EVOLUTION AND DEVELOPMENT OF THE SYMPATHETIC NERVOUS SYSTEM

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Table of Content

Contents

Table of figures

Zusammenfassung

Das Sympathische Nervensystem der Gnathostomata ist ein Teil des autonomen Nervensystems und ist an der unwillentlichen Steuerung der inneren Organe und der Aufrechterhaltung der Homöosthase des Körpers beteiligt. Dabei stimuliert es durch seine ergotrope Wirkung die Stressreaktionen des Körpers.

Die Neuronen des Sympathikus sind Derivate der Neuralleiste. Die Zellen der Neuralleiste sind pluripotent und dadurch gekennzeichten, dass sie im Laufe der Embryonalentwicklung durch den Organismus wandern und dabei die Anlage verschiedener Gewebe bilden, wie zum Beispiel sensorische Neurone, Neurone des autonomen Nervensystems, Glia, Melanozyten, Nebennierenmark und außerdem im Kopfbereich Knochen, Knorpel, Bindegewebe und Muskeln.

Neunaugen sind Vertreter der Agnatha und somit basale, kieferlose Vertebraten, die kambrischen Fossilien ähneln. Neunaugen besitzen Neuralleistenzellen und auch die meisten Neuralleistenderivate, aber ihnen fehlt der sympathische Grenzstrang – Truncus sympathicus. Obwohl ein Truncus sympathicus nicht vorhanden ist, wurden verschiedene Zelltypen beschrieben, welche in Neunaugen sympathische Funktionen übernehmen.

Um das Verständnis der Evolution und der Entwicklung des Sympathischen Nervensystems zu erhellen, wurden Antikörperfärbungen, Zellmarkierungen mittels DiI und *in-situ*-Hybridisierungen durchgeführt. Anfärbungen mittels eines pan-neuronalen Antikörpers und Zellmarkierungen mit DiI bestätigten die Existenz von Spinalganglien und enterischen Ganglien in Neunaugen. *In-situ*-Hybridisierungen in Neunaugenembryonen mit spezifischen Sonden für Markergene des Sympathischen Nervensystems, Ascl1, dHand und Phox2b, zeigten die Expression dieser Gene in verschiedenen Geweben in denen auch eine

entsprechende Expression bei Gnathostomata bekannt ist. Jedoch wurde keine Expression in Bereichen, in denen sich bei Gnathostomata Strukturen des Sympathischen Nervensystems befinden, detektiert. Insgesamt wurde mit keiner der drei angewandten Methoden sympathische Ganglienzellen oder Zellen, die als Vorläuferzellen des Sympathikus angesehen werden könnten, gefunden.

Das Fehlen eines Truncus sympathicus in Neunaugen führte zu der Hypothese, dass eine cis-regulatorische Umregulierung bereits vorhandener Gene zu der Entstehung des Sympatischen Nervensystems in Wirbeltieren beigetragen haben könnte. Zur Überprüfung dieser Hypothese wurden drei konservierte nicht-kodierende Elemente mithilfe von Reporter-Expressions-Experimentes in Haushuhn und Neunage verglichen. Ein konserviertes nicht-kodierendes Element, welches proximal im Phox2b-Promoter liegt, führte zu Expression im Haushuhn. Im Gegensatz dazu war dieses Element bei dem Test der drei Elemente in Neunaugen inaktiv. Allerdings war in Neunaugen von den drei getesteten Elementen eine andere Sequenz im ersten Intron aktiv. Überraschenderweise führten die zwei unterschiedlichen Elemente in beiden Arten zu Expression in vergleichbaren Geweben. Keins der drei getesteten Elemente zeigte Expression in sympathischen Neuronen oder Ganglien. Das Interspezies-Experiment hat gezeigt, dass Regionen aus dem Genom des Haushuhn in der Lage sind, in Neunaugen Transkriptionsfaktoren zu rekrutieren und Expression zu erzeugen.

Diese Arbeit bestätigt, dass sowohl der Truncus sympathikus, als auch andere periphere sympathische Neuronen in Neunaugen fehlen. Es ist eine plausible Annahme, dass Änderungen der cis-regulatorischen Verschaltungen zu der evolutiven Rekrutierung von Neuralleistenzellen zu Vorläufern des Sympathischen Nervensystems führten. Im Folgenden wird

anhand der neuen Erkenntnisse und der bereits vorhanden Literatur die Evolution des Sympathischen Nervensystems dikutiert.

Abstract

In Gnathostomata, the sympathetic nervous system is a branch of the autonomic nervous system and it is responsible for the unconscious control of the inner organs. It mainly conveys stress responses, the so called “flight or fight” reaction.

Sympathetic neurons are derivatives of the neural crest, a pluripotent embryological cell lineage that has migratory capabilities. Neural crest cells give rise to various tissues, for example sensory neurons, autonomic neurons, glia, melanocytes, cells of the adrenal medulla as well as the bone, cartilage, connective tissue and muscle cells of the head.

Lampreys are jawless basal vertebrates, Agnatha, that resemble Cambrian era fossils. Lampreys do have neural crest cells and most of their derivatives, but lack a commissural sympathetic chain. Despite the absence of a definite sympathetic ganglion chain, different cell types of endocrine cells were reported to implement sympathetic functions in lampreys.

To shed light on the evolution and development of the sympathetic nervous system, immunohistochemistry, DiI cell tracing and *in-situ* hybridization experiments were carried out. Staining with a pan-neuronal antibody as well as the DiI cell labeling in lampreys confirmed the presence of dorsal root and enteric ganglia. *In-situ* hybridizations with probes against the sympathetic nervous system marker genes Ascl1, dHand and Phox2b, visualized transcripts of these genes in various tissues that correspond to the known expression of these genes in gnathostomes, except for expression in sites where sympathetic ganglia would be expected. None of the three different techniques detected sympathetic ganglia cells or cells that could be regarded as sympathetic precursor cells.

The absence of a commissural sympathetic chain in lampreys led to the hypothesis that redeployment of genes within the gene regulatory network for sympathetic neuron differentiation contributed to the appearance of the sympathetic nervous system. To test this hypothesis the cis-regulatory activity of three conserved non-coding elements was compared in lamprey and chicken using a reporter expression assay. A conserved non-coding element lying directly proximal to the chicken Phox2b promoter showed expression in the chicken. In contrast, this element was silent when the same three sequences were tested in lamprey. The only element which gave rise to expression in lamprey was a different one, which is spanning the first intron. Surprisingly the two different conserved non-coding elements drove expression in corresponding tissues in both species. None of the tested elements showed expression in sympathetic neurons or ganglia. The interspecies experiment showed that chicken genomic regions can successfully recruit transcription factors and drive expression in the lamprey.

This study confirms that a commissural sympathetic chain and peripheral sympathetic neurons are absent in lampreys. It is a plausible scenario that changes in cis-regulatory linkages led to the evolutionary deployment of neural crest cells as precursors for sympathetic neurons.

Hence, the evolution of the sympathetic nervous system is discussed based on the presented results and in the light of previous publications.

General Introduction

Lampreys

Lampreys are agnathans (jawless vertebrates). Together with hagfishes they comprise a sister group to the gnathostomes (jawed vertebrates) also called “cyclostomes” referring to their round mouths and the absence of jaws. Although controversy remains on the question if lampreys and hagfishes are monophyletic (Cyclostome hypothesis) or if lampreys are closer to the gnathostomes (Vertebrate hypothesis) then the hagfishes (Kuraku, Hoshiyama et al. 1999; Osorio and Retaux 2008). It is mostly anatomical data suggesting a closer relationship between lampreys and gnathostomes, but upcoming and growing genetic evidence seems to support a monophyly of hagfishes and lampreys (Heimberg, Cowper-Sal-lari et al. 2010).

Today there are 38 known lamprey species. The most studied species are the sea lamprey *Petromyzon marinus* and the Japanese lamprey *Lethenteron japonicum* (Shimeld and Donoghue 2012). Lampreys have an eel-like body shape and they possess many essential vertebrate characteristics. The 360-million-year-old fossil from the Devonian, *Priscomyzon riniensis* (Gess, Coates et al. 2006), is the oldest definite lamprey fossil discovered so far. Fossil records for Agnatha date back to the early Cambrian (Gess, Coates et al. 2006; Janvier 2006) and fossils that are morphologically lamprey-like were dated back 540 million-years to 510 million-years (D-G. Shu 1999). The fossils prove the parasitic lifestyle of lampreys at least for the last 360 million years (Gess, Coates et al. 2006).

The lifecycle of sea lampreys is very peculiar (Figure 1). The embryos grow to a larval stage called ammocoetes, worm-like larva that burrow themselves in sediments of slow streaming fresh water streams with only

their head above the ground to filter-feed from the streaming water. Initially the ammocoetes were thought to be an independent species which was called *Ammocoetes branchialis*. The larval stage lasts for 3 to 8 years and ends with the metamorphosis (3 – 10 month) to the adult lamprey. The adult lampreys then migrate into lakes or the sea and live for 1 - 4 years before they migrate up the streams to spawn and die (Osorio and Retaux 2008). Until their migration into freshwater streams for spawning most lamprey species live as ectoparasites on fish (Shimeld and Donoghue 2012). There are a few lamprey species that are living exclusively in freshwater and that do not feed as ectoparasites. The European brook lamprey *Lampetra planeri* for example stays in the freshwater streams as a filter feeding ammocoete and stops feeding during and after the metamorphose (Hardisty 1951).

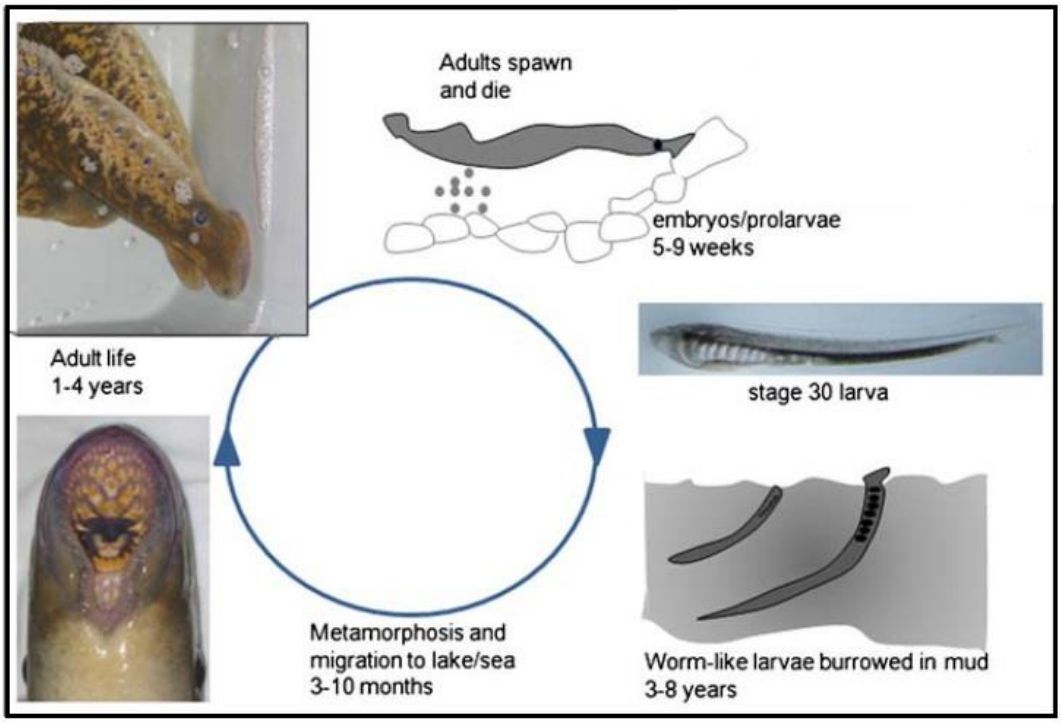

Figure 1: Lifecycle of the sea lamprey (*Petromyzon marinus*) (Osorio and Retaux 2008)

Despite their vertebrate characteristics lampreys lack some key features like paired appendages, the commissural sympathetic chain and jaws (Sauka-Spengler and Bronner-Fraser 2008).

