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3017139&tool=pmcentrez&rendertype=abstract.

Curran, Kevin, James A Lister, Gary R Kunkel, Andrew Prendergast, M David, and David W Raible. 2011. "Interplay Between Foxd3 and Mitf Regulates Cell Fate Plasticity in the Zebrafish Neural Crest" 344 (1): 107–118. doi:10.1016/j.ydbio.2010.04.023.Interplay.

Curran, Kevin, David W Raible, and James A Lister. 2010. "Foxd3 Controls Melanophore Specification in Zebrafish Neural Crest by Regulation of Mitf" 332 (2): 408–417. doi:10.1016/j.ydbio.2009.06.010.Foxd3.

Davidson, Eric H, and Douglas H Erwin. 2006. "Gene Regulatory Networks and the Evolution of Animal Body Plans." *Science (New York, N.Y.)* 311 (5762) (February 10): 796–800. doi:10.1126/science.1113832. http://www.ncbi.nlm.nih.gov/pubmed/16469913.

Davies, a M. 1994. "The Role of Neurotrophins During Successive Stages of Sensory Neuron Development." *Progress in Growth Factor Research* 5 (3) (January): 263–89. http://www.ncbi.nlm.nih.gov/pubmed/7888634.

Deardorff, M a, C Tan, J P Saint-Jeannet, and P S Klein. 2001. "A Role for Frizzled 3 in Neural Crest Development." *Development (Cambridge, England)* 128 (19) (October): 3655–63. http://www.ncbi.nlm.nih.gov/pubmed/11585792.

Delmas, Patrick, Jizhe Hao, and Lise Rodat-Despoix. 2011. "Molecular Mechanisms of Mechanotransduction in Mammalian Sensory Neurons." *Nature Reviews. Neuroscience* 12 (3) (March): 139–53. doi:10.1038/nrn2993. http://www.ncbi.nlm.nih.gov/pubmed/21304548.

Delsuc, Frédéric, Henner Brinkmann, Daniel Chourrout, and Hervé Philippe. 2006. "Tunicates and Not Cephalochordates Are the Closest Living Relatives of Vertebrates." *Nature* 439 (7079): 965–8. doi:10.1038/nature04336. http://www.ncbi.nlm.nih.gov/pubmed/16495997.

Denes, Alexandru S, Gáspár Jékely, Patrick R H Steinmetz, Florian Raible, Heidi Snyman, Benjamin Prud'homme, David E K Ferrier, Guillaume Balavoine, and Detlev Arendt. 2007. "Molecular Architecture of Annelid Nerve Cord Supports Common Origin of Nervous System Centralization in Bilateria." *Cell* 129 (2) (May 20): 277–88. doi:10.1016/j.cell.2007.02.040. http://www.ncbi.nlm.nih.gov/pubmed/17448990.

Detrich, H. William, III, Monte Westerfield, and Leonard I. Zon. 2010. *The Zebrafish: Cellular and Developmental Biology, Part 1 (Google eBook)*. Academic Press. http://books.google.com/books?id=rgD7ABqAt2MC&pgis=1.

Dickinson, Mary E, Mark A J Selleck, Andrew P Mcmahon, and Marianne Bronner-fraser. 1995. "Dorsalization of the Neural Tube by the Non-neural Ectoderm" 2106: 2099–2106.

Dorsett, D. a. 1964. "The Sensory and Motor Innervation of Nereis." *Proceedings of the Royal Society B: Biological Sciences* 159 (977) (March 17): 652–667. doi:10.1098/rspb.1964.0024. http://rspb.royalsocietypublishing.org/cgi/doi/10.1098/rspb.1964.0024.

Dorsky, R I, R T Moon, and D W Raible. 1998. "Control of Neural Crest Cell Fate by the Wnt Signalling Pathway." *Nature* 396 (6709) (November 26): 370–3. doi:10.1038/24620. http://www.ncbi.nlm.nih.gov/pubmed/9845073.

Dottori, M, M K Gross, P Labosky, and M Goulding. 2001. "The Winged-helix Transcription Factor Foxd3 Suppresses Interneuron Differentiation and Promotes Neural Crest Cell Fate." *Development (Cambridge, England)* 128 (21) (November): 4127–38. http://www.ncbi.nlm.nih.gov/pubmed/11684651.

Le Douarin, N M, and M a Teillet. 1973. "The Migration of Neural Crest Cells to the Wall of the Digestive Tract in Avian Embryo." *Journal of Embryology and Experimental Morphology* 30 (1) (August): 31–48. http://www.ncbi.nlm.nih.gov/pubmed/4729950.

Le Douarin, N., and C. Kalcheim. 1999. "The Neural Crest." http://books.google.de/books/about/The_Neural_Crest.html?id=wvt9sgGbHFwC&redir_esc=y.

Le Douarin, Nicole M, and Elisabeth Dupin. 2003. "Multipotentiality of the Neural Crest." *Current Opinion in Genetics & Development* 13 (5) (October): 529–536. doi:10.1016/j.gde.2003.08.002. http://dx.doi.org/10.1016/j.gde.2003.08.002.

Le Douarin, Nicole M., and Marie-Aimée M. Teillet. 1974. "Experimental Analysis of the Migration and Differentiation of Neuroblasts of the Autonomic Nervous System and of Neurectodermal Mesenchymal Derivatives, Using a Biological Cell Marking Technique." *Developmental Biology* 41 (1) (November): 162–184. doi:10.1016/0012-1606(74)90291-7. http://dx.doi.org/10.1016/0012-1606(74)90291-7.

Dufour, Héloïse D, Zoubida Chettouh, Carole Deyts, Renaud de Rosa, Christo Goridis, Jean-Stéphane Joly, and Jean-François Brunet. 2006. "Precraniate Origin of Cranial Motoneurons." *Proceedings of the National Academy of Sciences of the United States of America* 103 (23) (June 6): 8727–32. doi:10.1073/pnas.0600805103. http://www.pnas.org/content/103/23/8727.full.

Dykes, Iain M, Jason Lanier, S Raisa Eng, and Eric E Turner. 2010. "Brn3a Regulates Neuronal Subtype Specification in the Trigeminal Ganglion by Promoting Runx Expression During Sensory Differentiation." *Neural Development* 5: 3. http://discovery.ucl.ac.uk/1321077/.
Dykes, Iain M, Lynne Tempest, Su-In Lee, and Eric E Turner. 2011. "Brn3a and Islet1 Act Epistatically to Regulate the Gene Expression Program of Sensory Differentiation." *Journal of Neuroscience* 31 (27): 9789–9799. http://discovery.ucl.ac.uk/1316987/.
D'Alessio, M, and M Frasch. 1996. "Msh May Play a Conserved Role in Dorsoventral Patterning of the Neuroectoderm and Mesoderm." *Mechanisms of Development* 58 (1-2): 217–231. http://www.ncbi.nlm.nih.gov/pubmed/8887329.
D'Autréaux, Fabien, Eva Coppola, Marie-Rose Hirsch, Carmen Birchmeier, and Jean-François Brunet. 2011. "Homeoprotein Phox2b Commands a Somatic-to-visceral Switch in Cranial Sensory Pathways." *Proceedings of the National Academy of Sciences of the United States of America* 108 (50) (December 13): 20018–23. doi:10.1073/pnas.1110416108. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3250195&tool=pmcentrez&rendertype=abstract.
Ebbesson, S O. 1980. "The Parcellation Theory and Its Relation to Interspecific Variability in Brain Organization, Evolutionary and Ontogenetic Development, and Neuronal Plasticity." *Cell and Tissue Research* 213 (2): 179–212. http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=7459999.
Ebbesson, Sven O. E. 1984. "Evolution and Ontogeny of Neural Circuits." *Behavioral and Brain Sciences* 7 (03) (September 1): 321–331. http://journals.cambridge.org/abstract_S0140525X00018379.
Edgar, Robert C. 2004. "MUSCLE: Multiple Sequence Alignment with High Accuracy and High Throughput." *Nucleic Acids Research* 32 (5): 1792–1797. http://www.ncbi.nlm.nih.gov/pubmed/15034147.
Elworthy, Stone, Jorge P Pinto, Anna Pettifer, M Leonor Cancela, and Robert N Kelsh. 2005. "Phox2b Function in the Enteric Nervous System Is Conserved in Zebrafish and Is Sox10-dependent." *Mechanisms of Development* 122 (5) (May): 659–69. doi:10.1016/j.mod.2004.12.008. http://www.ncbi.nlm.nih.gov/pubmed/15817223.
Erickson, C A, T D Duong, and K W Tosney. 1992. "Descriptive and Experimental Analysis of the Dispersion of Neural Crest Cells Along the Dorsolateral Path and Their Entry into Ectoderm in the Chick Embryo." *Developmental Biology* 151 (1) (May): 251–72. http://www.ncbi.nlm.nih.gov/pubmed/1577191.
Ericson, J, P Rashbass, a Schedl, S Brenner-Morton, a Kawakami, V van Heyningen, T M Jessell, and J Briscoe. 1997. "Pax6 Controls Progenitor Cell Identity and Neuronal Fate in Response to Graded Shh Signaling." *Cell* 90 (1) (July 11): 169–80. http://www.ncbi.nlm.nih.gov/pubmed/9230312.
Ernsberger, Uwe. 2009. "Role of Neurotrophin Signalling in the Differentiation of Neurons from Dorsal Root Ganglia and Sympathetic Ganglia." *Cell and Tissue Research* 336 (3) (June): 349–84. doi:10.1007/s00441-009-0784-z. http://www.ncbi.nlm.nih.gov/pubmed/19387688.
Essex, L J, R Mayor, and M G Sargent. 1993. "Expression of Xenopus Snail in Mesoderm and Prospective Neural Fold Ectoderm." *Developmental Dynamics : an Official Publication of the American Association of Anatomists* 198 (2) (October): 108–22. doi:10.1002/aja.1001980205. http://www.ncbi.nlm.nih.gov/pubmed/8305705.
Exposito, J Y, and R Garrone. 1990. "Characterization of a Fibrillar Collagen Gene in Sponges Reveals the Early Evolutionary Appearance of Two Collagen Gene Families." *Proceedings of the National Academy of Sciences of the United States of America* 87 (17) (September): 6669–73. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=54598&tool=pmcentrez&rendertype=abstract.
Faas, Laura, and Harry V Isaacs. 2009. "Overlapping Functions of Cdx1, Cdx2, and Cdx4 in the Development of the Amphibian Xenopus Tropicalis." *Developmental Dynamics : an Official Publication of the American Association of Anatomists* 238 (4) (April): 835–52. doi:10.1002/dvdy.21901. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2701559&tool=pmcentrez&rendertype=abstract.
Fekany-Lee, K, E Gonzalez, V Miller-Bertoglio, and L Solnica-Krezel. 2000. "The Homeobox Gene Bozozok Promotes Anterior Neuroectoderm Formation in Zebrafish Through Negative Regulation of BMP2/4 and Wnt Pathways." *Development (Cambridge, England)* 127 (11) (June): 2333–45. http://www.ncbi.nlm.nih.gov/pubmed/10804176.
Fernandez-Fuentes, Narcis, Brajesh K Rai, Carlos J Madrid-Aliste, J Eduardo Fajardo, and András Fiser. 2007. "Comparative Protein Structure Modeling by Combining Multiple Templates and Optimizing Sequence-to-structure Alignments." *Bioinformatics* 23 (19): 2558–2565. http://www.ncbi.nlm.nih.gov/pubmed/17823132.
Fernández-López, Blanca, Verona Villar-Cerviño, Silvia M Valle-Maroto, Antón Barreiro-Iglesias, Ramón Anadón, and María Celina Rodicio. 2012. "The Glutamatergic Neurons in the Spinal Cord of the Sea Lamprey: An in Situ Hybridization and Immunohistochemical Study." Ed. Shawn Hochman. *PloS One* 7 (10) (January): e47898. doi:10.1371/journal.pone.0047898. http://dx.plos.org/10.1371/journal.pone.0047898.
Filippi, Alida, Natascia Tiso, Gianluca Deflorian, Elisabetta Zecchin, Marino Bortolussi, and Francesco Argenton. 2005. "The Basic Helix-loop-helix Olig3 Establishes the Neural Plate Boundary of the Trunk and Is Necessary for Development of the Dorsal Spinal Cord." *Proceedings of the National*

Academy of Sciences of the United States of America 102 (12) (March 22): 4377–82. doi:10.1073/pnas.0407284102. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=549604&tool=pmcentrez&rendertype=abstract.