As basal vertebrates, lampreys and hagfishes occupy a phylogenetic position, which is critical for understanding evolution of vertebrate traits. However, lampreys offer a significant advantage for developmental studies over hagfishes due to the accessibility and ease of obtaining embryos for experimental manipulation (Holland 2007; Shimeld and Donoghue 2012).

There are two different staging tables available for lamprey embryos, the one of Tahara for *Lampetra reissneri* (Tahara 1988) and the one of Piavis for *Petromyzon marinus* (Piavis 1961). Piavis categorized the early development starting from the ovulated but unfertilized egg to the first larval stage into 19 stages. His stages are defined by morphological

hallmarks. The 19 Piavis stages occur over a period of 35 – 40 days and are therefore not detailed enough for genetic studies. In this study lamprey embryos are staged by “embryonic days” (E) meaning the days after fertilization of the egg.

Neural Crest

Neural crest cells are a vertebrate specific population of migratory and multipotent precursors. They were first identified in the developing chicken embryo by Wilhelm His in 1868 (His 1868). The extant Agnatha, lampreys and hagfishes also possess neural crest cells (McCauley and Bronner-Fraser 2006; Ota, Kuraku et al. 2007).

Neural crest cells undergo 4 phases (Figure 2), induction, delamination, migration and, differentiation. The induction phase was traditionally thought to start at the time of neural tube closure, but is now proven to start at early gastrula stage and last until the closure of the neural tube. The induction phase is characterized by two important steps. First, the early induction phase when the prospective neural crest cells are induced during the open neural plate stage and second, the late phase that is required for neural crest cells to maintain their identity after the neural tube closure (Aybar and Mayor 2002; Stuhlmiller and Garcia-Castro 2012).

After closure of the neural tube the neural crest cells start to delaminate in a rostral to caudal pattern (Sauka-Spengler and Bronner-Fraser 2008). The delamination of neural crest cells from the neural epithelium requires an epithelial - mesenchymal transition. This is a process of drastic changes in cell shape and cell-cell interactions. From the formation of an epithelium in which cells have an apical-basal polarity and strong cell-cell interactions via tight junctions, cadherin based adherens junctions and gap junctions, delaminating neural crest cells change to cells with no

apical-basal cell polarity, but filopodia and the ability to interact with the extracellular matrix (Duband, Monier et al. 1995; Hay 2005; Radisky 2005).

The neural crest cells that have undergone the epithelial – mesenchymal transition start their migration along defined pathways throughout the embryos body. The mechanism of path finding varies between the different subpopulations of the neural crest. The mechanisms employed involve cell-cell attraction, cell-cell inhibition, repellent signals from the surrounding tissues as well as attracting signals from target tissues (Clay and Halloran 2010).

After reaching their target sites neural crest cells start differentiating. Neural crest cells give rise to a multitude of differentiated cell types, for example sensory neurons, autonomic neurons, glia, melanocytes, cells of the adrenal medulla as well as the bone, cartilage, connective tissue and muscle cells of the head (Le Douarin 1982; Graham 2003).

The exact time point of fate restriction towards certain derivatives varies between the different neural crest cells. Even before migration starts the pool of migrating cells consists of pluripotent neural crest stem cells as well as fate restricted progenitors (Mundell and Labosky 2011). The exact niche for neural crest cell pluripotency and differentiation is under intensive investigation since adult neural crest stem cells could be of great therapeutic value (Delfino-Machin, Chipperfield et al. 2007).

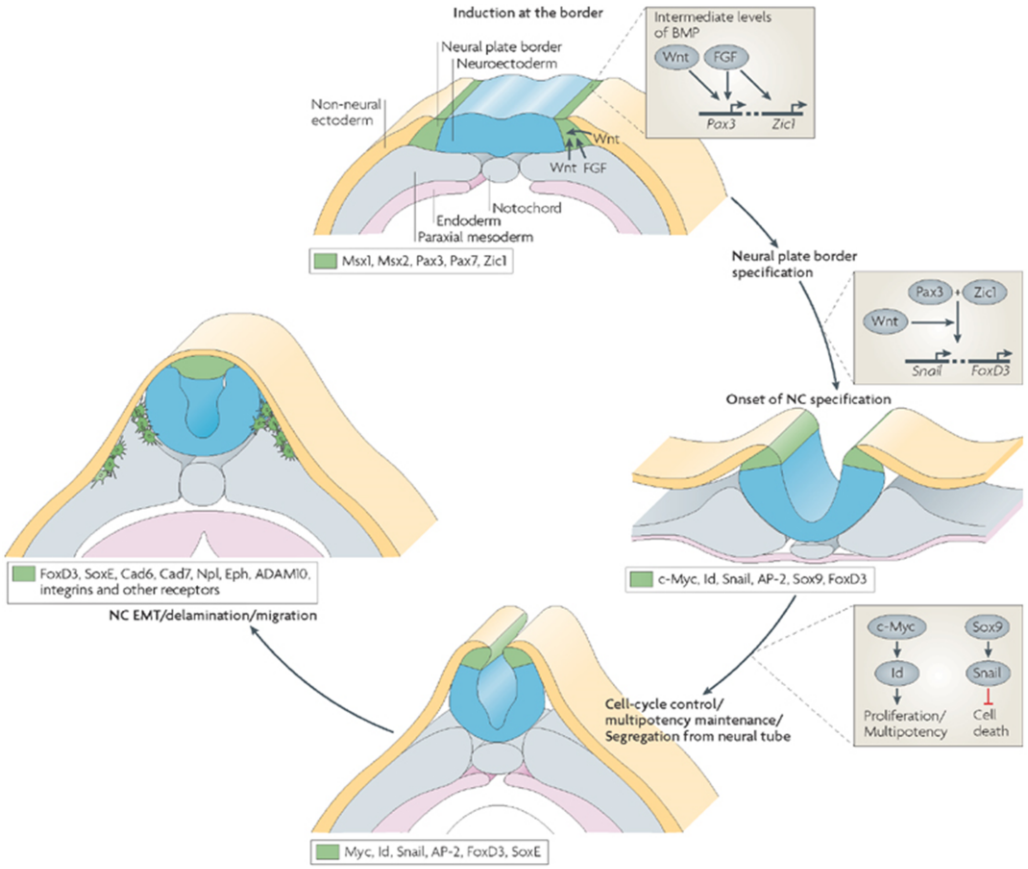

Figure 2: Developmental steps of neural crest formation (Sauka-Spengler and Bronner-Fraser 2008)

The neural crest is subdivided into different populations namely the cranial, the vagal including the cardiac which is sometimes regarded as an independent population and the trunk neural crest. Some researchers also regard the lumbo-sacral neural crest as an individual neural crest population (Kuo and Erickson 2010) citations). All populations of neural crest cells migrate along distinct and defined pathways and give rise to specific cell types.

The cranial crest cells migrate predominantly between the ectoderm and the underlying mesoderm which is called the dorsolateral pathway

(Tosney 1982; Gans and Northcutt 1983). The most obvious derivatives of the cranial crest are the facial skeleton, cartilage and connective tissue of the head as well as its contribution to the fore- and midbrain, but it also gives rise to melanocytes, neurons and glial cells (Noden 1978; Le Douarin, Brito et al. 2007).

The vagal neural crest contributes to the neurons and glia cells of the peripheral nervous system and melanocytes, too (Reedy, Faraco et al. 1998; Reedy, Faraco et al. 1998; Kuo and Erickson 2011) and it is the main population that gives rise to the neurons and glia cells of the enteric nervous system. For a long time the vagal neural crest was thought to be the sole population of neural crest cells that gives rise to enteric neurons (Yntema and Hammond 1954; Le Douarin and Teillet 1973; Epstein, Mikawa et al. 1994; Burns, Champeval et al. 2000; Burns and Le Douarin 2001; Burns, Delalande et al. 2002).

The cardiac crest is a subpopulation of the vagal neural crest. It appears in chick from the post-otic brain to somite level three and the name is derived from the major contribution of the cardiac crest to formation of the outflow tract as well as the septation of the heart and the parasympathetic innervation of the heart. Additional derivatives are the smooth muscle tunics of the great arteries, the connective tissue of the thymus, the thyroid and parathyroid, the articopulmonary septum and melanocytes (Kuo and Erickson 2010).

The trunk neural crest gives rise to sensory and sympathetic ganglia, the adrenal medulla and melanocytes (Bronner 2012). The neural crest cells of the trunk migrate along two main routes, the cells contributing to the dorsal root and sympathetic ganglia migrate along the ventromedial path whereas the melanocytes use the dorsolateral path underneath the ectoderm (Theveneau and Mayor 2011).

The lumbo-sacral crest is a subpopulation of the neural crest, arising caudally to the somite 28 in chick that was mainly drawn attention to when

it was discovered to give rise to enteric neurons. The in vivo contribution and relevance still needs to be elucidated, but Burns and Le Douarin (Burns and Douarin 1998) have shown that in major portions of the intestine there is less than 1 neuron out of 1000 of lumbo-sacral neural crest origin as opposed to vagal neural crest origin.

The neural crest gives rise to a plethora of important tissues and structures. All impairments of these neural crest derivatives are categorized as neurochristopathies including malformations and cancers (Etchevers, Amiel et al. 2006). Among the most common neurochristopathies in pediatric practice are impairments of the autonomic nervous system including Hirschsprung's disease and neuroblastoma. Neuroblastoma is the most common extracranial malignant tumor in childhood with an incidence of 10 per 1 million children and these tumors arise from the sympathetic nervous system and the adrenal medulla (Maris and Matthay 1999). Hirschsprung's disease is a malformation of the enteric nervous system characterized by an absence of neurons in the distal-most part of the gut. The incidence is 1 in 5000 live births (Amiel, Sproat-Emison et al. 2008). Neurochristopathies are as manifold as the neural crest derivatives and include birth defects of the facial structures, like oral clefts or Waardenburg syndrome (Kouskoura, Fragou et al. 2011), defects of cardiac crest formation like congenital heart disease (Stoller and Epstein 2005) or reduced vagal neural crest migration leading to Hirschsprung's disease (Sullivan 1996). Misregulation of the gene regulatory network that forms the neural crest often leads to the formation of cancer like neuroblastoma or schwannoma (Etchevers, Amiel et al. 2006).

In evolution bona fide neural crest cells allowed for the "new head" as initially described by Gans and Northcutt as the key evolutionary step of vertebrates to develop from filter feeding to a predatory lifestyle (Gans and Northcutt 1983; Northcutt 2005).

Previous studies have shown that lampreys possess neural crest cells and most neural crest derivatives (Braun 1996; Northcutt 1996; McCauley and Bronner-Fraser 2006; Osorio and Retaux 2008), like cartilage, pigment cells and neurons.

Sauka-Spengler and Bronner-Fraser carried out a comprehensive study on the gene regulatory network of the lamprey neural crest and compared it with the one in gnathostomes (Figure 3). Interestingly, there is a strong conservation in deployment of factors and regulatory modules at the level of patterning signals, neural plate border specifiers and neural crest specifiers. At the level of the neural crest effector genes and for some neural crest specifier genes differences were observed. A shared proximal part of the gene regulatory network in lampreys and gnathostomes suggests a conservation since the split of the two infraphyla which is dated 500 million years ago (Sauka-Spengler and Bronner-Fraser 2008).

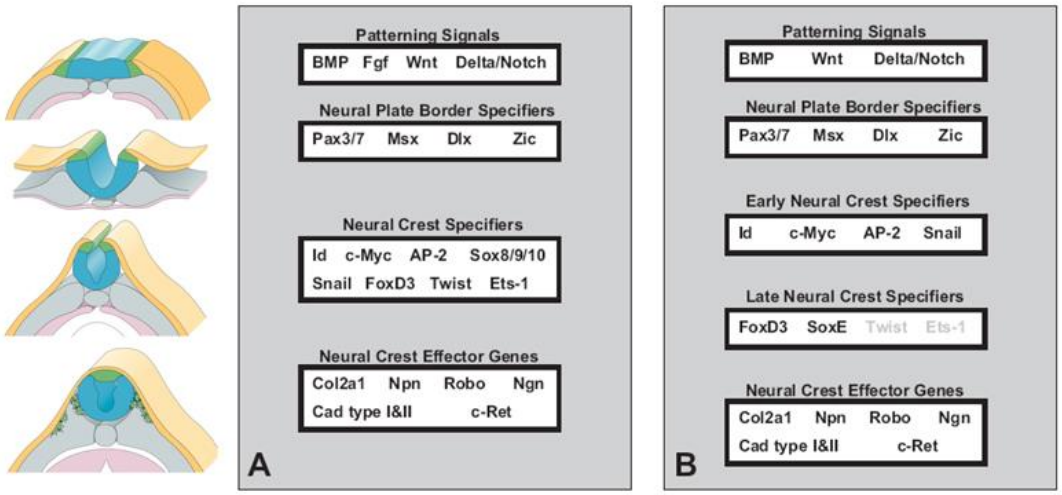

Figure 3: The neural crest gene regulatory network in gnathostomes (A) and lamprey (B) (Sauka-Spengler and Bronner-Fraser 2008). The gene regulatory network is comprised of spatially and temporally distinct regulatory modules.

The sympathetic nervous system

The peripheral nervous system (PNS) of jawed vertebrates is subdivided into a somatic and an autonomic nervous system. The somatic nervous system is described as the “voluntary nervous system”, the autonomic nervous system in contrast is the “visceral nervous system” and it can be further subdivided into the parasympathetic, sympathetic and enteric nervous system. Nowadays the enteric nervous system is often regarded as an independent nervous system, also called the second brain, because it can carry out some of its functions in the absence of input from the CNS (Gershon 2005). They innervate peripheral structures of the body via motor neurons running from the central nervous system (CNS) to the effector tissues and relay information back via sensory neurons to the central nervous system (Figure 4). The autonomic nervous system uses two groups of motor neurons for transmitting signals from the CNS to the effector. The first group is made of the preganglionic neurons that emanate from the CNS and run into a ganglion. In the ganglion they relay the stimulus to the postganglionic neuron which runs into the effector tissue. In the parasympathetic nervous system both, the pre- and postganglionic neurons use acetylcholine as a neurotransmitter. However, nowadays a few exceptions are discovered, for example a few postganglionic parasympathetic neurons that use nitric oxide as their neurotransmitter (Balligand, Kobzik et al. 1995). In the sympathetic nervous system acetylcholine is used in pre- and postganglionic neurons only for the innervation of the sweat glands. The other sympathetic preganglionic neurons use acetylcholine, too, but the postganglionic neurons use noradrenaline as their neurotransmitter. The use of noradrenaline as primary neurotransmitter in the PNS is unique to the sympathetic nervous system. Another exception in the sympathetic nervous system is the innervation of the adrenal gland. It is directly

innervated by a preganglionic fiber without going through a ganglion and therefore the adrenal gland also receives an acetylcholine signal. Through sympathetic stimulation, the adrenal gland itself secretes noradrenaline and adrenaline into the bloodstream. Therefore it can be regarded as a paraganglionic structure which systemically activates catecholamine receptors throughout the body.

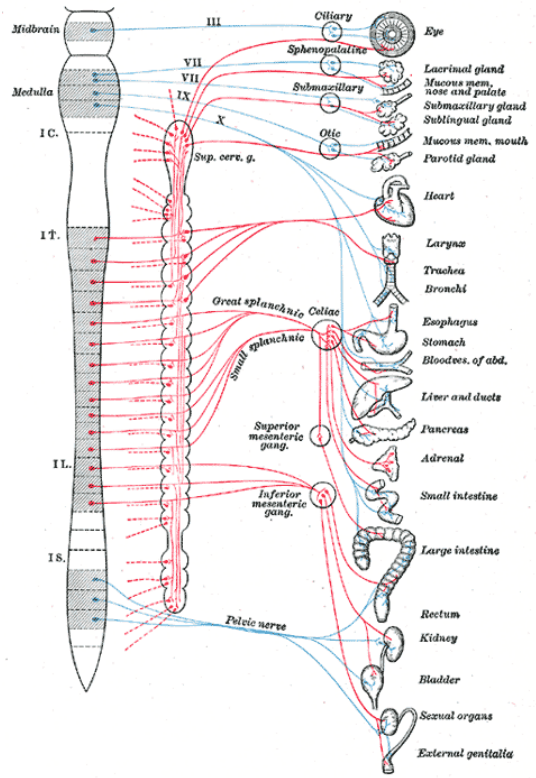

Figure 4: Drawing of the sympathetic (red) and parasympathetic (blue) nervous system from Grays Anatomy plate 839 (Gray 1918).

Sympathetic ganglia form a chain of ganglia alongside the spinal cord that extends from the upper neck down to the coccyx and is situated ventral and lateral to the spinal cord. Innervation to the commissural chain of

ganglia always comes from the spinal nerves of the spinal cord and not from the encephalon (Gray 1918).
All of these sensory, motor and autonomic ganglia are mainly neural crest derived features, except for a contribution of cranial placodes to the sensory ganglia of the head (D'Amico-Martel and Noden 1983).

Gene regulatory networks

One concept of how morphological structures are established in ontogenesis describes developmental processes as the result of the formation of gene regulatory networks (GRN). In the era of genomics and proteomics large datasets describing regulatory states are being generated that enable to unravel the complex interdependence of genes and the important role of cis-regulatory regions (Davidson and Erwin 2006). The observation that one gene is often deployed in the formation of various embryological traits deprecated the “one gene one trait” idea. Amongst other factors, for example chromatin structure, posttranslational regulation or epigenetics, the GRN theory is a widely accepted concept to explain how one gene functions in different spatial and temporal contexts. Therefore, genes have to be able to respond to different transcription factors.
GRNs are defined by such cis-regulatory linkages of transcription factor genes, which ultimately define the regulatory states of embryonic tissues (Davidson 2006). These networks have a modular structure comprised by sub-circuits in different hierarchical order. The sub-circuits that are most resistant to evolutionary changes are called “kernels” and consist of highly conserved regulatory interactions. Kernels lay the basis for cis-regulatory linkages in the development of embryological structures. The regulation and interdependence in a kernel is so sensitive that a single non-

functional prevents the formation of the particular body part. Sub-circuits that are in the periphery of the GRN can be variable, for example the sub-circuit that controls pigmentation (Silver, Hou et al. 2006).

Generally there are two different modes through which GRNs are altered to form new or more specialized traits during the course of evolution. One way to form a new trait is the augmentation of new genes and gene interactions into a pre-existing GRN. The second mode is the redeployment of an entire GRN sub-circuit for a new function. These sub-circuits of GRNs bear special evolutionary relevance. They are called "GRN plug-ins" and are defined as sub-circuits that are frequently redeployed in different parts of the GRN, but keep their internal structure (Erwin and Davidson 2009). Two examples for this second mechanism are described by Reed et al. and by Dinwiddie et al. (Dinwiddie and Rachootin 2011; Reed, Papa et al. 2011). Both found a new trait formed by the redeployment of the GRN sub-circuit around the *optix* gene. Reed et al. found that the *optix* containing sub-circuit underlying eye formation is also activated in a different spatio-temporal context, controlling the red pigmentation of the butterfly (*Heliconius*) wing. Dinwiddie and colleagues discovered that this sub-circuit around the master regulator *optix* is responsible for the formation of ommatidia on the tip of the wings of the females of extinct midges (*Eohelea petrunkevitchi*).

The GRN in embryos of the lamprey underlying the formation and differentiation of neural crest cells is remarkably similar to that of higher vertebrates. Sauka-Spengler and Bronner-Fraser could demonstrate that the proximal portion of the neural crest GRN, the patterning signals, the border specifiers, the neural plate specifiers as well as most of the neural crest specifiers are conserved between lamprey and chicken (Figure 3). There are four prominent signaling molecules that represent the patterning signals in gnathostomes, namely BMP, FGF, Wnt and Delta/Notch. The three genes BMP, Wnt and Delta/Notch were tested in the lamprey and

found to carry out equivalent functions. Guérin et al. (Guerin, d'Aubenton-Carafa et al. 2009) describe the early expression of lamprey FGFs in detail and these fit in with a pattern signaling role for FGF in lamprey, too. The four analyzed genes of the border specifier gene regulatory network are the same in lamprey and gnathostomes, namely Pax3/7, Msx, Dlx and Zic.

The neural crest specifiers are transcription factors that render the cells *bona fide* neural crest progenitors conveying the ability to delaminate and migrate. Id, c-Myc, AP-2 and Snail, as the early neural crest specifiers and FoxD3, SoxE and with concessions also Twist and Ets-1, as the late neural crest specifiers are members of a conserved gene regulatory network. The neural crest effector genes analyzed by Sauka-Spengler and Bronner-Fraser (Sauka-Spengler and Bronner-Fraser 2008), Col2a1, Npn, Robo, Ngn, Cad type I&II and c-ret, are present in a pattern temporally similar to the one in gnathostomes, but the interactions in this GRN sub-circuit differ.

These differences become apparent among the neural crest derivatives, as lampreys lack some key neural crest structures including dentine, bone and sympathetic chain ganglia (Baker 2008; Sauka-Spengler and Bronner-Fraser 2008; Martin, Bumm et al. 2009).

There are a few well described transcription factors that are required for the formation of a sympathetic nervous system in developing embryos (Figure 5). These can be used as markers for sympathetic neurons. The transcription factors include Phox2b, Ascl1, and dHand (Huber 2006).

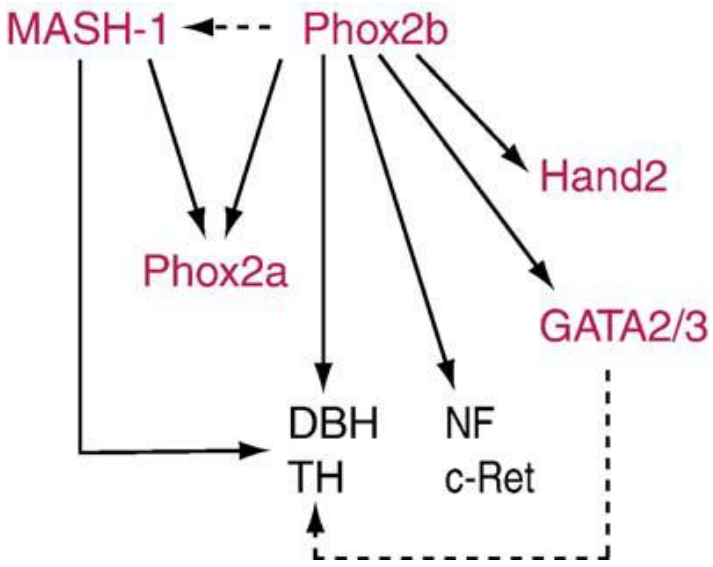

Figure 5: The gene regulatory network of sympathoadrenal cells (Huber 2006). These interactions are inferred from gene knockout studies. The dashed lines depict a required interaction for maintenance.

Ascl1 (achaete-scute homolog 1 formerly Ash1) is a bHLH transcription factor generally required for development of autonomic neurons. It induces expression of pan-neuronal genes in neural crest precursor cells of the peripheral nerve, but does not specify subtype specific expression of tyrosine hydroxylase (TH) or dopamine-β-hydroxylase (DBH), the enzymes responsible for the catalyzing synthesis of the neurotransmitter nor-epinephrine (Lo, Johnson et al. 1991).

Phox2b is a homeodomain transcription factor and it is also required for autonomic neurogenesis and, in a feedback loop, is required for maintenance of Ascl1 expression (Pattyn, Morin et al. 1997; Rychlik, Hsieh et al. 2005).

dHand, a bHLH transcription factor and another determinant of the sympathetic lineage, promotes the proliferation and differentiation of sympathetic neuron precursors into noradrenergic neurons. Over-

expression of dHand upregulates Phox2b, TH and DBH. Its own expression depends on Phox2b, but not Ascl1 (Schmidt, Lin et al. 2009).