Fischer, Albrecht, and Adriaan Dorresteijn. 2004. "The Polychaete Platynereis Dumerilii (Annelida): a Laboratory Animal with Spiralian Cleavage, Lifelong Segment Proliferation and a Mixed Benthic/pelagic Life Cycle." *BioEssays : News and Reviews in Molecular, Cellular and Developmental Biology* 26 (3) (March): 314–25. doi:10.1002/bies.10409. http://www.ncbi.nlm.nih.gov/pubmed/14988933.

Fischer, Antje Hl, Thorsten Henrich, and Detlev Arendt. 2010. "The Normal Development of Platynereis Dumerilii (Nereididae, Annelida)." *Frontiers in Zoology* 7 (January): 31. doi:10.1186/1742-9994-7-31. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3027123&tool=pmcentrez&rendertype=abstract.

Fritzsch, B, I Fariñas, and L F Reichardt. 1997. "Lack of Neurotrophin 3 Causes Losses of Both Classes of Spiral Ganglion Neurons in the Cochlea in a Region-specific Fashion." *Journal of Neuroscience* 17 (16): 6213–6225. http://www.ncbi.nlm.nih.gov/pubmed/9236232.

Fritzsch, B, and R G Northcutt. 1993. "Cranial and Spinal Nerve Organization in Amphioxus and Lampreys: Evidence for an Ancestral Craniate Pattern." *Acta Anatomica* 148 (2-3): 96–109. http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=8109201.

Furlong, Rebecca F, and Anthony Graham. 2005. "Vertebrate Neurogenin Evolution: Long-term Maintenance of Redundant Duplicates." *Development Genes and Evolution* 215 (12) (December): 639–44. doi:10.1007/s00427-005-0023-x. http://www.ncbi.nlm.nih.gov/pubmed/16220265.

Gammill, Laura S, and Marianne Bronner-Fraser. 2003. "Neural Crest Specification: Migrating into Genomics." *Nature Reviews. Neuroscience* 4 (10) (October): 795–805. doi:10.1038/nrn1219. http://dx.doi.org/10.1038/nrn1219.

Gammill, Laura S, Constanza Gonzalez, Chenghua Gu, and Marianne Bronner-Fraser. 2006. "Guidance of Trunk Neural Crest Migration Requires Neuropilin 2/semaphorin 3F Signaling." *Development (Cambridge, England)* 133 (1) (January): 99–106. doi:10.1242/dev.02187. http://www.ncbi.nlm.nih.gov/pubmed/16319111.

Gans, C, and R G Northcutt. 1983. "Neural Crest and the Origin of Vertebrates: a New Head." *Science* 220 (4594): 268–273. http://www.ncbi.nlm.nih.gov/pubmed/17732898.

Gavalas, a, P Trainor, L Ariza-McNaughton, and R Krumlauf. 2001. "Synergy Between Hoxa1 and Hoxb1: The Relationship Between Arch Patterning and the Generation of Cranial Neural Crest." *Development (Cambridge, England)* 128 (15) (August): 3017–27. http://www.ncbi.nlm.nih.gov/pubmed/11532923.

Gessert, Susanne, Daniel Maurus, and Michael Kühl. 2008. "Repulsive Guidance Molecule A (RGM A) and Its Receptor Neogenin During Neural and Neural Crest Cell Development of Xenopus Laevis." *Biology of the Cell / Under the Auspices of the European Cell Biology Organization* 100 (11) (November): 659–73. doi:10.1042/BC20080023. http://www.ncbi.nlm.nih.gov/pubmed/18479252.

Gibson, A W, and R D Burke. 1985. "The Origin of Pigment Cells in Embryos of the Sea Urchin Strongylocentrotus Purpuratus." *Developmental Biology* 107 (2): 414–419. http://www.ncbi.nlm.nih.gov/pubmed/3972163.

Giovannone, Dion, Michelle Reyes, Rachel Reyes, Lisa Correa, Darwin Martinez, Hannah Ra, Gustavo Gomez, et al. 2012. "Slits Affect the Timely Migration of Neural Crest Cells via Robo Receptor." *Developmental Dynamics : an Official Publication of the American Association of Anatomists* 241 (8) (August): 1274–88. doi:10.1002/dvdy.23817. http://www.ncbi.nlm.nih.gov/pubmed/22689303.

Glass, David J, Elizabeth D Apel, Sonal Shah, David C Bowen, Thomas M DeChiara, Trevor N Stitt, Joshua R Sanes, and George D Yancopoulos. 1997. "Kinase Domain of the Muscle-specific Receptor Tyrosine Kinase (MuSK) Is Sufficient for Phosphorylation but Not Clustering of Acetylcholine Receptors: Required Role for the MuSK Ectodomain?" *Proceedings of the National Academy of Sciences of the United States of America* 94 (16): 8848–8853. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=23162&tool=pmcentrez&rendertype=abstract.

González-Estévez, C, T Momose, W J Gehring, and E Saló. 2003. "Transgenic Planarian Lines Obtained by Electroporation Using Transposon-derived Vectors and an Eye-specific GFP Marker." *Proceedings of the National Academy of Sciences of the United States of America* 100 (24): 14046–14051. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=283543&tool=pmcentrez&rendertype=abstract.

Goodrich, J T, P Bernd, D Sherman, and M D Gershon. 1980. "Phylogeny of Enteric Serotonergic Neurons." *The Journal of Comparative Neurology* 190 (1) (March 1): 15–28. doi:10.1002/cne.901900103. http://www.ncbi.nlm.nih.gov/pubmed/6966633.

Groves, A K, and M Bronner-Fraser. 1999. "Neural Crest Diversification." *Current Topics in Developmental Biology* 43 (January): 221–58. http://www.ncbi.nlm.nih.gov/pubmed/9891888.

Guder, C, I Philipp, T Lengfeld, H Watanabe, B Hobmayer, and T W Holstein. 2006. "The Wnt Code: Cnidarians Signal the Way." *Oncogene* 25 (57) (December 4): 7450–60. doi:10.1038/sj.onc.1210052. http://www.ncbi.nlm.nih.gov/pubmed/17143289.

Guindon, Stéphane, Jean-François Dufayard, Vincent Lefort, Maria Anisimova, Wim Hordijk, and Olivier Gascuel. 2010. "New Algorithms and Methods to Estimate Maximum-likelihood Phylogenies: Assessing the Performance of PhyML 3.0." *Systematic Biology* 59 (3) (May): 307–21. doi:10.1093/sysbio/syq010. http://www.ncbi.nlm.nih.gov/pubmed/20525638.

Guindon, Stéphane, Franck Lethiec, Patrice Duroux, and Olivier Gascuel. 2005. "PHYML Online—a Web Server for Fast Maximum Likelihood-based Phylogenetic Inference." *Nucleic Acids Research* 33 (Web Server issue): W557–W559. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1160113&tool=pmcentrez&rendertype=abstract.

Gunthorpe, Martin J, Christopher D Benham, Andrew Randall, and John B Davis. 2002. "The Diversity in the Vanilloid (TRPV) Receptor Family of Ion Channels." *Trends in Pharmacological Sciences* 23 (4) (April): 183–91. http://www.ncbi.nlm.nih.gov/pubmed/11931994.

Habuchi, Satoshi, Hidekazu Tsutsui, Anna B Kochaniak, Atsushi Miyawaki, and Antoine M Van Oijen. 2008. "mKikGR, a Monomeric Photoswitchable Fluorescent Protein." Ed. Shuguang Zhang. *PLoS ONE* 3 (12): 9. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2592542&tool=pmcentrez&rendertype=abstract.

Hager, G, and C N David. 1997. "Pattern of Differentiated Nerve Cells in Hydra Is Determined by Precursor Migration." *Development (Cambridge, England)* 124 (2) (January): 569–76. http://www.ncbi.nlm.nih.gov/pubmed/9053332.

Haldin, Caroline E, and Carole LaBonne. 2010. "SoxE Factors as Multifunctional Neural Crest Regulatory Factors." *The International Journal of Biochemistry & Cell Biology* 42 (3) (March): 441–4. doi:10.1016/j.biocel.2009.11.014. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2826508&tool=pmcentrez&rendertype=abstract.

Hallböök, F, L G Lundin, and K Kullander. 1998. "Lampetra Fluviatilis Neurotrophin Homolog, Descendant of a Neurotrophin Ancestor, Discloses the Early Molecular Evolution of Neurotrophins in the Vertebrate Subphylum." *The Journal of Neuroscience : the Official Journal of the Society for Neuroscience* 18 (21) (November 1): 8700–11. http://www.ncbi.nlm.nih.gov/pubmed/9786977.

Hari, Lisette, Véronique Brault, Maurice Kléber, Hye-Youn Lee, Fabian Ille, Rainer Leimeroth, Christian Paratore, Ueli Suter, Rolf Kemler, and Lukas Sommer. 2002. "Lineage-specific Requirements of Beta-catenin in Neural Crest Development." *The Journal of Cell Biology* 159 (5) (December 9): 867–80. doi:10.1083/jcb.200209039. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2173383&tool=pmcentrez&rendertype=abstract.

Haun, R S, I M Serventi, and J Moss. 1992. "Rapid, Reliable Ligation-independent Cloning of PCR Products Using Modified Plasmid Vectors." *BioTechniques* 13 (4) (October): 515–8. http://www.ncbi.nlm.nih.gov/pubmed/1362067.

Hausen, Harald. 2007. "Lateral Organs in Sedentary Polychaetes (Annelida) – Ultrastructure and Phylogenetic Significance of an Insufficiently Known Sense Organ" 39 (January): 23–39.

Henion, P D, a S Garner, T H Large, and J a Weston. 1995. "trkC-mediated NT-3 Signaling Is Required for the Early Development of a Subpopulation of Neurogenic Neural Crest Cells." *Developmental Biology* 172 (2) (December): 602–13. doi:10.1006/dbio.1995.8054. http://www.ncbi.nlm.nih.gov/pubmed/8612975.

Hernandez-Lagunas, Laura, Irene F Choi, Takao Kaji, Peter Simpson, Candice Hershey, Yi Zhou, Len Zon, Mark Mercola, and Kristin Bruk Artinger. 2005. "Zebrafish Narrowminded Disrupts the Transcription Factor Prdm1 and Is Required for Neural Crest and Sensory Neuron Specification." *Developmental Biology* 278 (2) (February 15): 347–57. doi:10.1016/j.ydbio.2004.11.014. http://www.ncbi.nlm.nih.gov/pubmed/15680355.

Higashijima, S, T Michiue, Y Emori, and K Saigo. 1992. "Subtype Determination of Drosophila Embryonic External Sensory Organs by Redundant Homeo Box Genes BarH1 and BarH2." *Genes & Development* 6 (6): 1005–1018. http://www.genesdev.org/cgi/doi/10.1101/gad.6.6.1005.

Hirsch, M R, M C Tiveron, F Guillemot, J F Brunet, and C Goridis. 1998. "Control of Noradrenergic Differentiation and Phox2a Expression by MASH1 in the Central and Peripheral Nervous System." *Development (Cambridge, England)* 125 (4) (February): 599–608. http://www.ncbi.nlm.nih.gov/pubmed/9435281.

Hjerling-Leffler, Jens, Mona Alqatari, Patrik Ernfors, and Martin Koltzenburg. 2007. "Emergence of Functional Sensory Subtypes as Defined by Transient Receptor Potential Channel Expression." *The Journal of Neuroscience : the Official Journal of the Society for Neuroscience* 27 (10) (March 7): 2435–43. doi:10.1523/JNEUROSCI.5614-06.2007. http://www.ncbi.nlm.nih.gov/pubmed/17344381.

Hjerling-Leffler, Jens, Frédéric Marmigère, Mikael Heglind, Anna Cederberg, Martin Koltzenburg, Sven Enerbäck, and Patrik Ernfors. 2005. "The Boundary Cap: a Source of Neural Crest Stem Cells That Generate Multiple Sensory Neuron Subtypes." *Development Cambridge England* 132 (11): 2623–2632. http://www.ncbi.nlm.nih.gov/pubmed/15872002.

Hjorth, Jensen, and Brian Key. 2002. "Development of Axon Pathways in the Zebrafish Central Nervous System." *The International Journal of Developmental Biology* 46 (4) (January): 609–19. http://www.ncbi.nlm.nih.gov/pubmed/12141449.