Evolution of the sympathetic nervous system

It has been reported that lampreys lack the commissural sympathetic chain that all gnathostomes possess (Figure 6) (Nicol 1952). While anatomical data on the absence of a chain of sympathetic ganglia in lampreys is convincing it is still unknown what type of sympathetic cells or what level of sympathetic precursors they do possess. Lampreys are reported to have direct sympathetic innervation from fibers, analogous to the preganglionic fibers, to the terminal plexus. This mode of innervation is similar to the situation in amphioxus (Johnels 1956). Additionally some authors argue that lampreys and hagfishes have chromaffin-like cells along the blood vessels, the heart and the cloaca, but their interpretation of these cells varies.

Chromaffin cells were historically given their name for their affinity to chrome that was used as a stain. These cells are catecholaminergic endocrine cells. Johnels proposed that these cells are innervated via the ventral and dorsal roots and are therefore analogous to postganglionic neurons (Johnels 1956), whereas Gibbins stated that a connection to the CNS is not certain yet and that the chromaffin cells might represent more distant evolutionary precursors to the gnathostome sympathetic nervous system (Gibbins 1994).

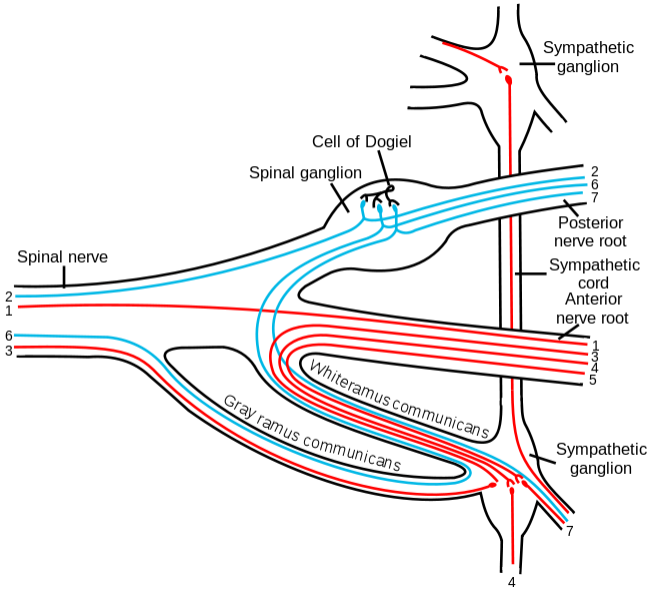

Figure 6: Drawing of the structure of a typical spinal nerve from Grays Anatomy plate 799 (Gray 1918). 1. Somatic efferent 2. Somatic afferent 3.,4.,5. Sympathetic efferent 6.,7. Sympathetic afferent

Several findings suggest the existence of cells from the sympathetic lineage in lampreys: the presence of several neurotransmitters, most importantly acetylcholine and noradrenaline as well as DNA sequence data from lampreys that uncovers the presence of two adrenergic receptors, specifically a beta-adrenoreceptor A and a beta-adrenoreceptor B. Beta-adrenergic receptors are a class of G-protein coupled receptors that are targeted by catecholamines, especially adrenalin and noradrenaline. This highly relates the beta-adrenergic receptors to the sympathetic nervous system in gnathostomes (Scofield, Deupree et al. 2002).

Owsiannikof (1883) and Lignon (1979) reported a sympathetic ganglion comprised of small intensely fluorescent cells (SIF cells) adjacent to the heart in adult lampreys (Owsiannikof 1883; Lignon 1979). SIF cells posses endocrine function by their capability to store and release catecholamines

from their granules and they can function as interneurons (Elfvin, Lindh et al. 1993). In vertebrates the vagus nerve, a nerve of the parasympathetic nervous system, innervates almost all internal organs including the heart. The heart of hagfishes is an exception as it is aneural, no innervation from the parasympathetic or sympathetic nervous system could be detected (Jensen 1965). The heart of larval lamprey lacks the vagal innervation observed in the adult (Carlson 1906). The vagus nerve is cardioinhibitory in all gnathostomes, but not in lampreys. There are different hypotheses to explain this finding. One hypothesis claims that the vagus nerve is innervating a sympathetic ganglion adjacent to the lamprey heart (Burnstock 1969) or chromaffin cells inside the heart (Fänge 1963) and thus induces the release of cardioexcitatory catecholamines. Another hypothesis to explain this finding was proposed by Fänge et al. (Fänge 1963), by suggesting that the vagus nerve of lampreys could contain adrenergic nerve fibers, as it does in Myxine. Or the adrenergic fibers could be distinct from the vagus nerve, but enter the vagi from the spinal outflow as suggested by Peters (Peters 1963). Otorii reported that acetylcholine accelerates the isolated heart of a lamprey (*Entosphenus*), whereas adrenaline retards heart rate frequency (Otorii 1953) thus explaining the excitatory attribute of the vagus nerve in lamprey. This finding was confirmed in *Lampretra fluviatilis* and *Lampetra planeri* by Augustinsson et al. (Augustinsson, Fange et al. 1956). Itina (Itina 1959) proposed that the lamprey heart is accelerated by the vagus innervation, because the cardiac muscle resembles skeletal muscles of higher vertebrates more closely than their striated cardiac muscles and therefore acetylcholine would have an excitatory effect.

A hypothesis about tissues carrying out other sympathetic functions in lampreys suggests the presence of chromaffin cells in the wall of blood vessels, the kidneys and the urogenital ducts (Huber 2006). Chromaffin cells differentiate from the sympathoadrenal lineage of neural crest cells

and carry out endocrine functions. They can be distinguished morphologically from sympathetic neurons by the presence of large chromaffin granules for storage and release of catecholamines (Coupland 1972; Coupland and Tomlinson 1989) and the inability to grow neurites.
SIF cells, chromaffin cells and sympathetic neurons are derived from the same lineage of neural crest cells and therefore it is a plausible hypothesis that there was an evolutionary shift from SIF cells and chromaffin cells towards sympathetic neurons.

Aim of the thesis

This study aims to challenge the finding that lampreys lack a commissural sympathetic chain with methods of modern cellular and molecular biology and examine the presence of potential sympathetic precursors. The last studies on this topic were carried out decades ago and they were mostly anatomical observations. With new techniques on hand the finding that sympathetic ganglia are absent in lamprey and the level of potential sympathetic precursors can be reinvestigated.
A first strategy is to employ immunohistochemistry. Doing antibody stainings for known pan-neuronal markers, like neurofilament, can help identify neuronal structures even if the cells were scarcely scattered and therefore hard to find by anatomical observations.
A second strategy to unravel if sympathetic innervation is present in lampreys or absent is to do cell tracing studies of neural crest cells using the lipophilic dye DiI (1,1',di-octadecyl-3,3,3'3'-tetramethylindocarbocyanine perchlorate). Sympathetic neurons are derivatives of the trunk neural crest and these cells can be specifically labeled and followed during development using DiI.

A third strategy is to do *in-situ* hybridizations for genes known to be important for the establishment of sympathetic neurons, like Ascl1, dHand and Phox2b. For the formation of sympathetic neurons all three genes are required and thus sympathetic neurons can only form in locations that co-express them.

A different approach to the topic is the comparison of the activity of gene regulatory sequences driving the expression of a sympathetic neuron marker gene in a gnathostome and lamprey. Therefore this study aims to find conserved non-coding elements in the sympathetic nervous system marker gene Phox2b that drive expression in sympathetic neurons and to compare these to the corresponding regions in the lamprey genome. Also the relevant sequences are tested in interspecies experiments to unravel if they can drive expression in the lamprey.

Results

Neurofilament staining in the trunk region of lamprey

Previous studies showed that lampreys posses most of the neural crest derivatives, but lack the commissural sympathetic chain. To unravel if neuronal structures that could be sympathetic neurons form in lampreys, antibody stainings for the neuronal marker neurofilament-m were carried out. Neurofilaments are special cytoskeletal elements exclusive to neurons, similar to the intermediate filaments of other cells. There are three types of neurofilaments: NF-L, NF-M and NF-H, the light, medium and heavy chain respectively. These three subtypes assemble as obligate heteropolymers and they are most abundant in the long axons of neurons (Lee and Cleveland 1996).

Lampreys were fixed at embryonic day 16 (E16) or E33. Afterwards they were cryosectioned and the trunk sections were stained with anti-neurofilament-m antibody. The results are displayed in Figure 7.

At E16 there is neurofilament-m staining in the neural tube, the dorsal root ganglia, paired structures above the neural tube and also staining in the gut, which may reflect enteric ganglia. There is no neurofilament antibody staining in the vicinity of the dorsal aorta where sympathetic ganglia would be expected.

In sections of older embryos (E33) the sites of neurofilament-m staining are comparable to the sites observed in E16 embryos. There is staining of the neural tube, the dorsal root ganglia, which appear to be bigger at E33 and staining of potential enteric ganglia. The paired dorsal structures are not visible at E33. A dorsal branch of the spinal nerve growing into the periphery of the dorsal fin becomes visible. At this stage, there is also no neuronal staining in the proximity of the dorsal aorta where sympathetic neurons accumulate in gnathostomes to form sympathetic ganglia.

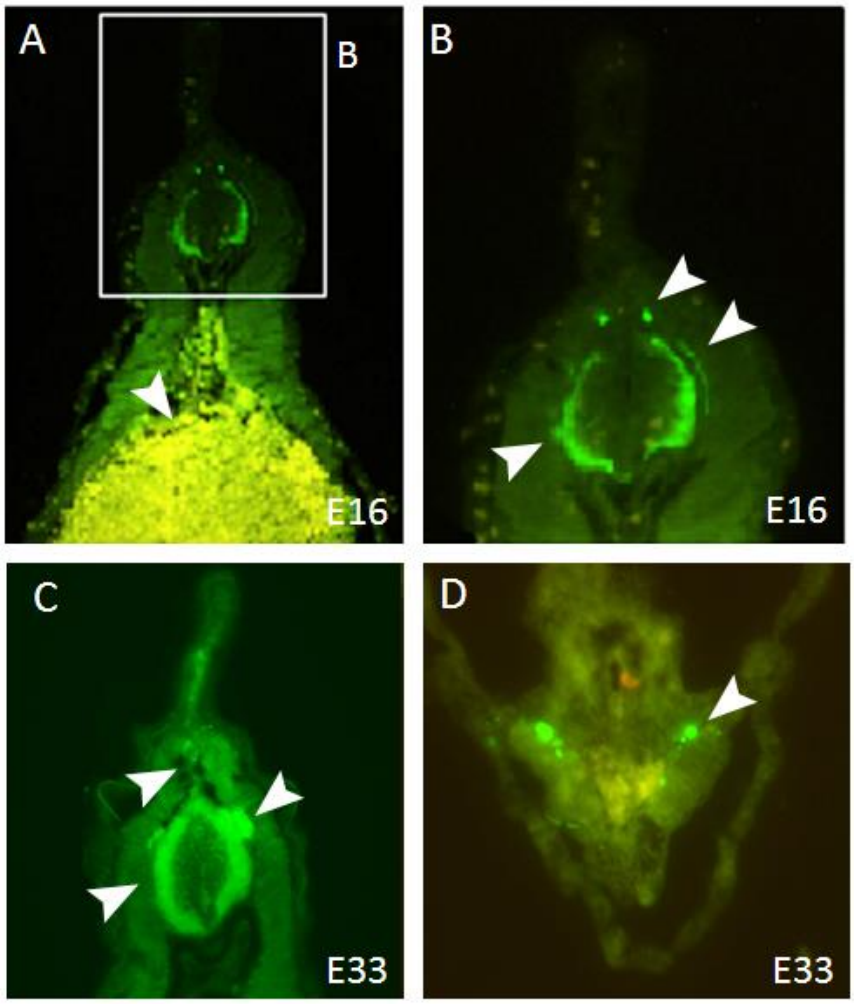

Figure 7: Antibody staining of lamprey embryos on E16 and E33 with the neurofilament-m antibody. On E16 the neurofilament-m antibody detects the neural tube and potential enteric ganglia. The latter cannot be determined with certainty due to the strong autoflourescence of the yolk (A arrow). Further, a paired structure above the neural tube and dorsal root ganglia (B) are detected. On E33 the antibody also detects dorsal root ganglia, that seem to be larger in size than on E16, the neural tube (C) and enteric neurons (D), but the paired structure visible in the E16 embryo is not detected anymore. Additionally on E33 peripheral nerve growth is visible in the dorsal fin.

DiI labeling of trunk neural crest in lamprey

In lamprey, as well as in teleosts the neural tube forms by secondary neurulation. A solid rod-like structure, the neural keel, transforms by cavitation into a tube. At E5, the neural rod elevates, gradually detaching from the dorsal epithelium and the first distinguishable neural crest precursors occur at this time. Cavitation starts at E6, when the head morphologically extends and becomes visible. At the same time neural crest primordia appear as bulges on the dorsal side of the newly formed neural tube (Damas 1944; Lowery and Sive 2004; Sauka-Spengler, Meulemans et al. 2007).

In gnathostomes, trunk neural crest cells migrate along two major pathways, dorsolaterally to form melanocytes and ventrally to give rise to neuronal and neuroendocrine derivatives. For example, ventrally migrating cells differentiate into sensory and sympathetic ganglia, as well as adrenal chromaffin cells and peripheral glia (Le Douarin 1982). Vagal neural crest cells migrate ventrolaterally to contribute to dorsal root and sympathetic ganglia, as well as undergoing extremely long migrations to form the entire enteric nervous system of the gut.

To trace the neural crest derivatives in the lamprey, DiI was injected into the lumen of the neural tube of E 6.5 to E7 lamprey embryos. The migration of labeled cells was examined at E8 to E34. The labeled cells contributed to several neural crest derivatives at the trunk and vagal levels. These include dorsal root ganglia, the mesenchymal cells of the fin, as well as enteric ganglia (Figure 8). However, no structures resembling sympathetic ganglia were observed at any stage. These results definitively show that lamprey neural crest cells fail to condense into structures similar to sympathetic ganglia during embryonic development.

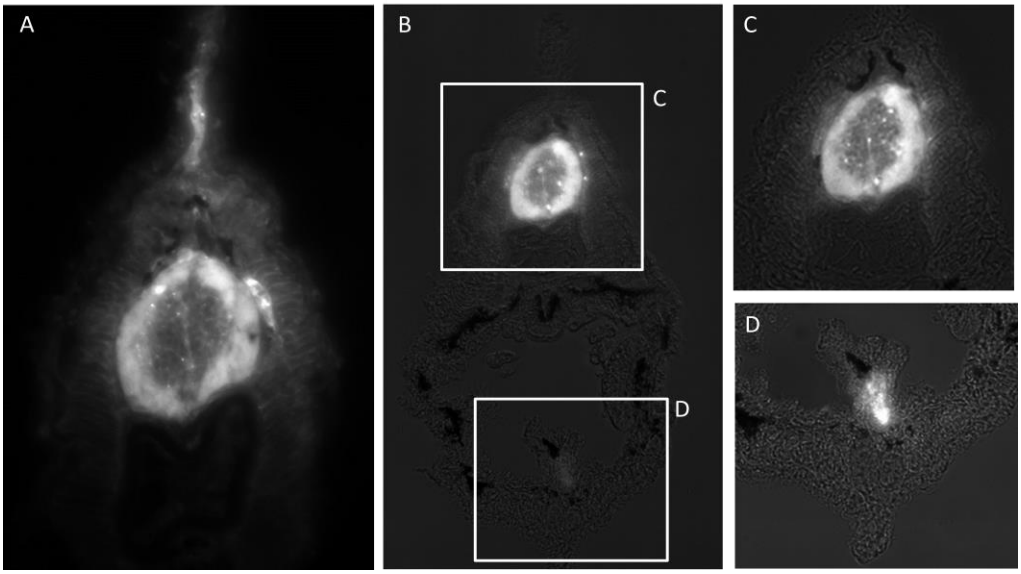

Figure 8: DiI injection into the neural tube labels fin, dorsal root ganglia and enteric neurons. (A) and (B) show a section of a E34 lamprey embryo that was injected with DiI at E6 into the neural tube. In (A) there are DiI labeled neural crest cells migrating into the fin where they form mesenchymal cells of the fin and there also can be observed DiI labeled cells forming dorsal root ganglia. The close-up (C) shows DiI labeled neural crest cells forming dorsal root ganglia and (D) neural crest cells migrating into the gut to form the enteric nervous system.

Gene expression analysis of sympathetic marker genes in chicken and lamprey embryos

A gene regulatory network of complex interactions underlies the formation of the nervous system. Some major players of the gene regulatory network underlying the formation of the sympathetic nervous system are known already, like dHand, Ascl1 and Phox2b. The transcription factor Ascl1 is expressed in precursors of sympathetic and enteric neurons (Lo, Johnson et al. 1991). dHand is a transcription factor that is essential for proliferation differentiation of sympathetic neuron precursors (Schmidt, Lin et al. 2009) and Phox2b is a transcription factor that is required for

sympathetic, parasympathetic, and enteric neurogenesis (Pattyn, Morin et al. 1997; Rychlik, Hsieh et al. 2005). Therefore all sympathetic cells or tissues express these three genes.

The expression of Ascl1, dHand and Phox2b was analyzed in the chicken embryo (*Gallus gallus domesticus*) as an example of a vertebrate which forms a sympathetic nervous system (Figure 9). The expression patterns were analyzed at embryonic stage HH22 (Hamburger and Hamilton 1992), the stage at which the dorsal root ganglia and sympathetic ganglia have formed (Teillet, Kalcheim et al. 1987; Lallier and Bronner-Fraser 1988).

All three genes are expressed in a variety of tissues at that stage. Ascl1 is expressed in the midbrain, the diencephalon, the spinal cord and in the sympathetic ganglia and nerves.

dHand transcripts can be detected in the leg mesenchyme, in the wing mesenchyme, in the heart, in the anterior heartfield, in the pharyngeal arches and clefts and in the spinal and sympathetic ganglia and nerves.

Phox2b expression can be visualized in the cranial ganglia, in the hindbrain, in the spinal cord and in the sympathetic ganglia and nerves.

All three genes, Ascl1, dHand and Phox2b, are co-expressed in the sympathetic ganglia and nerves at HH22.

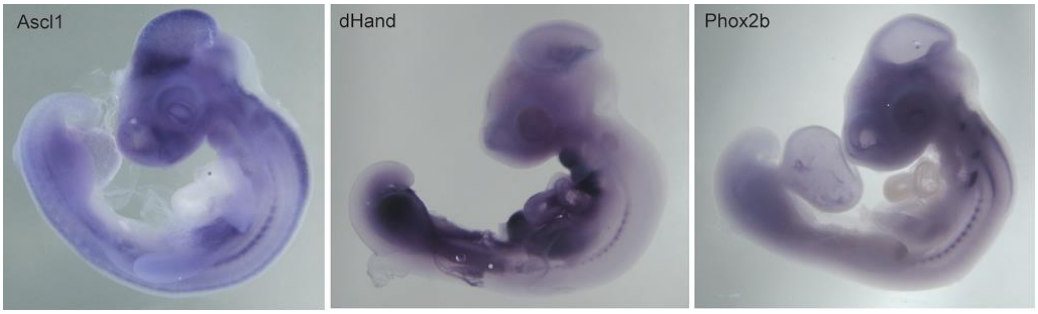

Figure 9: The expression of the three marker genes for the sympathetic nervous system, Ascl1, dHand and Phox2b was analyzed at embryonic stage HH22 (Hamburger and Hamilton 1992). Ascl1 is expressed in the diencephalon, in the spinal cord, in the midbrain, in the sympathetic ganglia and nerves. dHand is expressed in the leg and wing mesenchyme, in the heart, in the pharyngeal arches and clefts, in the anterior heartfield and in the spinal sympathetic ganglia and nerves. Phox2b is expressed in the hindbrain, in the cranial ganglia, in the spinal cord and in the spinal sympathetic ganglia and nerves.

To examine the presence and deployment of these genes in lampreys during neural crest development, fragments of their lamprey orthologues were cloned using 5'RACE and used for *in-situ* hybridization.
The expression patterns of the lamprey orthologues of these genes were analyzed at various stages from E4 to E45 to unravel their deployment and find the commonalities and differences compared to the gene expression pattern in the chicken embryo. All three genes are expressed in lamprey embryos.
In-situ hybridization in lamprey embryos shows that the expression of dHand starts at E5 in the bilateral precursors that form the cardiac field. Beginning at E7 there is additional expression in the anterior portion of the ventral mesenchyme surrounding the endostyle (Figure 10). From E8 on the amount of transcript builds up in the entire ventral mesenchyme that surrounds the endostyle. By E12 in addition to the expression in the heart and the entire ventral mesenchyme, there is expression in the notochord.

dHand transcripts can also be detected in cardiac ganglia and the posterior mesoderm. Sectioning of the whole mounts revealed additional expression in the developing gut.

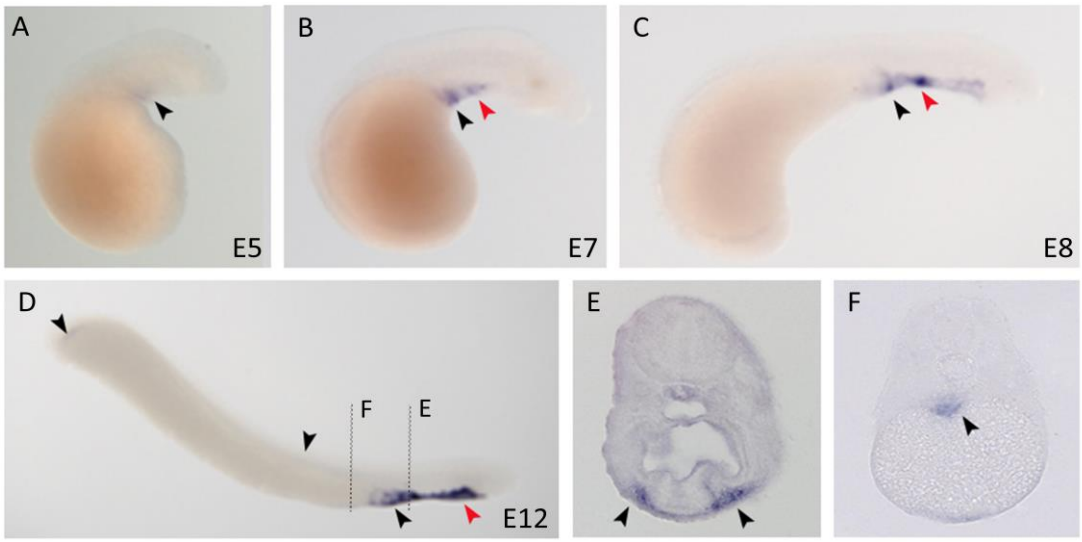

Figure 10: dHand *in-situ* hybridization of lamprey embryos at various stages. The first expression of dHand becomes visible on E5 (A) in the precursors of the cardiac field (arrow). From E7 onward there is additional expression in the ventral mesenchyme surrounding the endostyle (B red arrow, and E) that intensifies through E8 (C). On E12 (D) dHand is also expressed in the cardiac ganglia, posterior mesoderm and in the notochord. Additional transcripts of dHand in the developing gut were uncovered by sectioning (F).

Ascl1 starts to be expressed in the pituitary gland at E6. At E7 additional expression can be detected in the lens. On E11 transcripts could also be visualized in the trigeminal ganglion, the trigeminal nerve and in the notochord (Figure 11).

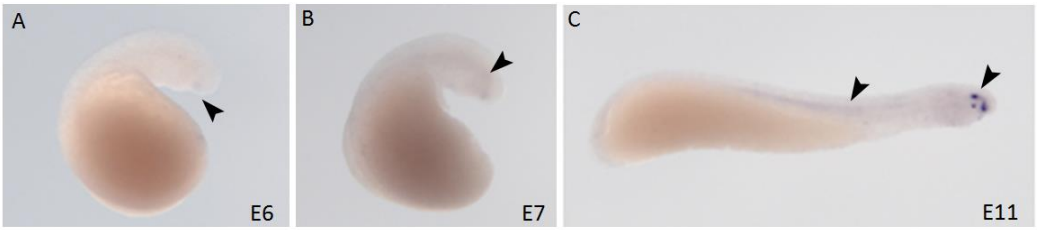

Figure 11: Ascl1 *in-situ* hybridization of lamprey embryos at various stages. On E6 Ascl1 transcripts are visible in the pituitary gland (A). Additional expression in the lens can be observed on E7 (B). From E11 onwards transcripts of Ascl1 can be also detected in the trigeminal ganglion and nerve and in the notochord (C).