Hobert, Oliver. 2008. "Regulatory Logic of Neuronal Diversity: Terminal Selector Genes and Selector Motifs." *Proceedings of the National Academy of Sciences of the United States of America* 105 (51)

(December 23): 20067–71. doi:10.1073/pnas.0806070105. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2629285&tool=pmcentrez&rendertype=abstract.
Holland, N D, G Panganiban, E L Henyey, and L Z Holland. 1996. "Sequence and Developmental Expression of AmphiDll, an Amphioxus Distal-less Gene Transcribed in the Ectoderm, Epidermis and Nervous System: Insights into Evolution of Craniate Forebrain and Neural Crest." *Development (Cambridge, England)* 122 (9) (September): 2911–20. http://www.ncbi.nlm.nih.gov/pubmed/8787764.
Horesh, D, T Sapir, F Francis, S G Wolf, M Caspi, M Elbaum, J Chelly, and O Reiner. 1999. "Doublecortin, a Stabilizer of Microtubules." *Human Molecular Genetics* 8 (9): 1599–1610. http://www.ncbi.nlm.nih.gov/pubmed/10441322.
Horn, C, and E A Wimmer. 2000. "A Versatile Vector Set for Animal Transgenesis." *Development Genes and Evolution* 210 (12): 630–637. http://www.ncbi.nlm.nih.gov/pubmed/11151300.
Hory-Lee, F, M Russell, R M Lindsay, and E Frank. 1993. "Neurotrophin 3 Supports the Survival of Developing Muscle Sensory Neurons in Culture." *Proceedings of the National Academy of Sciences of the United States of America* 90 (7) (April 1): 2613–7. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=46145&tool=pmcentrez&rendertype=abstract.
Hou, L, J J Panthier, and H Arnheiter. 2000. "Signaling and Transcriptional Regulation in the Neural Crest-derived Melanocyte Lineage: Interactions Between KIT and MITF." *Development (Cambridge, England)* 127 (24) (December): 5379–89. http://www.ncbi.nlm.nih.gov/pubmed/11076759.
Huang, E J, and L F Reichardt. 2001. "Neurotrophins: Roles in Neuronal Development and Function." *Annual Review of Neuroscience* 24 (January): 677–736. doi:10.1146/annurev.neuro.24.1.677. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2758233&tool=pmcentrez&rendertype=abstract.
Huang, Eric J, and Louis F Reichardt. 2003. "Trk Receptors: Roles in Neuronal Signal Transduction." *Annual Review of Biochemistry* 72 (January): 609–42. doi:10.1146/annurev.biochem.72.121801.161629. http://www.ncbi.nlm.nih.gov/pubmed/12676795.
Häming, Daniela, Marcos Simoes-Costa, Benjamin Uy, Jonathan Valencia, Tatjana Sauka-Spengler, and Marianne Bronner-Fraser. 2011. "Expression of Sympathetic Nervous System Genes in Lamprey Suggests Their Recruitment for Specification of a New Vertebrate Feature." *PloS One* 6 (10) (January): e26543. doi:10.1371/journal.pone.0026543. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3203141&tool=pmcentrez&rendertype=abstract.
Ibáñez, C F. 1998. "Emerging Themes in Structural Biology of Neurotrophic Factors." *Trends in Neurosciences* 21 (10): 438–444. http://www.ncbi.nlm.nih.gov/pubmed/9786342.
Inoue, Ken-ichi, Kosei Ito, Motomi Osato, Bernett Lee, Suk-Chul Bae, and Yoshiaki Ito. 2007. "The Transcription Factor Runx3 Represses the Neurotrophin Receptor TrkB During Lineage Commitment of Dorsal Root Ganglion Neurons." *The Journal of Biological Chemistry* 282 (33) (August 17): 24175–84. doi:10.1074/jbc.M703746200. http://www.ncbi.nlm.nih.gov/pubmed/17584746.
Inoue, Ken-ichi, Takashi Shiga, and Yoshiaki Ito. 2008. "Runx Transcription Factors in Neuronal Development." *Neural Development* 3 (January): 20. doi:10.1186/1749-8104-3-20. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2531103&tool=pmcentrez&rendertype=abstract.
Ip, Y T, M Levine, and E Bier. 1994. "Neurogenic Expression of Snail Is Controlled by Separable CNS and PNS Promoter Elements." *Development (Cambridge, England)* 120 (1) (January): 199–207. http://www.ncbi.nlm.nih.gov/pubmed/8119127.
Isshiki, T, M Takeichi, and A Nose. 1997. "The Role of the Msh Homeobox Gene During Drosophila Neurogenesis: Implication for the Dorsoventral Specification of the Neuroectoderm." *Development Cambridge England* 124 (16): 3099–3109. http://www.ncbi.nlm.nih.gov/pubmed/9272951.
Jackman, W R, J a Langeland, and C B Kimmel. 2000. "Islet Reveals Segmentation in the Amphioxus Hindbrain Homolog." *Developmental Biology* 220 (1) (April 1): 16–26. doi:10.1006/dbio.2000.9630. http://www.ncbi.nlm.nih.gov/pubmed/10720427.
Jacobson, M. 1981. "ROHON-BEARD 16-CELL FROG NEURON EMBRYO ' ORIGIN FROM" (1967): 918–922.
Jafar-Nejad, Hamed, Melih Acar, Riitta Nolo, Haluk Lacin, Hongling Pan, Susan M Parkhurst, and Hugo J Bellen. 2003. "Senseless Acts as a Binary Switch During Sensory Organ Precursor Selection." *Genes & Development* 17 (23): 2966–2978. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=289154&tool=pmcentrez&rendertype=abstract.
James L. Roberts, Nicholas C. Spitzer, Michael J. Zigmond , Susan K. McConnell, Floyd E. Bloom. 2008. *Fundamental Neuroscience (Google eBook)*. Academic Press. http://books.google.com/books?id=GOxrtYzmixcC&pgis=1.
Janssen, Ralf, Martine Le Gouar, Matthias Pechmann, Francis Poulin, Renata Bolognesi, Evelyn E Schwager, Corinna Hopfen, et al. 2010. "Conservation, Loss, and Redeployment of Wnt Ligands in Protostomes: Implications for Understanding the Evolution of Segment Formation." *BMC Evolutionary Biology* 10 (1) (January): 374. doi:10.1186/1471-2148-10-374.

http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3003278&tool=pmcentrez&rendertype=abstract.
Jarman, a P, Y Sun, L Y Jan, and Y N Jan. 1995. "Role of the Proneural Gene, Atonal, in Formation of Drosophila Chordotonal Organs and Photoreceptors." *Development (Cambridge, England)* 121 (7) (July): 2019–30. http://www.ncbi.nlm.nih.gov/pubmed/7635049.
Jeanmougin, François, Julie D Thompson, Manolo Gouy, Desmond G Higgins, and Toby J Gibson. 1998. "Multiple Sequence Alignment with Clustal X." *Trends in Biochemical Sciences* 23 (10): 403–405. doi:10.1016/S0968-0004(98)01285-7.
http://linkinghub.elsevier.com/retrieve/pii/S0968000498012857.
Jeffery, William R. 2006. "Ascidian Neural Crest-Like Cells : Phylogenetic Distribution , Relationship to Larval Complexity , and Pigment Cell Fate" (February): 3–4. doi:10.1002/jez.b.
———. 2007. "Chordate Ancestry of the Neural Crest: New Insights from Ascidians." *Seminars in Cell & Developmental Biology* 18 (4) (August): 481–91. doi:10.1016/j.semcdb.2007.04.005.
http://www.ncbi.nlm.nih.gov/pubmed/17509911.
Jeffery, William R, Takuto Chiba, Florian Razy Krajka, Carole Deyts, Nori Satoh, and Jean-Stéphane Joly. 2008. "Trunk Lateral Cells Are Neural Crest-like Cells in the Ascidian Ciona Intestinalis: Insights into the Ancestry and Evolution of the Neural Crest." *Developmental Biology* 324 (1) (December 1): 152–60. doi:10.1016/j.ydbio.2008.08.022.
http://www.ncbi.nlm.nih.gov/pubmed/18801357.
Jeffery, William R, Allen G Strickler, and Yoshiyuki Yamamoto. 2004. "Migratory Neural Crest-like Cells Form Body Pigmentation in a Urochordate Embryo." *Nature* 431 (7009) (October 7): 696–9. doi:10.1038/nature02975. http://www.ncbi.nlm.nih.gov/pubmed/15470430.
Jing, Lili, Laura R Gordon, Elena Shtibin, and Michael Granato. 2010. "Temporal and Spatial Requirements of unplugged/MuSK Function During Zebrafish Neuromuscular Development." Ed. Michael Hendricks. *PLoS ONE* 5 (1): 10.
http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2809748&tool=pmcentrez&rendertype=abstract.
John, Shinu a, and Lee Ann Garrett-Sinha. 2009. "Blimp1: a Conserved Transcriptional Repressor Critical for Differentiation of Many Tissues." *Experimental Cell Research* 315 (7) (April 15): 1077–84. doi:10.1016/j.yexcr.2008.11.015. http://www.ncbi.nlm.nih.gov/pubmed/19073176.
Jones, Frederick S, Chrissa Kioussi, Donald W Copertino, Pekka Kallunki, Brent D Holst, and Gerald M Edelman. 1997. "Barx2, a New Homeobox Gene of the Bar Class, Is Expressed in Neural and Craniofacial Structures During Development." *Proceedings of the National Academy of Sciences of the United States of America* 94 (6): 2632–2637.
http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=20140&tool=pmcentrez&rendertype=abstract.
Jékely, Gáspár. 2011. "Origin and Early Evolution of Neural Circuits for the Control of Ciliary Locomotion." *Proceedings. Biological Sciences / The Royal Society* 278 (1707) (March 22): 914–22. doi:10.1098/rspb.2010.2027.
http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3049052&tool=pmcentrez&rendertype=abstract.
Jékely, Gáspár, and Detlev Arendt. 2007. "Cellular Resolution Expression Profiling Using Confocal Detection of NBT/BCIP Precipitate by Reflection Microscopy." *BioTechniques* 42 (6) (June): 751–755. doi:10.2144/000112462. http://www.biotechniques.com/article/000112462.
Jékely, Gáspár, Julien Colombelli, Harald Hausen, Keren Guy, Ernst Stelzer, François Nédélec, and Detlev Arendt. 2008. "Mechanism of Phototaxis in Marine Zooplankton." *Nature* 456 (7220) (November 20): 395–9. doi:10.1038/nature07590.
http://www.ncbi.nlm.nih.gov/pubmed/19020621.
Kahn-Kirby, Amanda H, and Cornelia I Bargmann. 2006. "TRP Channels in C. Elegans." *Annual Review of Physiology* 68 (13) (January): 719–36. doi:10.1146/annurev.physiol.68.040204.100715.
http://www.ncbi.nlm.nih.gov/pubmed/16460289.
Kaltenbach, Stacy L, Jr-Kai Yu, and Nicholas D Holland. 2009. "The Origin and Migration of the Earliest-developing Sensory Neurons in the Peripheral Nervous System of Amphioxus." *Evolution & Development* 11 (2): 142–51. doi:10.1111/j.1525-142X.2009.00315.x.
http://www.ncbi.nlm.nih.gov/pubmed/19245546.
Kee, Yun, and Marianne Bronner-Fraser. 2005. "To Proliferate or to Die: Role of Id3 in Cell Cycle Progression and Survival of Neural Crest Progenitors." *Genes & Development* 19 (6) (March 15): 744–55. doi:10.1101/gad.1257405.
http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1065727&tool=pmcentrez&rendertype=abstract.
Kempf, H, C Linares, P Corvol, and J M Gasc. 1998. "Pharmacological Inactivation of the Endothelin Type A Receptor in the Early Chick Embryo: a Model of Mispatterning of the Branchial Arch Derivatives." *Development (Cambridge, England)* 125 (24) (December): 4931–41.
http://www.ncbi.nlm.nih.gov/pubmed/9811577.
Khudyakov, Jane, and Marianne Bronner-Fraser. 2009. "Comprehensive Spatiotemporal Analysis of Early Chick Neural Crest Network Genes." *Developmental Dynamics : an Official Publication of the American Association of Anatomists* 238 (3) (March): 716–23. doi:10.1002/dvdy.21881.
http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2650819&tool=pmcentrez&rendertype=abstract.

Kim, Janghwan, Yun Doo Chung, Dae-Young Park, SooKyung Choi, Dong Wook Shin, Heun Soh, Hye Won Lee, et al. 2003. "A TRPV Family Ion Channel Required for Hearing in Drosophila." *Nature* 424 (6944): 81–84. http://www.ncbi.nlm.nih.gov/pubmed/21204501.