Phox2b starts to be expressed in hindbrain motor neurons at E5. At E6 there appears additional expression in the ventral branchial mesenchyme. From E9 onwards transcripts can also be detected in epibranchial ganglia and cranial nerves. In contrast to the expression patterns of Ascl1 and dHand, which are limited to the head region and the heart, apart from the notochord expression, Phox2b is also present in vagal crest cells migrating posteriorly from E9 onwards. Phox2 starts to be expressed in the notochord at E11. At day 12, Phox2b expression can also be detected in the cardiac ganglia (Figure 12).

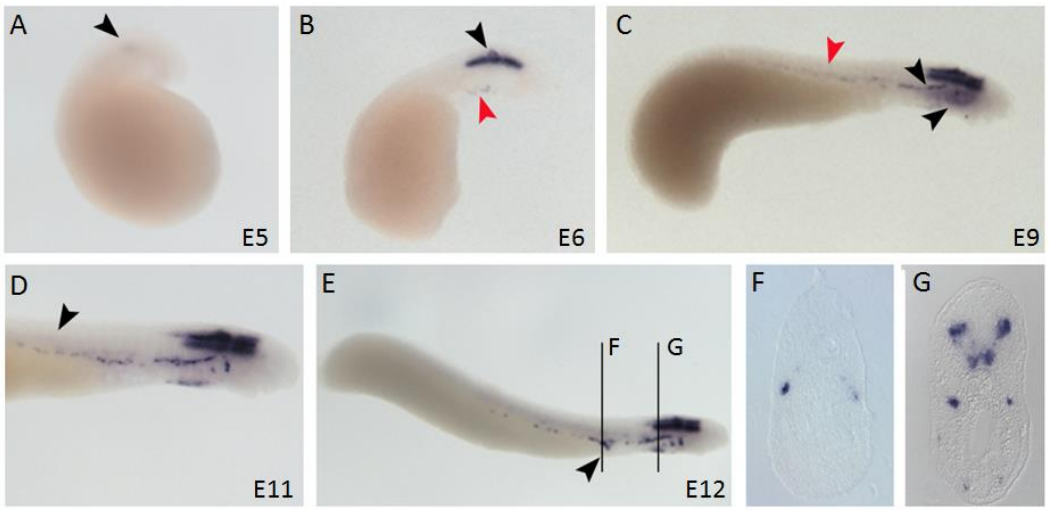

Figure 12: Phox2b *in-situ* hybridization of lamprey embryos at various stages. The first Phox2b transcripts can be observed in hindbrain motor neurons (A) on E5 followed by expression in the branchial mesenchyme (B red arrow) on E6. From E9 onwards there are additional transcripts detected in the epibranchial ganglia, cranial nerves and vagal crest cells migrating posteriorly (C, red arrow). Notochord expression of Phox2b starts on E11 (D) and is followed by expression in cardiac ganglia on E12 (E arrow). The section in (F) presents the Phox2b expression in the cardiac ganglia, whereas (G) is a section taken more anteriorly, showing the transcripts detected in the hindbrain motor neurons, the epibranchial ganglia and the ventral branchial mesenchyme.

There is no evidence for tissues corresponding to sympathetic ganglia in which dHand, Ascl1 and Phox2b are co-expressed. In fact, the only site in which all three genes are co-expressed is in the notochord of lamprey embryos. Their expression in the notochord starts at E12 for dHand and at E11 for Ascl1 and Phox2b.

The results show that the expression patterns of Ascl1, dHand and Phox2b largely overlap with the expression patterns in the chicken, but there is no expression found in structures resembling sympathetic ganglia

or nerves. Co-expression of all three genes is a requirement for a sympathetic phenotype of neurons in vertebrates.

Analysis of cis-regulatory changes of Phox2 expression

The three tested marker genes, for the formation of a sympathetic nervous system, are expressed in the lamprey, but not in the cooperative manner that leads to the formation of sympathetic ganglia in gnathostomes. This raised the question of which the underlying gene regulatory changes are that led to the redeployment of Ascl1, dHand and Phox2b into a new gene regulatory sub-circuit. To experimentally unravel this, conserved non-coding elements (CNEs also evolutionary conserved regions ECRs) associated with Phox2b were identified in the chicken. Sequences with regulatory functions have an evolutionary constraint and will be conserved across species (Aparicio, Morrison et al. 1995; Hardison 2000; Manzanares, Wada et al. 2000). Therefore, they can be detected by their strong sequence conservation across different species.

These sequences were inserted into a reporter vector. This reporter vector contained the CNE of interest followed by thymidine kinase promoter (pTK) as a constitutively active basal promoter and the sequence for enhanced green fluorescent protein (eGFP).

Three CNEs a that lie in the proximity of the chicken Phox2b promoter and which are highly conserved among vertebrates were tested for their capability to drive expression in chicken embryos. The three CNEs that are closest around the promoter are depicted in Figure 13. CNE 1 is a region of 3kb that lies 11.3kb upstream of the transcription start site, CNE 2 spans 3,2kb and lies directly upstream, adjacent to the Phox2b promoter whereas the CNE 3 has a length of 900bp and spans the first intron.

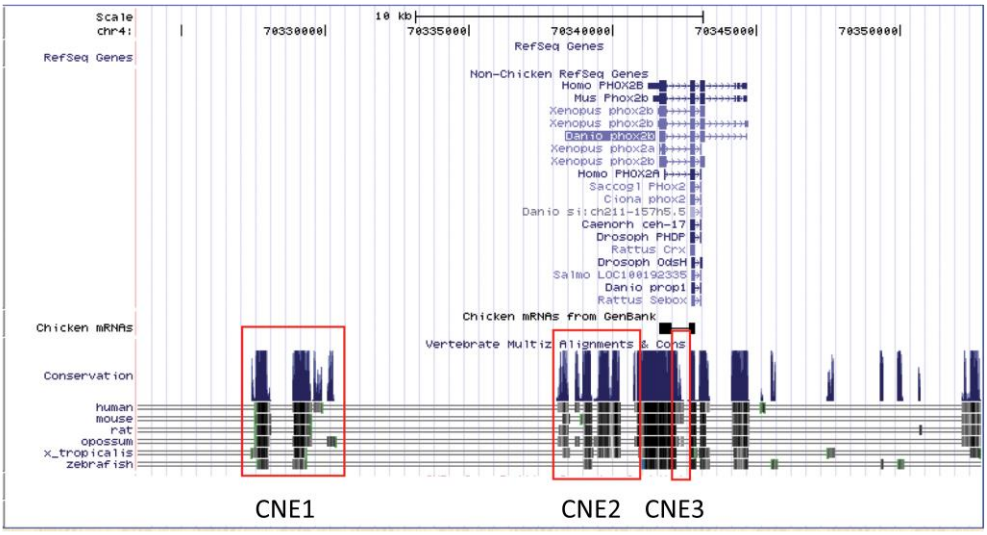

Figure 13: Screen shot of the Phox2b chicken locus in the UCSC genome browser. The red boxes mark the location of the three CNEs that were experimentally tested.

Transiently transgenic chicken can be obtained by injection of a plasmid followed by *in-ovo* electroporation. As a control for successful injection and electroporation a constitutively active H2B-cherry vector was co-injected.

The results are shown in Figure 14. Injecting the CNE 1 plasmid leads to no specific eGFP expression at the tested stages. The CNE that lies directly upstream of the promoter, CNE 2, drives eGFP expression in the cranial ganglia. So, expression of CNE 2 resembles the staining of cranial ganglia as seen in the Phox2b *in-situ* hybridizations (Figure 9).

CNE 3, which corresponds to the first intron, shows no expression in the chicken embryo at the analyzed stages.

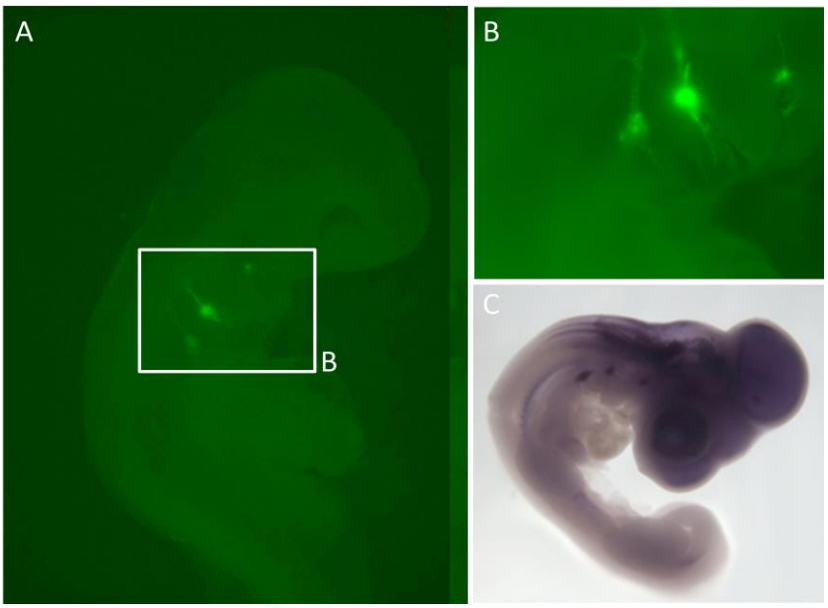

Figure 14: (A and B) show a HH22 chicken embryo that was injected with the CNE2-ptkEGFP reporter vector. The CNE2-ptkEGFP reporter drives expression in the cranial ganglia (B) matching parts of the Phox2b expression pattern known from *in-situ* hybridization at the same stage (C).

In a next step, the chicken CNEs 1, 2 and 3 were cloned into a vector suitable for lampreys (Parker, Piccinelli et al. 2011) in order to examine if the chicken sequence is capable of recruiting transcription factors in the lamprey. The principle of parsimony suggests that the expression of Phox2b in equivalent tissues in chicken and lampreys is due to a regulation by the same transcription factors.

This vector for lamprey injections is based on an I-sceI meganuclease system for genome integration. Genome integration is necessary in lampreys, because they do not tolerate electroporations. Also they only develop well, when they are injected at late one-cell stage or during two-cell stage. For these reasons it would result in a too high dilution of the injected plasmids during the development of the embryo up to the time points of interest without the genome integration. This technology is newly

developed for lamprey (Parker, Piccinelli et al. 2011) and opens up the possibility for various functional *in-vivo* studies in lamprey embryos.

The lamprey embryos were injected at late one-cell stage and monitored for expression of eGFP driven by the CNEs. Injection of CNE 1 and CNE 2 did not lead to an expression of eGFP in any tissues. Lampreys that are injected with CNE 3 show expression around the gills, the epibranchial ganglia and in the cranial nerves (Figure 15). The observed eGFP expression pattern matches parts of the Phox2b expression pattern in the lamprey (Figure 12). Also the expression of Phox2b in cranial ganglia is analogous in the chicken and in the lamprey. This expression in cranial ganglia is driven by two different chicken CNEs, when comparing chicken and lamprey.

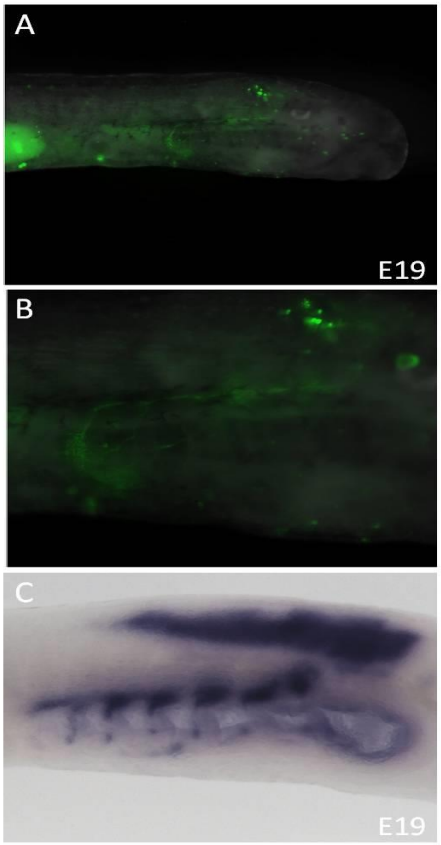

Figure 15: (A and B) show an E19 lamprey embryo that was injected with the CNE3-cfos-Iscel reporter vector driving expression in the epibranchial ganglia, around the gills and in the cranial nerves (B). (C) shows the head region of the Phox2b expression pattern visualized by *in-situ* hybridization.

Discussion

Morphological studies

Antibody staining of trunk sections of lamprey embryos at E16 and E33 with an antibody against neurofilament-m was carried out to detect all neuronal structures. The pan-neuronal marker did successfully stain the neural tube, dorsal root ganglia and enteric neurons. Therefore it is shown that the antibody successfully binds to neuronal structures in lampreys. There was no staining in the vicinity of the dorsal aorta were sympathetic ganglia would be expected to form. This finding supports the notion that lampreys lack a chain of sympathetic ganglia as reported in anatomical studies (Nicol 1952).

DiI injection into the neural tube of E6 lamprey embryos followed by cell tracking in the developing embryo revealed the neural crest contribution to different embryonic tissues and organs. Neural crest cells migrated to form dorsal root ganglia, mesenchymal cells of the fin and enteric neurons at E34. At no point were neural crest cells observed ventrally past the dorsal root ganglia, migrating in the direction of the dorsal aorta. Thus, DiI injections did not detect any sympathetic neurons, as they are known from higher vertebrates.

The results of the DiI injections are in agreement with the findings from the antibody staining with neurofilament-m. Neither DiI nor neurofilament staining were observed scattered throughout the lamprey body along the big vessels as was reported for the chromaffin cells (Gibbins 1994). This could be attributed to a technical drawback of the antibody staining. The expected scattered chromaffin cells could be masked by the very high autoflourescence of lamprey embryos. The DiI signal on the other hand fades very rapidly and is thus hard to detect in sections under the microscope, whereas the whole mount embryos are too opaque for the

expected individual cells to be observed. Another possible explanation of the experimental results could be that the chromaffin cells form during metamorphosis of the lamprey and are only present in the adult animal. This seems very unlikely, since there are no reports on dormant neural crest cells residing in the embryo, which could start a new wave of migration and differentiation during metamorphosis. If neural crest cells were to populate the wall of blood vessels already in the embryo and only start differentiating to chromaffin cells during metamorphosis, the DiI cell labeling should have uncovered these migrating neural crest cells.

Tracing sympathetic structures by marker gene expression

In-situ hybridization with probes against common sympathetic nervous system markers in chicken successfully detected sympathetic ganglia amongst other tissues.

At HH22 Ascl1 is expressed in the mesencephalon, the diencephalon, the spinal cord and in the sympathetic ganglia and nerves. dHand expression is observed in the mesenchyme of the legs and wings, in the heart and the anterior heartfield, in the pharyngeal arches and clefts as well as in the spinal and sympathetic ganglia and nerves.

Phox2b transcripts are detected in neuronal structures, namely the hindbrain, the cranial ganglia, in the spinal cord and in the sympathetic ganglia and nerves.

In-situ hybridization with probes for all three neuronal genes studied successfully detects expression in the sympathetic ganglia and nerves of chick at HH22 as expected. Therefore these genes are significant marker to analyze the nervous system present in lampreys.

Using probes for the lamprey homologues of Ascl1, dHand and Phox2b in an *in-situ* hybridization experiment revealed that all three genes are expressed in various tissues during lamprey development.

At E6 Ascl1 expression is detectable in the pituitary gland, in the lens (E7), in the anterior lip mesoderm (E10), in the notochord (E11) and in the cranial ganglion VI (E12).

dHand expression is detected in the heart (E5), in the ventral mesenchyme surrounding the endostyle (E7), in the notochord (E12) and in the cardiac ganglia (E14).

Phox2b transcripts are observed in the hindbrain motor neurons (E5), in the ventral branchial mesenchyme (E6), in the epibranchial ganglia and cranial nerves (E9). Phox2b is also expressed in the notochord (E11), in vagal neural crest cells that migrate posteriorly and in cardiac ganglia (E12).

At no time point during the development of lamprey embryos is there any expression of Ascl1, dHand or Phox2b in the vicinity of the dorsal aorta, where sympathetic ganglia would form. The only commonality between the expression patterns of Ascl1, dHand and Phox2b is the expression in the notochord from E11 on for Ascl1 and Phox2b and from E12 on for dHand.

The expression of dHand in ganglia adjacent to the heart of lamprey embryos is in agreement with the hypothesis of Lignon, that lampreys possess cardiac SIF cell aggregates that have sympathetic like endocrine function (Lignon 1979). On the other hand neither Ascl1 nor Phox2 are present in the same cells proving that these cells are not of definite sympathetic lineage.

The parasympathetic nervous system is supposed to be evolutionarily older than the sympathetic nervous system. It is believed that the parasympathetic nervous system, which is responsible for the physical homeostasis of the body, is a basal feature upon which the sympathetic

functions were added. The first sympathetic functions in evolution are reported in basal vertebrates, the cyclostomes, and consist of scattered chromaffin cells carrying out sympathetic functions in an “indirect” way. The chromaffin cells are described to release their catecholamines into the big vessels leading to a global sympathetic output. Gnathostomes are still capable of executing a global sympathetic response, but in addition direct sympathetic functions evolved, by sympathetic nerves that innervate specific organs or tissues (Shigei, Tsuru et al. 2001; Huber 2006). The chromaffin cells present in chicken and mice are dependent on the expression of Ascl1, dHand and Phox2b (Huber, Bruhl et al. 2002; Unsicker, Huber et al. 2005). At all the stages of lamprey development examined by *in-situ* hybridization no structure co-expressing all three genes is observed. The observed chromaffin cells in the lamprey heart must underlie a different genomic make-up than the analogous cells in gnathostomes. There is no evidence for the reported scattered chromaffin cells supposedly occurring throughout the lamprey body (Gibbins 1994) using gnathostome sympathoadrenal marker genes.

In conclusion it can be demonstrated that no structures homologous to a sympathetic ganglia chain form during the embryological development of lamprey, confirming earlier publications (Nicol 1952).

If the chromaffin cells or SIF cells described to be present in lamprey are direct precursors of a definite sympathetic nervous system is unclear. A more thorough investigation of the molecular identity of these cells is required to come to a conclusion on the evolutionary relationship between the chromaffin cells or SIF cells in the lamprey and true sympathetic neurons. The data presented suggests that a commissural sympathetic chain is an evolutionary novelty in the gnathostome lineage and that the redeployment of Ascl1, dHand and Phox2b into a new cis regulatory module might have facilitated this emergence.

Conserved non-coding elements from the Phox2b locus

CNEs from the chicken Phox2b gene locus were cloned into a reporter plasmid and injected into chicken embryos. Two of the three CNEs chosen showed no eGFP expression. These were the more distantly upstream CNE and the CNE spanning the first intron. The third CNE that lies directly adjacent to the Phox2b promoter led to eGFP expression in cranial nerves.

In lamprey on the other hand both the CNEs from the chicken genome that lie upstream of the transcription start site showed no expression of eGFP, but the CNE spanning the first intron shows expression around the gills, the epibranchial ganglia and in the cranial nerves. This demonstrates that the genomic sequence from the chicken is able to recruit transcription factors in the lamprey.

The fact that two different CNEs are responsible for expression in partially corresponding structures in different animals raises interesting questions. Spatio-temporal gene expression is thought to be strongly influenced by cis-regulatory sequences within the gene. Only the modular regulation of genes allows for their full flexibility in function. The fact that enhancers and silencers work in an additive manner makes evolutionary changes on those more likely. They change only subsets of the gene expression and are less likely to have global deleterious effects as mutations in the gene itself are likely to produce (Gaunt 2012). At this point the lamprey genomic sequence is not available yet. Therefore, it cannot be determined if the effect that two different CNEs drives expression in corresponding structures is due to the presence of different transcription factor binding sites in the sequence. Another possibility is that the variance in transcription factors present in the studied tissues and at the studied time points is the cause for the different activity of the CNEs tested in chicken and lamprey.

Evolution of the sympathetic nervous system

The differentiation of sympathetic neurons from neural crest cells is the result of a highly interdependent network of transcription factors (Figure 5). None of the transcription factors is expressed in locations relevant for sympathoadrenal tissues in the lamprey. Unfortunately, the full lamprey sequence of the three genes studied is not available yet. A major challenge in the ongoing sequencing project is the fact that lampreys undergo a programmed genome rearrangement between blastula and gastrula stage and eliminate >20% of their genome (Smith, Antonacci et al. 2009). Therefore the similarity of the Ascl1, dHand and Phox2b lamprey loci with the loci of gnathostomes is unacquainted.

Once the lamprey genome becomes available, the lamprey genome sequence will be of great value for further analysis of the transcription factor binding sites. A sequence alignment of the CNEs in chicken and lamprey would uncover the sequence variation of transcription factor binding sites.

Outlook

There is an array of very diverse experiments that would aid in describing the sympathetic state of lampreys more thoroughly. Cloning more CNEs from the Phox2b locus is required to uncover the region that drives expression in the sympathetic ganglia in chicken. 15 additional CNEs lie between the two neighboring genes of Phox2b. Once the element responsible for sympathetic expression is discovered, it could be broken down in smaller fragments and ultimately it would allow a fine mapping of the cis-regulatory linkages contained in the region in the chicken genome as compared to the lamprey genome. Sequence comparisons of the

correlating regions in various vertebrates and invertebrates are necessary steps to unravel the evolutionary steps of the makeup of the GRN underlying formation of the sympathetic system as it is present in gnathostomes.

As a next step the size of the CNEs should be reduced step wise. *In-silico* prediction of transcription factor binding sites can be employed to define potentially relevant binding sites. The functionality of these binding sites can be tested by inserting pointmutations. This would unravel if the expression in the cranial nerves of chicken and lamprey are driven by the same transcription factors. It could also help to find out which mutations led to the shift in use of the transcription factor binding sites.

Additionally the inverse interspecies experiment should be carried out. CNEs taken from the lamprey genome should be tested in the chicken. This experiment would allow testing for the lamprey CNE1, 2 and 3 expression behaviors in chicken and lamprey. Sequence alignment of these CNEs could also help uncover if the difference in expression stem from different transcription factor binding sites present in the CNE or different transcription factors being present in the tissue. Until sufficient sequence data from the lamprey genome is available, this cannot be unraveled *in-silico*.

Additionally, the loci of Ascl1 and dHand should be analyzed for CNEs and these should be tested in interspecies experiments, too.

As a next step the expression patterns of Ascl1, dHand and Phox2b at various stages should be analyzed in the cephalochordate amphioxus (*Branchiostoma lanceolatum*) in order to elucidate if the expression pattern in the lamprey is an ancestral pattern or if it is already an intermediate step in the evolution of the sympathetic nervous system. Amphioxus is a cephalochordate and the closest invertebrate which is experimentally accessible. So far, sequences matching Ascl1 and dHand,

but no sequence homologous to the Phox2b gene of vertebrates in the amphioxus genome can be found in GenBank.

Further analysis of the genomic make-up of lamprey chromaffin and/or SIF cells is necessary to analyze the details of the evolutionary relationship between these cells and definite sympathetic neurons. Chicken and mouse chromaffin cells are reported to express Ascl1, dHand and Phox2b (Huber, Bruhl et al. 2002; Unsicker, Huber et al. 2005), but these three genes were not expressed in the expected locations in the lamprey. One of the expected locations would be the heart (Fänge 1963) as it was previously reported to contain chromaffin cells. It appears that lamprey chromaffin cells are possibly an evolutionary precursor to gnathostome chromaffin cells. They do already have the same characteristic catecholamine containing granules and the affinity for chrome staining, but are made up from a different and unknown genetic repertoire. It would be very interesting to isolate these chromaffin cells from the lamprey heart and from gnathostomes respectively to perform molecular analysis on both lineages, for example a whole transcriptome analysis.