Kim, Myung Hee, Tomasz Cierpicki, Urszula Derewenda, Daniel Krowarsch, Yuanyi Feng, Yancho Devedjiev, Zbigniew Dauter, et al. 2003. "The DCX-domain Tandems of Doublecortin and Doublecortin-like Kinase." *Nature Structural Biology* 10 (5): 324–333. http://www.ncbi.nlm.nih.gov/pubmed/12692530.

Kim, a S, S a Anderson, J L Rubenstein, D H Lowenstein, and S J Pleasure. 2001. "Pax-6 Regulates Expression of SFRP-2 and Wnt-7b in the Developing CNS." *The Journal of Neuroscience : the Official Journal of the Society for Neuroscience* 21 (5) (March 1): RC132. http://www.ncbi.nlm.nih.gov/pubmed/11222670.

Knight, R D, G D Panopoulou, P W Holland, and S M Shimeld. 2000. "An Amphioxus Krox Gene: Insights into Vertebrate Hindbrain Evolution." *Development Genes and Evolution* 210 (10) (October): 518–21. doi:10.1007/s004270050341. http://www.ncbi.nlm.nih.gov/pubmed/11180801.

Kohashi, Tsunehiko, Natsuyo Nakata, and Yoichi Oda. 2012. "Effective Sensory Modality Activating an Escape Triggering Neuron Switches During Early Development in Zebrafish." *The Journal of Neuroscience : the Official Journal of the Society for Neuroscience* 32 (17) (April 25): 5810–20. doi:10.1523/JNEUROSCI.6169-11.2012. http://www.ncbi.nlm.nih.gov/pubmed/22539843.

Kollros, J J, and a M Bovbjerg. 1997. "Growth and Death of rohon-Beard Cells in Rana Pipiens and Ceratophrys Ornata." *Journal of Morphology* 232 (1) (April): 67–78. doi:10.1002/(SICI)1097-4687(199704)232:1<67::AID-JMOR4>3.0.CO;2-L. http://www.ncbi.nlm.nih.gov/pubmed/9068202.

Koltzenburg, M. 2004. "The Role of TRP Channels in Sensory Neurons." *Novartis Foundation Symposium* 260 (1528-2511 LA - eng PT - Journal Article PT - Review PT - Review, Tutorial RN - 0 (ANKTM1 protein, human) RN - 0 (Calcium Channels) RN - 0 (Calcium Channels, T-Type) RN - 0 (Cation Transport Proteins) RN - 0 (Ion Channels) RN - 0 (Neoplasm Proteins) RN - 0): 206–213; discussion 213–220, 277–279. http://discovery.ucl.ac.uk/1336020/.

Kramer, Ina, Markus Sigrist, Joriene C De Nooij, Ichiro Taniuchi, Thomas M Jessell, and Silvia Arber. 2006. "A Role for Runx Transcription Factor Signaling in Dorsal Root Ganglion Sensory Neuron Diversification." *Neuron* 49 (3): 379–393. http://www.ncbi.nlm.nih.gov/pubmed/16446142.

Kulakova, Milana, Nadezhda Bakalenko, Elena Novikova, Charles E Cook, Elena Eliseeva, Patrick R H Steinmetz, Roman P Kostyuchenko, et al. 2007. "Hox Gene Expression in Larval Development of the Polychaetes Nereis Virens and Platynereis Dumerilii (Annelida, Lophotrochozoa)." *Development Genes and Evolution* 217 (1) (January): 39–54. doi:10.1007/s00427-006-0119-y. http://www.ncbi.nlm.nih.gov/pubmed/17180685.

Kulesa, P M, and S E Fraser. 2000. "In Ovo Time-lapse Analysis of Chick Hindbrain Neural Crest Cell Migration Shows Cell Interactions During Migration to the Branchial Arches." *Development (Cambridge, England)* 127 (6) (March): 1161–72. http://www.ncbi.nlm.nih.gov/pubmed/10683170.

LaBonne, C, and M Bronner-Fraser. 2000. "Snail-related Transcriptional Repressors Are Required in Xenopus for Both the Induction of the Neural Crest and Its Subsequent Migration." *Developmental Biology* 221 (1) (May 1): 195–205. doi:10.1006/dbio.2000.9609. http://www.ncbi.nlm.nih.gov/pubmed/10772801.

Lacalli, Thurston C. 2006. "Prospective Protochordate Homologs of Vertebrate Midbrain and MHB, with Some Thoughts on MHB Origins." *International Journal of Biological Sciences* 2 (3) (January): 104–9. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1458436&tool=pmcentrez&rendertype=abstract.

Lai, Karen, Wing Sun, James P Correia, and Timothy E Kennedy. 2011. "Netrins : Versatile Extracellular Cues with Diverse Functions" 2169: 2153–2169. doi:10.1242/dev.044529.

Lakiza, Olga, Sarah Miller, Ashley Bunce, Eric Myung-Jae Lee, and David W McCauley. 2011. "SoxE Gene Duplication and Development of the Lamprey Branchial Skeleton: Insights into Development and Evolution of the Neural Crest." *Developmental Biology* 359 (1) (November 1): 149–61. doi:10.1016/j.ydbio.2011.08.012. http://www.ncbi.nlm.nih.gov/pubmed/21889937.

Lallemend, Francois, and Patrik Ernfors. 2012. "Molecular Interactions Underlying the Specification of Sensory Neurons." *Trends in Neurosciences* 35 (6) (June): 373–81. doi:10.1016/j.tins.2012.03.006. http://www.ncbi.nlm.nih.gov/pubmed/22516617.

Lallier, T E. 1991. "Cell Lineage and Cell Migration in the Neural Crest." *Annals of the New York Academy of Sciences* 615 (January): 158–71. http://www.ncbi.nlm.nih.gov/pubmed/2039141.

Lamborghini, J E. 1980. "Rohon-beard Cells and Other Large Neurons in Xenopus Embryos Originate During Gastrulation." *Journal of Comparative Neurology* 189 (2): 323–333. http://www.ncbi.nlm.nih.gov/pubmed/7364967.

———. 1987. "Disappearance of Rohon-Beard Neurons from the Spinal Cord of Larval Xenopus Laevis." *The Journal of Comparative Neurology* 264 (1) (October 1): 47–55. doi:10.1002/cne.902640105. http://www.ncbi.nlm.nih.gov/pubmed/3680623.

Landry, Marc, Rabia Bouali-Benazzouz, Salah El Mestikawy, Philippe Ravassard, and Frédéric Nagy. 2004. "Expression of Vesicular Glutamate Transporters in Rat Lumbar Spinal Cord, with a Note on Dorsal Root Ganglia." *Journal of Comparative Neurology* 468 (3): 380–394. http://www.ncbi.nlm.nih.gov/pubmed/14681932.

Laubichler, M. D. 2000. "Homology in Development and the Development of the Homology Concept." *Integrative and Comparative Biology* 40 (5) (October 1): 777–788. doi:10.1093/icb/40.5.777. http://icb.oxfordjournals.org/cgi/doi/10.1093/icb/40.5.777.

Lei, Qiubo, Yongsu Jeong, Kamana Misra, Shike Li, Alice K Zelman, Douglas J Epstein, and Michael P Matise. 2006. "Wnt Signaling Inhibitors Regulate the Transcriptional Response to Morphogenetic Shh-Gli Signaling in the Neural Tube": 325–337. doi:10.1016/j.devcel.2006.06.013.

Levanon, Ditsa, David Bettoun, Catherine Harris-Cerruti, Eilon Woolf, Varda Negreanu, Raya Eilam, Yael Bernstein, et al. 2002. "The Runx3 Transcription Factor Regulates Development and Survival of TrkC Dorsal Root Ganglia Neurons." *The EMBO Journal* 21 (13) (July 1): 3454–63. doi:10.1093/emboj/cdf370. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=125397&tool=pmcentrez&rendertype=abstract.

Levi-Montalcini, Rita, and Viktor Hamburger. 1951. "Selective Growth Stimulating Effects of Mouse Sarcoma on the Sensory and Sympathetic Nervous System of the Chick Embryo." *Journal of Experimental Zoology* 116 (2) (March): 321–361. doi:10.1002/jez.1401160206. http://doi.wiley.com/10.1002/jez.1401160206.

Levi-montalcini, Rita. 1987. "The Nerve Growth Factor: Thirty-five Years Later." *The EMBO Journal* 6 (9) (September): 2856. http://www.ncbi.nlm.nih.gov/pubmed/16453797.

Lewis, Katharine E, and Judith S Eisen. 2003. "From Cells to Circuits : Development of the Zebrafish Spinal Cord" 69 (April): 419–449. doi:10.1016/S0301-0082(03)00052-2.

Li, Wei, and Robert a Cornell. 2007. "Redundant Activities of Tfap2a and Tfap2c Are Required for Neural Crest Induction and Development of Other Non-neural Ectoderm Derivatives in Zebrafish Embryos." *Developmental Biology* 304 (1) (April 1): 338–54. doi:10.1016/j.ydbio.2006.12.042. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1904501&tool=pmcentrez&rendertype=abstract.

Liebl, D J, L Tessarollo, M E Palko, and L F Parada. 1997. "Absence of Sensory Neurons Before Target Innervation in Brain-derived Neurotrophic Factor-, Neurotrophin 3-, and TrkC-deficient Embryonic Mice." *The Journal of Neuroscience : the Official Journal of the Society for Neuroscience* 17 (23) (December 1): 9113–21. http://www.ncbi.nlm.nih.gov/pubmed/9364058.

Liem, K F, G Tremml, H Roelink, and T M Jessell. 1995. "Dorsal Differentiation of Neural Plate Cells Induced by BMP-mediated Signals from Epidermal Ectoderm." *Cell* 82 (6) (September 22): 969–79. http://www.ncbi.nlm.nih.gov/pubmed/7553857.

Litsiou, Anna, Sven Hanson, and Andrea Streit. 2005. "A Balance of FGF, BMP and WNT Signalling Positions the Future Placode Territory in the Head." *Development (Cambridge, England)* 132 (18) (September): 4051–62. doi:10.1242/dev.01964. http://www.ncbi.nlm.nih.gov/pubmed/16093325.

Liu, Ying, Amy W Helms, and Jane E Johnson. 2004. "Distinct Activities of Msx1 and Msx3 in Dorsal Neural Tube Development." *Development (Cambridge, England)* 131 (5) (March): 1017–28. doi:10.1242/dev.00994. http://www.ncbi.nlm.nih.gov/pubmed/14973289.

Lowery, Laura Anne, and Hazel Sive. 2004. "Strategies of Vertebrate Neurulation and a Re-evaluation of Teleost Neural Tube Formation" 121: 1189–1197. doi:10.1016/j.mod.2004.04.022.

Lu, Tsai-Ming, Yi-Jyun Luo, and Jr-Kai Yu. 2012. "BMP and Delta/Notch Signaling Control the Development of Amphioxus Epidermal Sensory Neurons: Insights into the Evolution of the Peripheral Sensory System." *Development (Cambridge, England)* 139 (11) (June): 2020–30. doi:10.1242/dev.073833. http://www.ncbi.nlm.nih.gov/pubmed/22535413.

Luo, Ting, Young-hoon Lee, and Thomas D Sargent. 2002. "Induction of Neural Crest in Xenopus by Transcription Factor AP2 ␣."

Ma, Qiufu, Carol Fode, Francois Guillemot, and David J Anderson. 1999a. "Control Two Distinct Waves of Neurogenesis in Developing Dorsal Root Ganglia": 1717–1728.

———. 1999b. "Neurogenin1 and Neurogenin2 Control Two Distinct Waves of Neurogenesis in Developing Dorsal Root Ganglia": 1717–1728.

Mackie, G O. 1970. "Neuroid Conduction and the Evolution of Conducting Tissues." *The Quarterly Review of Biology* 45 (4): 319–332. http://www.ncbi.nlm.nih.gov/pubmed/4395914.

Marlow, Heather, Eric Roettinger, Michiel Boekhout, and Mark Q Martindale. 2012. "Functional Roles of Notch Signaling in the Cnidarian Nematostella Vectensis." *Developmental Biology* 362 (2) (February 15): 295–308. doi:10.1016/j.ydbio.2011.11.012. http://www.ncbi.nlm.nih.gov/pubmed/22155407.

Marlow, Heather, Maria Antonietta Tosches, Raju Tomer, Patrick Steinmetz, Antonella Lauri, Tomas Larsson, and Detlev Arendt. "Larval Body Patterning and Apical Organs Are Conserved in Animal Evolution" 49 (0).

Marmigère, Frédéric, and Patrik Ernfors. 2007. "Specification and Connectivity of Neuronal Subtypes in the Sensory Lineage." *Nature Reviews. Neuroscience* 8 (2) (February): 114–27. doi:10.1038/nrn2057. http://www.ncbi.nlm.nih.gov/pubmed/17237804.

Maro, Géraldine S, Matthieu Vermeren, Octavian Voiculescu, Lisa Melton, James Cohen, Patrick Charnay, and Piotr Topilko. 2004. "Neural Crest Boundary Cap Cells Constitute a Source of Neuronal and Glial Cells of the PNS." *Nature Neuroscience* 7 (9) (September): 930–8. doi:10.1038/nn1299. http://www.ncbi.nlm.nih.gov/pubmed/15322547.

Marshall, Milnes. 1879. "The Morphology of the Vertebrate Olfactory Organ." *Quarterly J. Microscopical Science S2-19, 300.* (193).

Martínez, A, S Alcántara, V Borrell, J A Del Río, J Blasi, R Otal, N Campos, et al. 1998. "TrkB and TrkC Signaling Are Required for Maturation and Synaptogenesis of Hippocampal Connections." *Journal of Neuroscience* 18 (18): 7336–7350. http://www.ncbi.nlm.nih.gov/pubmed/9736654.

Matsunaga, Eiji, and Alain Chédotal. 2004. “Repulsive Guidance Molecule/neogenin: a Novel Ligand-receptor System Playing Multiple Roles in Neural Development.” *Development, Growth & Differentiation* 46 (6) (December): 481–6. doi:10.1111/j.1440-169x.2004.00768.x. http://www.ncbi.nlm.nih.gov/pubmed/15610137.
McCarthy, R a, and E D Hay. 1991. “Collagen I, Laminin, and Tenascin: Ultrastructure and Correlation with Avian Neural Crest Formation.” *The International Journal of Developmental Biology* 35 (4) (December): 437–52. http://www.ncbi.nlm.nih.gov/pubmed/1724907.
McCauley, David W. 2008. “SoxE, Type II Collagen, and Evolution of the Chondrogenic Neural Crest.” *Zoological Science* 25 (10) (October): 982–9. doi:10.2108/zsj.25.982. http://www.ncbi.nlm.nih.gov/pubmed/19267634.
McDougall, Carmel, Wei-Chung Chen, Sebastian M Shimeld, and David E K Ferrier. 2006. “The Development of the Larval Nervous System, Musculature and Ciliary Bands of Pomatoceros Lamarckii (Annelida): Heterochrony in Polychaetes.” *Frontiers in Zoology* 3 (1) (January): 16. doi:10.1186/1742-9994-3-16. http://www.frontiersinzoology.com/content/3/1/16.
McKeown, Sonja J, Vivian M Lee, Marianne Bronner-Fraser, Donald F Newgreen, and Peter G Farlie. 2005. “Sox10 Overexpression Induces Neural Crest-like Cells from All Dorsoventral Levels of the Neural Tube but Inhibits Differentiation.” *Developmental Dynamics : an Official Publication of the American Association of Anatomists* 233 (2) (June): 430–44. doi:10.1002/dvdy.20341. http://www.ncbi.nlm.nih.gov/pubmed/15768395.
Mccauley, David W, and Marianne Bronner-fraser. 2003. “Neural Crest Contributions to the Lamprey Head”: 2317–2327. doi:10.1242/dev.00451.
Medina, Diego L, Carla Sciarretta, Anna Maria Calella, Oliver Von Bohlen Und Halbach, Klaus Unsicker, and Liliana Minichiello. 2004. “TrkB Regulates Neocortex Formation Through the Shc/PLCgamma-mediated Control of Neuronal Migration.” *The The European Molecular Biology Organization Journal* 23 (19): 3803–3814. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=522798&tool=pmcentrez&rendertype=abstract.
Meinhardt, Hans. 2006. “Primary Body Axes of Vertebrates: Generation of a near-Cartesian Coordinate System and the Role of Spemann-type Organizer.” *Developmental Dynamics : an Official Publication of the American Association of Anatomists* 235 (11) (November): 2907–19. doi:10.1002/dvdy.20952. http://www.ncbi.nlm.nih.gov/pubmed/16958119.
Meulemans, Daniel, and Marianne Bronner-Fraser. 2007. “The Amphioxus SoxB Family: Implications for the Evolution of Vertebrate Placodes.” *International Journal of Biological Sciences* 3 (6): 356–364. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1950271&tool=pmcentrez&rendertype=abstract.
Meulemans, Daniel, and Marianne Bronner-fraser. 2007. “Insights from Amphioxus into the Evolution of Vertebrate Cartilage” (8). doi:10.1371/journal.pone.0000787.
Meyer, Emily J, Aissam Ikmi, and Matthew C Gibson. 2011. “Interkinetic Nuclear Migration Is a Broadly Conserved Feature of Cell Division in Pseudostratified Epithelia.” *Current Biology : CB* 21 (6) (March 22): 485–91. doi:10.1016/j.cub.2011.02.002. http://www.ncbi.nlm.nih.gov/pubmed/21376598.
Meyer, Néva P, and Elaine C Seaver. 2010. “Cell Lineage and Fate Map of the Primary Somatoblast of the Polychaete Annelid Capitella Teleta.” *Integrative and Comparative Biology* 50 (5) (November): 756–67. doi:10.1093/icb/icq120. http://www.ncbi.nlm.nih.gov/pubmed/21558238.
Miller, C T, T F Schilling, K Lee, J Parker, and C B Kimmel. 2000. “Sucker Encodes a Zebrafish Endothelin-1 Required for Ventral Pharyngeal Arch Development.” *Development (Cambridge, England)* 127 (17) (September): 3815–28. http://www.ncbi.nlm.nih.gov/pubmed/10934026.
Minichiello, L, and R Klein. 1996. “TrkB and TrkC Neurotrophin Receptors Cooperate in Promoting Survival of Hippocampal and Cerebellar Granule Neurons.” *Genes & Development* 10 (22) (November 15): 2849–58. http://www.ncbi.nlm.nih.gov/pubmed/8918886.
Minichiello, L, M Korte, D Wolfer, R Kühn, K Unsicker, V Cestari, C Rossi-Arnaud, H P Lipp, T Bonhoeffer, and R Klein. 1999. “Essential Role for TrkB Receptors in Hippocampus-mediated Learning.” *Neuron* 24 (2): 401–414. http://www.ncbi.nlm.nih.gov/pubmed/10571233.
Mirnics, K, and H R Koerber. 1995. “Prenatal Development of Rat Primary Afferent Fibers: I. Peripheral Projections.” *Journal of Comparative Neurology* 355 (4): 601–614. http://www.ncbi.nlm.nih.gov/pubmed/7636034.
Montelius, Andreas, Frédéric Marmigère, Christel Baudet, Jorge B Aquino, Sven Enerbäck, and Patrik Ernfors. 2007. “Emergence of the Sensory Nervous System as Defined by Foxs1 Expression.” *Differentiation; Research in Biological Diversity* 75 (5) (June): 404–17. doi:10.1111/j.1432-0436.2006.00154.x. http://www.ncbi.nlm.nih.gov/pubmed/17309606.
Moorman, S J. 2001. “Development of Sensory Systems in Zebrafish (Danio Rerio).” *ILAR Journal / National Research Council, Institute of Laboratory Animal Resources* 42 (4) (January): 292–8. http://www.ncbi.nlm.nih.gov/pubmed/11581521.
Moreno, E, and G Morata. 1999. “Caudal Is the Hox Gene That Specifies the Most Posterior Drosophile Segment.” *Nature* 400 (6747) (August 26): 873–7. doi:10.1038/23709. http://www.ncbi.nlm.nih.gov/pubmed/10476966.
Mori-Akiyama, Yuko, Haruhiko Akiyama, David H Rowitch, and Benoit de Crombrugghe. 2003. “Sox9 Is Required for Determination of the Chondrogenic Cell Lineage in the Cranial Neural Crest.” *Proceedings of the National Academy of Sciences of the United States of America* 100 (16) (August 5): 9360–5. doi:10.1073/pnas.1631288100.

http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=170923&tool=pmcentrez&rendertype=abstract.
Morris, Simon Conway, and John S. Peel. 2008. "The Earliest Annelids: Lower Cambrian Polychaetes from the Sirius Passet Lagerstätte, Peary Land, North Greenland." *Acta Palaeontologica Polonica* 53 (1) (January): 137–148. doi:10.4202/app.2008.0110. http://www.bioone.org/doi/abs/10.4202/app.2008.0110.
Morris-Kay, Gillian, and Seong-Seng Tan. 1987. "Mapping Cranial Neural Crest Cell Migration Pathways in Mammalian Embryos." *Trends in Genetics* 3 (null) (January): 257–261. doi:10.1016/0168-9525(87)90260-5. http://dx.doi.org/10.1016/0168-9525(87)90260-5.
Motter, Arianne L, and Gerard P Ahern. 2012. "TRPA1 Is a Polyunsaturated Fatty Acid Sensor in Mammals." *PLoS ONE* 7 (6): e38439. doi:10.1371/journal.pone.0038439. http://www.ncbi.nlm.nih.gov/pubmed/22723860.
Murakami, Yasunori, and Shigeru Kuratani. 2008. "Brain Segmentation and Trigeminal Projections in the Lamprey; with Reference to Vertebrate Brain Evolution." *Brain Research Bulletin* 75 (2-4) (March 18): 218–24. doi:10.1016/j.brainresbull.2007.10.057. http://www.ncbi.nlm.nih.gov/pubmed/18331874.
Muroyama, Yuko, Motoyuki Fujihara, Makoto Ikeya, Hisato Kondoh, and Shinji Takada. 2002. "Wnt Signaling Plays an Essential Role in Neuronal Specification of the Dorsal Spinal Cord." *Genes & Development* 16 (5) (March 1): 548–53. doi:10.1101/gad.937102. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=155351&tool=pmcentrez&rendertype=abstract.
Mutai, Hideki, and Stefan Heller. 2012. "Vertebrate and Invertebrate TRPV-like Mechanoreceptors." *Cell Calcium* 33 (5-6): 471–8. Accessed November 7. http://www.ncbi.nlm.nih.gov/pubmed/12765692.
Myers, Paul Z, Judith S Eisen, and Monte Westerfield. 1986. "Development Zebrafish and Axonal Outgrowth of Identified Motoneurons in The" 6 (August).
Müller, Monika C M. 2006. "Polychaete Nervous Systems: Ground Pattern and variations--cLS Microscopy and the Importance of Novel Characteristics in Phylogenetic Analysis." *Integrative and Comparative Biology* 46 (2) (April): 125–33. doi:10.1093/icb/icj017. http://www.ncbi.nlm.nih.gov/pubmed/21672729.
Nakao, T, and a Ishizawa. 1987. "Development of the Spinal Nerves in the Lamprey: I. Rohon-Beard Cells and Interneurons." *The Journal of Comparative Neurology* 256 (3) (February 15): 342–55. doi:10.1002/cne.902560304. http://www.ncbi.nlm.nih.gov/pubmed/3571509.
Nave, Klaus-Armin. 2010. "Myelination and the Trophic Support of Long Axons." *Nature Reviews Neuroscience* 11 (4): 275–283. http://www.ncbi.nlm.nih.gov/pubmed/20216548.
Ng, Teclise, Fengwei Yu, and Sudipto Roy. 2006. "A Homologue of the Vertebrate SET Domain and Zinc Finger Protein Blimp-1 Regulates Terminal Differentiation of the Tracheal System in the Drosophila Embryo." *Development Genes and Evolution* 216 (5): 243–252. http://www.ncbi.nlm.nih.gov/pubmed/16506071.
Nielsen, Morten, Claus Lundegaard, Ole Lund, and Thomas Nordahl Petersen. 2010. "CPHmodels-3.0--remote Homology Modeling Using Structure-guided Sequence Profiles." *Nucleic Acids Research* 38 (Web Server issue) (July): W576–81. doi:10.1093/nar/gkq535. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2896139&tool=pmcentrez&rendertype=abstract.
Nikitina, Natalya, Leslie Tong, and Marianne E Bronner. 2011. "Ancestral Network Module Regulating Prdm1 Expression in the Lamprey Neural Plate Border." *Developmental Dynamics : an Official Publication of the American Association of Anatomists* 240 (10) (October): 2265–71. doi:10.1002/dvdy.22720. http://www.ncbi.nlm.nih.gov/pubmed/21932309.
Nolo, R, L A Abbott, and H J Bellen. 2000. "Senseless, a Zn Finger Transcription Factor, Is Necessary and Sufficient for Sensory Organ Development in Drosophila." *Cell* 102 (3): 349–362. http://www.ncbi.nlm.nih.gov/pubmed/10975525.
Northcutt, R. Glenn, and Carl Gans. 1983. "The Genesis of Neural Crest and Epidermal Placodes: A Reinterpretation of Vertebrate Origins." *The Quarterly Review of Biology* 58 (1) (March): 1. doi:10.1086/413055. http://www.journals.uchicago.edu/doi/abs/10.1086/413055.
Nowotschin, Sonja, and Anna-Katerina Hadjantonakis. 2009. "Use of KikGR a Photoconvertible Green-to-red Fluorescent Protein for Cell Labeling and Lineage Analysis in ES Cells and Mouse Embryos." *BMC Developmental Biology* 9: 49. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2872819&tool=pmcentrez&rendertype=abstract.
Ockel, M, G R Lewin, and Y a Barde. 1996. "In Vivo Effects of Neurotrophin-3 During Sensory Neurogenesis." *Development (Cambridge, England)* 122 (1) (January): 301–7. http://www.ncbi.nlm.nih.gov/pubmed/8565842.
Ohtani, Kaoru, Tuoya Yao, Mari Kobayashi, Rie Kusakabe, Shigeru Kuratani, and Hiroshi Wada. 2008. "Expression of Sox and Fibrillar Collagen Genes in Lamprey Larval Chondrogenesis with Implications for the Evolution of Vertebrate Cartilage." *Journal of Experimental Zoology. Part B, Molecular and Developmental Evolution* 310 (7) (November 15): 596–607. doi:10.1002/jez.b.21231. http://www.ncbi.nlm.nih.gov/pubmed/18702077.
Olesnicky, Eugenia, Laura Hernandez-Lagunas, and Kristin Bruk Artinger. 2010. "Prdm1a Regulates Sox10 and Islet1 in the Development of Neural Crest and Rohon-Beard Sensory Neurons." *Genesis (New York, N.Y. : 2000)* 48 (11) (November): 656–66. doi:10.1002/dvg.20673.

http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3119337&tool=pmcentrez&rendertype=abstract.
Van Otterloo, Eric, Wei Li, Aaron Garnett, Maria Cattell, Daniel Meulemans Medeiros, and Robert a Cornell. 2012. "Novel Tfap2-mediated Control of soxE Expression Facilitated the Evolutionary Emergence of the Neural Crest." *Development (Cambridge, England)* 139 (4) (February): 720–30. doi:10.1242/dev.071308. http://www.ncbi.nlm.nih.gov/pubmed/22241841.
Pani, Ariel M, Erin E Mullarkey, Jochanan Aronowicz, Stavroula Assimacopoulos, Elizabeth a Grove, and Christopher J Lowe. 2012. "Ancient Deuterostome Origins of Vertebrate Brain Signalling Centres." *Nature* 483 (7389) (March 15): 289–94. doi:10.1038/nature10838. http://www.ncbi.nlm.nih.gov/pubmed/22422262.
Park, Byung-Yong, Chang-Soo Hong, Jamie R Weaver, Elizabeth M Rosocha, and Jean-Pierre Saint-Jeannet. 2012. "Xaml1/Runx1 Is Required for the Specification of Rohon-Beard Sensory Neurons in Xenopus." *Developmental Biology* 362 (1) (February 1): 65–75. doi:10.1016/j.ydbio.2011.11.016. http://www.ncbi.nlm.nih.gov/pubmed/22173066.
Pasini, Andrea, Aldine Amiel, Ute Rothbächer, Agnès Roure, Patrick Lemaire, and Sébastien Darras. 2006. "Formation of the Ascidian Epidermal Sensory Neurons: Insights into the Origin of the Chordate Peripheral Nervous System." *PLoS Biology* 4 (7) (July): e225. doi:10.1371/journal.pbio.0040225. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1481523&tool=pmcentrez&rendertype=abstract.
Patterson, C. 1988. "Homology in Classical and Molecular Biology." *Molecular Biology and Evolution* 5 (6) (November): 603–25. http://www.ncbi.nlm.nih.gov/pubmed/3065587.
Patthey, Cédric, Thomas Edlund, and Lena Gunhaga. 2009. "Wnt-regulated Temporal Control of BMP Exposure Directs the Choice Between Neural Plate Border and Epidermal Fate." *Development (Cambridge, England)* 136 (1) (January): 73–83. doi:10.1242/dev.025890. http://www.ncbi.nlm.nih.gov/pubmed/19060333.
Pattyn, a, M Hirsch, C Goridis, and J F Brunet. 2000. "Control of Hindbrain Motor Neuron Differentiation by the Homeobox Gene Phox2b." *Development (Cambridge, England)* 127 (7) (April): 1349–58. http://www.ncbi.nlm.nih.gov/pubmed/10704382.
Perris, R, D Krotoski, and M Bronner-Fraser. 1991. "Collagens in Avian Neural Crest Development: Distribution in Vivo and Migration-promoting Ability in Vitro." *Development (Cambridge, England)* 113 (3) (November): 969–84. http://www.ncbi.nlm.nih.gov/pubmed/1821863.
Pettersen, Eric F, Thomas D Goddard, Conrad C Huang, Gregory S Couch, Daniel M Greenblatt, Elaine C Meng, and Thomas E Ferrin. 2004. "UCSF Chimera--a Visualization System for Exploratory Research and Analysis." *Journal of Computational Chemistry* 25 (13): 1605–1612. doi:10.1002/jcc.20084. http://www.ncbi.nlm.nih.gov/pubmed/15264254.
Pfeffer, P L, T Gerster, K Lun, M Brand, and M Busslinger. 1998. "Characterization of Three Novel Members of the Zebrafish Pax2/5/8 Family: Dependency of Pax5 and Pax8 Expression on the Pax2.1 (noi) Function." *Development (Cambridge, England)* 125 (16) (August): 3063–74. http://www.ncbi.nlm.nih.gov/pubmed/9671580.
Pourquié, Olivier. 2009. *Hox Genes (Google eBook)*. Academic Press. http://books.google.com/books?id=OPBKAkPirqwC&pgis=1.
Pratt, Kara G, and Carlos D Aizenman. 2009. "Multisensory Integration in Mesencephalic Trigeminal Neurons in Xenopus Tadpoles." *Journal of Neurophysiology* 102 (1): 399–412. doi:10.1152/jn.91317.2008.
Puelles, Luis, and John L R Rubenstein. 2003. "Forebrain Gene Expression Domains and the Evolving Prosomeric Model." *Trends in Neurosciences* 26 (9) (September): 469–76. doi:10.1016/S0166-2236(03)00234-0. http://dx.doi.org/10.1016/S0166-2236(03)00234-0.
Pulido, D, S Campuzano, T Koda, J Modolell, and M Barbacid. 1992. "Dtrk, a Drosophila Gene Related to the Trk Family of Neurotrophin Receptors, Encodes a Novel Class of Neural Cell Adhesion Molecule." *The EMBO Journal* 11 (2) (February): 391–404. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=556467&tool=pmcentrez&rendertype=abstract.
Purschke, Gunter. 2005. "Sense Organs in Polychaetes (Annelida)." *Hydrobiologia*: 53–78.
Raff, R A. 2000. "Evo-devo: The Evolution of a New Discipline." *Nature Reviews. Genetics* 1 (1) (October): 74–9. doi:10.1038/35049594. http://dx.doi.org/10.1038/35049594.
Raff, R a, and B J Sly. 2000. "Modularity and Dissociation in the Evolution of Gene Expression Territories in Development." *Evolution & Development* 2 (2): 102–13. http://www.ncbi.nlm.nih.gov/pubmed/11258388.
Raible, D W, a Wood, W Hodsdon, P D Henion, J a Weston, and J S Eisen. 1992. "Segregation and Early Dispersal of Neural Crest Cells in the Embryonic Zebrafish." *Developmental Dynamics : an Official Publication of the American Association of Anatomists* 195 (1) (September): 29–42. doi:10.1002/aja.1001950104. http://www.ncbi.nlm.nih.gov/pubmed/1292751.
Raible, Florian, Kristin Tessmar-Raible, Kazutoyo Osoegawa, Patrick Wincker, Claire Jubin, Guillaume Balavoine, David Ferrier, et al. 2005. "Vertebrate-type Intron-rich Genes in the Marine Annelid Platynereis Dumerilii." *Science (New York, N.Y.)* 310 (5752) (November 25): 1325–6. doi:10.1126/science.1119089. http://www.ncbi.nlm.nih.gov/pubmed/16311335.
Ramoino, Paola, and Mario Pestarino. 2012. "A Neurochemical Map of the Developing Amphioxus Nervous System." doi:10.1186/1471-2202-13-59.

De Robertis E M & Sasai 1996. “De Robertis & Sasai 1996 A Common Plan for DV Patterning in Bilateria.pdf.”
Robinson, V, a Smith, a M Flenniken, and D G Wilkinson. 1997. “Roles of Eph Receptors and Ephrins in Neural Crest Pathfinding.” *Cell and Tissue Research* 290 (2) (November): 265–74. http://www.ncbi.nlm.nih.gov/pubmed/9321688.
Roots, Betty I. 2008. “The Phylogeny of Invertebrates and the Evolution of Myelin.” *Neuron Glia Biology* 4 (2): 101–109. http://www.ncbi.nlm.nih.gov/pubmed/19508741.
Rossi, Christy Cortez, Laura Hernandez-Lagunas, Chi Zhang, Irene F Choi, Letitia Kwok, Michael Klymkowsky, and Kristin Bruk Artinger. 2008. “Rohon-Beard Sensory Neurons Are Induced by BMP4 Expressing Non-neural Ectoderm in Xenopus Laevis.” *Developmental Biology* 314 (2) (February 15): 351–61. doi:10.1016/j.ydbio.2007.11.036. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2262044&tool=pmcentrez&rendertype=abstract.
Rossi, Christy Cortez, Takao Kaji, and Kristin Bruk Artinger. 2009. “Transcriptional Control of Rohon-Beard Sensory Neuron Development at the Neural Plate Border.” *Developmental Dynamics : an Official Publication of the American Association of Anatomists* 238 (4) (April): 931–43. doi:10.1002/dvdy.21915. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2755227&tool=pmcentrez&rendertype=abstract.
Roure, Agnès, Ute Rothbächer, François Robin, Eva Kalmar, Giustina Ferone, Clément Lamy, Caterina Missero, Ferenc Mueller, and Patrick Lemaire. 2007. “A Multicassette Gateway Vector Set for High Throughput and Comparative Analyses in Ciona and Vertebrate Embryos.” *PloS One* 2 (9) (January): e916. doi:10.1371/journal.pone.0000916. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1976267&tool=pmcentrez&rendertype=abstract.
Roy, Sudipto, and Teclise Ng. 2004. “Blimp-1 Specifies Neural Crest and Sensory Neuron Progenitors in the Zebrafish Embryo” 14: 1772–1777. doi:10.1016/j.
Rychel, Amanda L, and Billie J Swalla. 2007. “Development and Evolution of Chordate Cartilage.” *Journal of Experimental Zoology Part B Molecular and Developmental Evolution* 308 (3): 325–335. http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=17358002.
Rykunov, Dmitry, Elliot Steinberger, Carlos J Madrid-Aliste, and András Fiser. 2009. “Improved Scoring Function for Comparative Modeling Using the M4T Method.” *Journal of Structural and Functional Genomics* 10 (1): 95–99.
Sakai, Daisuke, Takashi Suzuki, Noriko Osumi, and Yoshio Wakamatsu. 2006. “Cooperative Action of Sox9, Snail2 and PKA Signaling in Early Neural Crest Development.” *Development (Cambridge, England)* 133 (7) (April): 1323–33. doi:10.1242/dev.02297. http://www.ncbi.nlm.nih.gov/pubmed/16510505.
Sambrook, J, E F Fritsch, and T Maniatis. 2001. *Molecular Cloning: A Laboratory Manual*. Ed. Jan Argentine. *Cold Spring Harbor Laboratory*. Vol. 3. Cold Spring Harbor Laboratory Press. doi:10.1128/AEM.71.8.4602. www.molecularcloning.com.
Santiago, Alicia, and Carol a Erickson. 2002. “Ephrin-B Ligands Play a Dual Role in the Control of Neural Crest Cell Migration.” *Development (Cambridge, England)* 129 (15) (August): 3621–32. http://www.ncbi.nlm.nih.gov/pubmed/12117812.
Sasai, N, K Mizuseki, and Y Sasai. 2001. “Requirement of FoxD3-class Signaling for Neural Crest Determination in Xenopus.” *Development (Cambridge, England)* 128 (13) (July): 2525–36. http://www.ncbi.nlm.nih.gov/pubmed/11493569.
Sasakura, Yasunori, Satoko Awazu, Shota Chiba, and Nori Satoh. 2003. “Germ-line Transgenesis of the Tc1 ¯Mariner Superfamily Transposon Minos in Ciona Intestinalis” 100 (13): 7726–7730.
Sauka-Spengler, Tatjana, and Marianne Bronner-Fraser. 2008a. “Insights from a Sea Lamprey into the Evolution of Neural Crest Gene Regulatory Network.” *The Biological Bulletin* 214 (3) (June): 303–14. http://www.ncbi.nlm.nih.gov/pubmed/18574106.
———. 2008b. “A Gene Regulatory Network Orchestrates Neural Crest Formation.” *Nature Reviews. Molecular Cell Biology* 9 (7) (July): 557–68. doi:10.1038/nrm2428. http://www.ncbi.nlm.nih.gov/pubmed/18523435.
Sauka-spengler, Tatjana, Daniel Meulemans, Matthew Jones, and Marianne Bronner-fraser. 2007. “Article Ancient Evolutionary Origin of the Neural Crest Gene Regulatory Network” (September): 405–420. doi:10.1016/j.devcel.2007.08.005.
Schneider, Stephan Q, and Bruce Bowerman. 2007. “beta-Catenin Asymmetries After All Animal/vegetal- Oriented Cell Divisions in Platynereis Dumerilii Embryos Mediate Binary Cell-fate Specification.” *Developmental Cell* 13 (1) (July): 73–86. doi:10.1016/j.devcel.2007.05.002. http://www.ncbi.nlm.nih.gov/pubmed/17609111.
Schubert, Michael, Nicholas D Holland, Vincent Laudet, and Linda Z Holland. 2006. “A Retinoic acid-Hox Hierarchy Controls Both Anterior/posterior Patterning and Neuronal Specification in the Developing Central Nervous System of the Cephalochordate Amphioxus.” *Developmental Biology* 296 (1) (August 1): 190–202. doi:10.1016/j.ydbio.2006.04.457. http://www.ncbi.nlm.nih.gov/pubmed/16750825.
Schultz, Jörg, Frank Milpetz, Peer Bork, and Chris P Ponting. 1998. “SMART, a Simple Modular Architecture Research Tool: Identification of Signaling Domains.” *Proceedings of the National Academy of Sciences of the United States of America* 95 (11): 5857–5864.

http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=34487&tool=pmcentrez&rendertype=abstract.
Schweigreiter, Rüdiger, Betty I Roots, Christine E Bandtlow, and Robert M Gould. 2006. "Understanding Myelination Through Studying Its Evolution." *International Review of Neurobiology* 73 (06) (January): 219–73. doi:10.1016/S0074-7742(06)73007-0. http://www.ncbi.nlm.nih.gov/pubmed/16737906.
Selleck, M A, and M Bronner-Fraser. 1995. "Origins of the Avian Neural Crest: The Role of Neural Plate-epidermal Interactions." *Development (Cambridge, England)* 121 (2) (February): 525–38. http://www.ncbi.nlm.nih.gov/pubmed/7768190.
Shimizu, Takashi, Young-Ki Bae, and Masahiko Hibi. 2006. "Cdx-Hox Code Controls Competence for Responding to Fgfs and Retinoic Acid in Zebrafish Neural Tissue." *Development (Cambridge, England)* 133 (23) (December): 4709–19. doi:10.1242/dev.02660. http://www.ncbi.nlm.nih.gov/pubmed/17079270.
Shu, D. 2008. "Cambrian Explosion: Birth of Tree of Animals." *Gondwana Research* 14 (1-2) (August): 219–240. doi:10.1016/j.gr.2007.08.004. http://linkinghub.elsevier.com/retrieve/pii/S1342937X07001694.
Sigrist, Christian J A, Lorenzo Cerutti, Edouard De Castro, Petra S Langendijk-Genevaux, Virginie Bulliard, Amos Bairoch, and Nicolas Hulo. 2010. "PROSITE, a Protein Domain Database for Functional Characterization and Annotation." *Nucleic Acids Research* 38 (Database issue): D161–D166. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2808866&tool=pmcentrez&rendertype=abstract.
Simionato, Elena, Pierre Kerner, Nicolas Dray, Martine Le Gouar, Valérie Ledent, Detlev Arendt, and Michel Vervoort. 2008. "Atonal- and Achaete-scute-related Genes in the Annelid Platynereis Dumerilii: Insights into the Evolution of Neural basic-Helix-Loop-Helix Genes." *BMC Evolutionary Biology* 8 (January): 170. doi:10.1186/1471-2148-8-170. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2435551&tool=pmcentrez&rendertype=abstract.
Skromne, Isaac, Dean Thorsen, Melina Hale, Victoria E Prince, and Robert K Ho. 2007. "Repression of the Hindbrain Developmental Program by Cdx Factors Is Required for the Specification of the Vertebrate Spinal Cord." *Development (Cambridge, England)* 134 (11) (June): 2147–58. doi:10.1242/dev.002980. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2804982&tool=pmcentrez&rendertype=abstract.
Soo, Kenneth, Meredith P. O'Rourke, Poh-Lynn Khoo, Kirsten a. Steiner, Nicole Wong, Richard R. Behringer, and Patrick P.L. Tam. 2002. "Twist Function Is Required for the Morphogenesis of the Cephalic Neural Tube and the Differentiation of the Cranial Neural Crest Cells in the Mouse Embryo." *Developmental Biology* 247 (2) (July): 251–270. doi:10.1006/dbio.2002.0699. http://linkinghub.elsevier.com/retrieve/pii/S0012160602906998.
Sossin, Wayne S. 2006. "Tracing the Evolution and Function of the Trk Superfamily of Receptor Tyrosine Kinases" 2006: 145–156. doi:10.1159/000094084.
Srivastava, Mansi, Claire Larroux, Daniel R Lu, Kareshma Mohanty, Jarrod Chapman, Bernard M Degnan, and Daniel S Rokhsar. 2010. "Early Evolution of the LIM Homeobox Gene Family." *BMC Biology* 8 (January): 4. doi:10.1186/1741-7007-8-4. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2828406&tool=pmcentrez&rendertype=abstract.
Steiner, Aaron B, Mark J Engleka, Qun Lu, Eileen C Piwarzyk, Sergey Yaklichkin, Julie L Lefebvre, James W Walters, Liliam Pineda-Salgado, Patricia A Labosky, and Daniel S Kessler. 2006. "FoxD3 Regulation of Nodal in the Spemann Organizer Is Essential for Xenopus Dorsal Mesoderm Development." *Development Cambridge England* 133 (24): 4827–4838. http://www.ncbi.nlm.nih.gov/pubmed/17092955.
Steinmetz, Patrick R H, Roman P Kostyuchenko, Antje Fischer, and Detlev Arendt. 2011. "The Segmental Pattern of Otx, Gbx, and Hox Genes in the Annelid Platynereis Dumerilii." *Evolution & Development* 13 (1): 72–9. doi:10.1111/j.1525-142X.2010.00457.x. http://www.ncbi.nlm.nih.gov/pubmed/21210944.
Steinmetz, Patrick R H, Rolf Urbach, Nico Posnien, Joakim Eriksson, Roman P Kostyuchenko, Carlo Brena, Keren Guy, Michael Akam, Gregor Bucher, and Detlev Arendt. 2010. "Six3 Demarcates the Anterior-most Developing Brain Region in Bilaterian Animals." *EvoDevo* 1 (1) (January): 14. doi:10.1186/2041-9139-1-14. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3025827&tool=pmcentrez&rendertype=abstract.
Stephens, R M, D M Loeb, T D Copeland, T Pawson, L A Greene, and D R Kaplan. 1994. "Trk Receptors Use Redundant Signal Transduction Pathways Involving SHC and PLC-gamma 1 to Mediate NGF Responses." *Neuron* 12 (3): 691–705. http://www.ncbi.nlm.nih.gov/pubmed/8155326.
Stewart, Rodney A, Brigitte L Arduini, Stephane Berghmans, Rani E George, John P Kanki, Paul D Henion, and A Thomas Look. 2006. "Zebrafish Foxd3 Is Selectively Required for Neural Crest Specification, Migration and Survival." *Developmental Biology* 292 (1): 174–188. http://www.ncbi.nlm.nih.gov/pubmed/16499899.

Stolt, C Claus, and Michael Wegner. 2010. "SoxE Function in Vertebrate Nervous System Development." *The International Journal of Biochemistry & Cell Biology* 42 (3) (March): 437–40. doi:10.1016/j.biocel.2009.07.014. http://dx.doi.org/10.1016/j.biocel.2009.07.014.

Sun, Yunfu, Iain M Dykes, Xingqun Liang, S Raisa Eng, Sylvia M Evans, and Eric E Turner. 2008. "A Central Role for Islet1 in Sensory Neuron Development Linking Sensory and Spinal Gene Regulatory Programs." *Nature Neuroscience* 11 (11) (November): 1283–93. doi:10.1038/nn.2209. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2605652&tool=pmcentrez&rendertype=abstract.

Suzuki, Takashi, Daisuke Sakai, Noriko Osumi, Hiroshi Wada, and Yoshio Wakamatsu. 2006. "Sox Genes Regulate Type 2 Collagen Expression in Avian Neural Crest Cells": 477–486. doi:10.1111/j.1440-169x.2006.00886.x.

"TRPV Family Ion Channels and Other Molecular Components Required for Hearing and Proprioception in Drosophila." 2007. CRC Press. http://www.ncbi.nlm.nih.gov/books/NBK5253/#top.

Tanaka, Hideomi, Yasuhiro Nojima, Wataru Shoji, Miki Sato, Ryoko Nakayama, Toshio Ohshima, and Hitoshi Okamoto. 2011. "Islet1 Selectively Promotes Peripheral Axon Outgrowth in Rohon-Beard Primary Sensory Neurons." *Developmental Dynamics : an Official Publication of the American Association of Anatomists* 240 (1) (January): 9–22. doi:10.1002/dvdy.22499. http://www.ncbi.nlm.nih.gov/pubmed/21108318.

Taneyhill, Lisa a, Edward G Coles, and Marianne Bronner-Fraser. 2007. "Snail2 Directly Represses cadherin6B During Epithelial-to-mesenchymal Transitions of the Neural Crest." *Development (Cambridge, England)* 134 (8) (April): 1481–90. doi:10.1242/dev.02834. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2595139&tool=pmcentrez&rendertype=abstract.

Teillet, M a, C Kalcheim, and N M Le Douarin. 1987. "Formation of the Dorsal Root Ganglia in the Avian Embryo: Segmental Origin and Migratory Behavior of Neural Crest Progenitor Cells." *Developmental Biology* 120 (2) (April): 329–47. http://www.ncbi.nlm.nih.gov/pubmed/3549390.

Tessarollo, L. 1998. "Pleiotropic Functions of Neurotrophins in Development." *Cytokine & Growth Factor Reviews* 9 (2) (June): 125–37. http://www.ncbi.nlm.nih.gov/pubmed/9754707.

Tessmar-Raible, Kristin, Patrick Steinmetz, Heidi Snyman, Monika Hassel, and Detlev Arendt. 2005. "Fluorescent Two-color Whole Mount in Situ Hybridization in Platynereis Dumerilii (Polychaeta, Annelida), an Emerging Marine Molecular Model for Evolution and Development." *BioTechniques* 39 (4) (October): 460–464. doi:10.2144/000112023. http://www.biotechniques.com/article/05394BM02.

Tomer, Raju, Alexandru S Denes, Kristin Tessmar-Raible, and Detlev Arendt. 2010. "Profiling by Image Registration Reveals Common Origin of Annelid Mushroom Bodies and Vertebrate Pallium." *Cell* 142 (5) (September 3): 800–9. doi:10.1016/j.cell.2010.07.043. http://www.ncbi.nlm.nih.gov/pubmed/20813265.

Trainor, Paul a. 2003. "Making Headway: The Roles of Hox Genes and Neural Crest Cells in Craniofacial Development." *TheScientificWorldJournal* 3 (April 14): 240–64. doi:10.1100/tsw.2003.11. http://www.ncbi.nlm.nih.gov/pubmed/12806110.

Ullrich-Lüter, Esther M, Sam Dupont, Enrique Arboleda, Harald Hausen, and Maria Ina Arnone. 2011. "Unique System of Photoreceptors in Sea Urchin Tube Feet." *Proceedings of the National Academy of Sciences of the United States of America* 108 (20) (May 17): 8367–72. doi:10.1073/pnas.1018495108. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3100952&tool=pmcentrez&rendertype=abstract.

Vagnetti, D, and R M Farnesi. 1978. "Morphological and Ultrastructural Study of the Ventral Nerve Cord in Branchiobdella Pentodonta Whitman (Annelida, Oligochaeta)." *The Journal of Comparative Neurology* 178 (2) (March 15): 365–82. doi:10.1002/cne.901780210. http://www.ncbi.nlm.nih.gov/pubmed/627629.

Villanueva, Sandra, Alvaro Glavic, Pablo Ruiz, and Roberto Mayor. 2002. "Posteriorization by FGF, Wnt, and Retinoic Acid Is Required for Neural Crest Induction." *Developmental Biology* 241 (2) (January 15): 289–301. doi:10.1006/dbio.2001.0485. http://www.ncbi.nlm.nih.gov/pubmed/11784112.

Vincent, Stéphane D, N Ray Dunn, Roger Sciammas, Miriam Shapiro-Shalef, Mark M Davis, Kathryn Calame, Elizabeth K Bikoff, and Elizabeth J Robertson. 2005. "The Zinc Finger Transcriptional Repressor Blimp1/Prdm1 Is Dispensable for Early Axis Formation but Is Required for Specification of Primordial Germ Cells in the Mouse." *Development (Cambridge, England)* 132 (6) (March): 1315–25. doi:10.1242/dev.01711. http://www.ncbi.nlm.nih.gov/pubmed/15750184.

Vitt, U a, S Y Hsu, and a J Hsueh. 2001. "Evolution and Classification of Cystine Knot-containing Hormones and Related Extracellular Signaling Molecules." *Molecular Endocrinology (Baltimore, Md.)* 15 (5) (May): 681–94. http://www.ncbi.nlm.nih.gov/pubmed/11328851.

Voiculescu, O, E Taillebourg, C Pujades, C Kress, S Buart, P Charnay, and S Schneider-Maunoury. 2001. "Hindbrain Patterning: Krox20 Couples Segmentation and Specification of Regional Identity." *Development (Cambridge, England)* 128 (24) (December): 4967–78. http://www.ncbi.nlm.nih.gov/pubmed/11748134.

Volosin, Marta, Christy Trotter, Andrea Cragnolini, Rajappa S Kenchappa, Matthew Light, Barbara L Hempstead, Bruce D Carter, and Wilma J Friedman. 2008. "Induction of Proneurotrophins and Activation of p75NTR-mediated Apoptosis via Neurotrophin Receptor-interacting Factor in Hippocampal Neurons After Seizures." *The Journal of Neuroscience : the Official Journal of the Society*

for Neuroscience 28 (39) (September 24): 9870–9. doi:10.1523/JNEUROSCI.2841-08.2008. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2578816&tool=pmcentrez&rendertype=abstract.
Votano, JR, M Parham, and LH Hall. 2004. "The Neural Crest and Neural Crest Cells in Vertebrate Development and Evolution." *Springer*. http://onlinelibrary.wiley.com/doi/10.1002/cbdv.200490137/abstract.
Vujovic, S, S Henderson, N Presneau, E Odell, T S Jacques, R Tirabosco, C Boshoff, and a M Flanagan. 2006. "Brachyury, a Crucial Regulator of Notochordal Development, Is a Novel Biomarker for Chordomas." *The Journal of Pathology* 209 (2) (June): 157–65. doi:10.1002/path.1969. http://www.ncbi.nlm.nih.gov/pubmed/16538613.
Wada, H. 2001. "Origin and Evolution of the Neural Crest: a Hypothetical Reconstruction of Its Evolutionary History." *Development Growth Differentiation* 43 (5): 509–520. http://www.ncbi.nlm.nih.gov/pubmed/11576168.
Wada, H, H Saiga, N Satoh, and P W Holland. 1998. "Tripartite Organization of the Ancestral Chordate Brain and the Antiquity of Placodes: Insights from Ascidian Pax-2/5/8, Hox and Otx Genes." *Development (Cambridge, England)* 125 (6) (March): 1113–22. http://www.ncbi.nlm.nih.gov/pubmed/9463358.
Wang, G, and S A Scott. 1999. "Independent Development of Sensory and Motor Innervation Patterns in Embryonic Chick Hindlimbs." *Developmental Biology* 208 (2): 324–336. http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=10191048.
Webster, M J, M M Herman, J E Kleinman, and C Shannon Weickert. 2006. "BDNF and trkB mRNA Expression in the Hippocampus and Temporal Cortex During the Human Lifespan." *Gene Expression Patterns GEP* 6 (8): 941–951. http://www.ncbi.nlm.nih.gov/pubmed/16713371.
Wei, Zheng, Robert C Angerer, and Lynne M Angerer. 2011. "Direct Development of Neurons Within Foregut Endoderm of Sea Urchin Embryos." *Proceedings of the National Academy of Sciences of the United States of America* 108 (22) (May 31): 9143–7. doi:10.1073/pnas.1018513108. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3107264&tool=pmcentrez&rendertype=abstract.
Westerfield, Monte, V Mcmurray, and Judith S Eisen. 1986. "Identified Motoneurons Zebrafish and Their Innervation of Axial Muscles in The" 6 (August): 2267–2277.
Whiting, H P. 1948. "Nervous Structure of the Spinal Cord of the Young Larval Brook-lamprey." *The Quarterly Journal of Microscopical Science* 89 (Pt. 4) (December): 359–83. http://www.ncbi.nlm.nih.gov/pubmed/18109176.
Williams, J a, a Barrios, C Gatchalian, L Rubin, S W Wilson, and N Holder. 2000. "Programmed Cell Death in Zebrafish Rohon Beard Neurons Is Influenced by TrkC1/NT-3 Signaling." *Developmental Biology* 226 (2) (October 15): 220–30. doi:10.1006/dbio.2000.9860. http://www.ncbi.nlm.nih.gov/pubmed/11023682.
Wilson, Karen H S. 2009. "The Genome Sequence of the Protostome Daphnia Pulex Encodes Respective Orthologues of a Neurotrophin, a Trk and a p75NTR: Evolution of Neurotrophin Signaling Components and Related Proteins in the Bilateria." *BMC Evolutionary Biology* 9 (January): 243. doi:10.1186/1471-2148-9-243. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2772990&tool=pmcentrez&rendertype=abstract.
Wilson, Stephen W, and Corinne Houart. 2004. "Early Steps in the Development of the Forebrain." *Developmental Cell* 6 (2) (February): 167–81. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2789258&tool=pmcentrez&rendertype=abstract.
Winberg, M L, L Tamagnone, J Bai, P M Comoglio, D Montell, and C S Goodman. 2001. "The Transmembrane Protein Off-track Associates with Plexins and Functions Downstream of Semaphorin Signaling During Axon Guidance." *Neuron* 32 (1): 53–62. http://www.ncbi.nlm.nih.gov/pubmed/11604138.
Wittbrodt, Joachim. 2007. "New Genes in the Evolution of the Neural Crest Differentiation." doi:10.1186/gb-2007-8-3-r36.
Woodhoo, Ashwin, and Lukas Sommer. 2008. "Development of the Schwann Cell Lineage: From the Neural Crest to the Myelinated Nerve." *Glia* 56 (14): 1481–90. doi:10.1002/glia.20723. http://www.ncbi.nlm.nih.gov/pubmed/18803317.
Wurst, W, and L Bally-Cuif. 2001. "Neural Plate Patterning: Upstream and Downstream of the Isthmic Organizer." *Nature Reviews. Neuroscience* 2 (2) (February): 99–108. doi:10.1038/35053516. http://www.ncbi.nlm.nih.gov/pubmed/11253000.
Yan, Y L, K Hatta, B Riggleman, and J H Postlethwait. 1995. "Expression of a Type II Collagen Gene in the Zebrafish Embryonic Axis." *Developmental Dynamics : an Official Publication of the American Association of Anatomists* 203 (3) (July): 363–76. doi:10.1002/aja.1002030308. http://www.ncbi.nlm.nih.gov/pubmed/8589433.
Yoda, Akinori, Isao Oishi, and Yasuhiro Minami. 2003. "Expression and Function of the Ror-family Receptor Tyrosine Kinases During Development: Lessons from Genetic Analyses of Nematodes, Mice, and Humans." *Journal of Receptor and Signal Transduction Research* 23 (1): 1–15. http://informahealthcare.com/doi/abs/10.1081/RRS-120018757.

Young, H M, C J Hearn, P G Farlie, A J Canty, P Q Thomas, and D F Newgreen. 2001. "GDNF Is a Chemoattractant for Enteric Neural Cells." *Developmental Biology* 229 (2) (January 15): 503–16. doi:10.1006/dbio.2000.0100. http://www.ncbi.nlm.nih.gov/pubmed/11150245.

Yu, Jr-Kai, Nicholas D Holland, and Linda Z Holland. 2002. "An Amphioxus Winged Helix/forkhead Gene, AmphiFoxD: Insights into Vertebrate Neural Crest Evolution." *Developmental Dynamics an Official Publication of the American Association of Anatomists* 225 (3): 289–297. http://www.ncbi.nlm.nih.gov/pubmed/12412011.

Yu, Jr-kai, Daniel Meulemans, Sonja J Mckeown, and Marianne Bronner-fraser. 2008. "Insights from the Amphioxus Genome on the Origin of Vertebrate Neural Crest" (626): 1127–1132. doi:10.1101/gr.076208.108.18.

Yu, Weiying, Kristen Mcdonnell, Makoto M Taketo, and C Brian Bai. 2008. "Wnt Signaling Determines Ventral Spinal Cord Cell Fates in a Time-dependent Manner" 3696: 3687–3696. doi:10.1242/dev.021899.

Zamboni, L, and M Stefanini. 2012. "On the Configuration of the Plasma Membrane of the Mature Spermatozoon." *Fertility and Sterility* 19 (4): 570–9. Accessed November 7. http://www.ncbi.nlm.nih.gov/pubmed/4873201.

Zhang, Jing, Julie L Lefebvre, Shuxia Zhao, and Michael Granato. 2004. "Zebrafish Unplugged Reveals a Role for Muscle-specific Kinase Homologs in Axonal Pathway Choice." *Nature Neuroscience* 7 (12): 1303–1309. http://dx.doi.org/10.1038/nn1350.

Zhu, Bangfu, Jenny A Pennack, Peter Mcquilton, Manuel G Forero, Kenji Mizuguchi, Ben Sutcliffe, Chun-jing Gu, Janine C Fenton, and Alicia Hidalgo. 2008. "Drosophila Neurotrophins Reveal a Common Mechanism for Nervous System Formation" 6 (11). doi:10.1371/journal.pbio.0060284.

Additional references:

Conway Morris S: The Crucible of Creation. The Burgess Shale and the Rise of Animals. Oxford: Oxford University Press; 1998, 2006

COOHILL, G. E., 1914. J. comp. Neurol., 24, 161. 1929- Anatomy and the Problem of Behaviour, Cambridge (University Press)

De Beer, G. R. (1938). Embryology and evolution. In Evolution :Essays on Aspects of Evolutionary Biology Presented to Professor E. S.Goodrich on his Seventieth Birthday (ed. G. R. de Beer), pp. 59–78.The Clarendon Press, Oxford.

Kimmel, C.B. and Westerfield, M. (1990) Primary neurons of the zebrafish. In Signal and Sense, G.M. Edelman, W.E. Gall, and M.W. Cowan, eds., New York: Wiley-Liss 561-588.

Levi-Montalcini, R. (1975) NGF: an unchartered route. In The Neuroscience, Paths of Discovery, pp. 245–265, MIT Press.

Parker, George Howard , 1919, The elementary nervous system, Philadelphia, London J.B. Lippincott Company

Remane, A. 1952. Die Grundlagen des natürlichen Systems, der vergleichenden Anatomie und der Phylogenetik.. Akademische Verlagsgesellschaft, Leipzig.

Retzius, G. 1891. Zur Kenntniss des Centralnervensystems von Amphioxus lanceolatus. Biol. Unters. 2: 29–42.

Thorogood P. Review of developmental and evolutionary aspects of the neural crest. Trends Neurosci. 1989;12:38–39.