In this thesis, all experiments were carried out in embryos/larva of the lamprey, but also additional studies in the adult lampreys are required to shed light on the evolution of the sympathetic nervous system. Moreover, the neurophysiological work needs to be repeated. The data published so far is highly arguable and contrasting. It would be crucial to unequivocally find out if the lamprey heart is indeed accelerated by acetylcholine and retarded by adrenaline as it was proposed earlier (Otorii 1953; Augustinsson, Fange et al. 1956). A different explanation of the results would be that the heart in those studies was accelerated because the acetylcholine induced the chromaffin cells in the heart to release their catecholamines. The fact that lamprey hearts do not react to added adrenaline might be explained by their phosphorylase state (Nayler and Howells 1965). Nayler and Howells suggested that the action of

adrenaline on a heart usually requires a transformation of the phosphorylase from the b form into the a form. They claimed that in lampreys endogenous amines keep most of the phosphorylase in the a form, thus rendering the lamprey heart insensitive to additionally added adrenalins. Most of these different and non-coherent hypotheses were carried out decades ago and should be reevaluated with modern methods.

Conclusions

The study presented confirmed the absence of a commissural sympathetic chain in lamprey embryos. How the sympathetic ganglia evolved remains elusive. It could be demonstrated that it is feasible to use a chicken sequence to recruit transcription factors in a lamprey and drive expression in neurons. Finding the CNEs that drive expression in the chicken sympathetic ganglia will be the key to analyze which different transcription factors are employed.

It appears that the chromaffin cells present in the lamprey are even more distantly related to those of gnathostomes than initially thought. They microscopically resemble gnathostome chromaffin cells, but they do not express the three marker genes that are crucial to the differentiation of chromaffin cells in gnathostomes. Therefore, lampreys hold a key position for the understanding of the sympathetic nervous system evolution.

The sympathetic nervous system is an impressive new feature of gnathostomes and it is crucial to their predatory lifestyle and adaptation capabilities. The evolutionarily older enteric nervous system is already present in lampreys as well as the parasympathetic nervous system that is responsible for the homeostasis. It is an exciting idea that changes in cis-linkages led to the formation of a new GRN circuit, allowing neural crest

cells to differentiate into sympathetic neurons. It would be fascinating to uncover defined mutations that underlie the formation of this milestone in the vertebrate evolution.

Material and Methods

Buffers and solutions

All chemicals are obtained from Sigma-Aldrich (St Louis, Missouri, USA) if not otherwise specified.

Bleaching solution

0.5%	SSC
10%	H_2O_2
5%	CH_3NO

Blocking solution for antibody staining

5%	donkey serum (Lampire Biological Laboratories, Pipersville, Pennsylvania, USA) in PBSTr

Blocking solution for chicken *in-situ* hybridization

10ml	sheep serum (Lampire Biological Laboratories, Pipersville, Pennsylvania, USA)
10ml	Boehringer Blocking Reagent (Hoffmann - La Roche AG, Basel, Switzerland)
30ml	MAB

Blocking solution for lamprey *in-situ* hybridization

20%	sheep serum (Lampire Biological Laboratories, Pipersville, Pennsylvania, USA)
2%	Boehringer blocking reagent in MABT

Hybridization solution

50% CH_3NO

1.3x SSC pH5

5mM EDTA pH8

200µg/ml tRNA

0.2% Tween-20

0.5% CHAPS

100µg/ml Heparin

ddH_2O

5x MAB(T)

58g Maleic Acid

43.5g NaCl

(1ml Tween-20)

Tris-base until pH7.5

ddH_20 ad 1l

MEM salts

1M MOPS pH7.4

20mM EGTA

10mM MgSO4

ddH_2O

MEMFA

10ml 16%Formaldehyde, MeOH free

4ml MEM salts

ddH_2O ad 40ml

10x MMR

1M	NaCl
10mM	$MgSO_4$
20mM	KCl
1mM	EDTA
50mM	HEPES
20mM	$CaCl \cdot 2H_2O$

adjust to pH 7.8

NTMT

5M	NaCl
1M	Tris pH9.5
2M	$MgCl_2$
10%	Tween-20
	ddH_2O

PBS

8g	NaCl
0.2g	KCl
1.44g	Na_2HPO_4
0.24g	KH_2PO_4
	ddH_2O ad 1l

adjust to pH to 7.4

PBSTr

8g NaCl

0.2g KCl

1.44g Na_2HPO_4

0.24g KH_2PO_4

0.5% Triton-X 100

ddH_2O ad 1l

adjust pH to 7.4

PBSTw (DEPC)

8g NaCl

0.2g KCl

1.44g Na_2HPO_4

0.24g KH_2PO_4

0.1% Tween-20

ddH_2O ad 1l

adjust pH to 7.4

0.1% DEPC (incubate >3hours and autoclave)

Ringer solution

6.5g NaCl

0.42g KCl

0.25g $CaCl_2$

1mol $NaHCO_3$

ddH_2O ad 1l

20x SSC

3M NaCl

300mM $Na_3C_6H_5O_7$

pH7.0

Antibodies

Neurofilament-m	Invitrogen (Carlsbad, California, USA) Cat # 13-0700
Alexa 488 IgG2a	Invitrogen (Carlsbad, California, USA) Cat # A21131
DIG-AP	Hoffmann-La Roche AG (Basel, Switzerland) Cat # 11093274910

Primerlist

All primers were obtained from Integrated DNA Technologies, Inc (Coralville, Iowa, USA).

ptk fwd	5’ GTGCCAGAACATTTCTCTAT
ptk rev	5’ GCAGCAAGCTTACTTAGATC
Phox2bCNE1_Asp718fwd	5’ TTTGGTACCGCACGGGCTGAAATTAGAGT
Phox2bCNE1_BglIIrev	5’ TTTAGATCTCCCTATAAGCCAGGAATAGCC
Phox2bCNE2_Asp718fwd	5’ TTTGGTACCCTGTGACTCAGACGCAGCTC
Phox2bCNE2_BglIIrev	5’ TTTAGATCTCCTATCGCTGATTCCTGCAT
Phox2bCNE3_Asp718fwd	5’ TTTGGTACCCAGGGACCACCAGAGCAG
Phox2bCNE3_BglIIrev	5’ TTTAGATCTGTTTCCGCTTCTCGTTCAAA

Lamprey husbandry and fertilizations

Lamprey husbandry was conducted according to the protocol given by Nikitina et al. (Nikitina, Bronner-Fraser et al. 2009) and is accredited by the Institutional Animal Care and Use Committee (IACUC, Title: ”Neural Crest Development in *Petromyzon Marinus*”, Animal Protocol Application

number: #1436 – 08). Mature lampreys were shipped from the Hammond Bay Biological Station (Millersburg, Michigan, USA).

Antibody staining

Immunostaining of lamprey embryos was performed according to the protocol given by Nikitina et al. (Nikitina, Bronner-Fraser et al. 2009).
Lamprey sections were degelatinized in 42°C PBS for 10 minutes and subsequently washed in PBSTr 2 times for 5 minutes each. Then, the sections were blocked in 10% goat serum (Lampire Biological Laboratories, Pipersville, Pennsylvania, USA) in PBSTr at 4°C for 5 hours. These sections were then incubated with the neurofilament-m antibody (Invitrogen, Carlsbad, California, USA) at a dilution of 1:200 in blocking solution overnight at 4°C in a humid chamber. Afterwards the sections were washed 5 times for 10 minutes each with PBSTr. The secondary antibody, Alexa-488 anti mouse IgG2a, was diluted 1:1000 in blocking solution and it was incubated on the sections for 2 hours at room temperature in a humid chamber.
Subsequently the excess antibody was washed of 3 times for 10 minutes each with PBSTr followed by 2 washes for 10 minutes each with PBS.
The sections were mounted with Permafluor (Beckman Coulter, Brea, California, USA). Before applying the Permafluor the sections were dipped into distilled water a few times to remove the salts. Mounted sections were allowed to dry.

DiI injections

DiI injections were carried out following the protocol published by Nikitina et al. (Nikitina, Bronner-Fraser et al. 2009). Lamprey embryos were

dechorionated in 0.1x MMR at E5 – E7 and placed into agarose-coated petridishes. DiI (Invitrogen, Carlsbad, California, USA) was diluted 0.5µg/ml in 0.3M sucrose. The DiI solution was filled into glass needles and injected into the lumen of the neural tube. Embryos were tested by fluorescence microscopy (axioscope2 Plus fluorescence microscope, Zeiss, Oberkochen, Germany) for successful injection. The correctly injected embryos were raised in petridishes containing 0.1x MMR at 18°C. Migration of labeled cells was monitored on a daily basis. Embryos that had reached the desired stage of development were fixed in 4% paraformaldehyde in PBS for 1 hour at room temperature.

Chicken *in-situ* hybridizations

Chicken probes for Ascl1, dHand and Phox2b were kindly provided by Hermann Rohrer (Max-Planck-Institut für Hirnforschung, Frankfurt, Germany).

Chicken embryos fixed in 100% methanol were gradually washed to PBSTw (DEPC), followed by two washes in PBSTw (DEPC) for 10 minutes each. The embryos were incubated for 3 hours in pre-warmed Hyb solution at 70°C. Subsequently the embryos were incubated overnight with the pre-warmed probe in Hyb solution at 70°C. Unbound probe was washed off with Hyb solution 2 times for 15 minutes each at 70°C, followed by 4 washes for 45 minutes. The next washing step is carried out using Hyb solution and MABT mixed 1:1 at 70°C once for 30 minutes. Thereafter the embryos were washed four times for 30 minutes each with MABT at room temperature. The MABT was then replaced with blocking solution and the embryos are incubated for 3 to 4 hours. As a next step the blocking solution was exchanged for Anti-digoxigenin-AP antibody (Hoffmann - La Roche AG, Basel, Switzerland) diluted 1:2000 in

blocking solution which was applied over night at 4°C. Excess antibody was removed with several washing steps with MABT at room temperature, 2 times for 5 minutes, 2 times for 30 minutes and 6 times for 1 hour each, followed by a wash with MABT over night at 4°C. Afterwards the embryos were washed in NTMT 2 times for 30 minutes and then NTMT was exchanged to NBT/BCIP (Hoffmann - La Roche AG, Basel, Switzerland) in NTMT according to the manufacturer's manual. As soon as the desired intensity of staining was reached, embryos were washed 3 times for 5 minutes in PBS and re-fixed in 4% paraformaldehyde in PBS over night at 4°C.

Chicken injections and electroporations

Fertilized chicken eggs were obtained from "McIntyre Poultry and fertile eggs" (Lakeside, California, USA).

The handling, injections and electroporation of chicken embryos is described in detail by Sauka-Spengler and Barembaum (Sauka-Spengler and Barembaum 2008).

Embryos were *in-ovo* injected and electroporated. The solution was injected into the neural tube at HH8 followed by immediate electroporation and the embryos were allowed to develop up to HH22.

The injection solution contained 2µg/µl of the ptk-EGFP vector (Uchikawa, Ishida et al. 2003) including one of the CNEs, 1µg/µl of the control vector pCI H2B-RFP (Betancur, Bronner-Fraser et al. 2010) and, 0.1% FD&C food dye blue no1 (Spectra Colors Corporation, Kearny, New Jersey, USA) in Ringer solution.

After reaching HH22 the embryos were removed from the egg and monitored using the Zeiss axioscope2 Plus fluorescence microscope (Zeiss, Oberkochen, Germany).

Lamprey *in-situ* probes

The orthologs of Ascl1, dHand and Phox2b were analyzed via a bioinformatic survey of the lamprey genome. Lamprey probes were obtained through RLM-RACE using the Invitrogen GeneRacer Kit (Invitrogen, Carlsbad, California, USA) according to the manual. Total RNA was extracted from lamprey embryos utilizing the Ambion RNAquous kit (Ambion, Austin, Texas, USA) and then dephosphorylated with calf intestine phosphatase (Hoffmann - La Roche AG, Basel, Switzerland). Afterwards the RNA was decapped using tobacco acid pyrophosphatase. The GeneRacer RNA oligo was ligated and reverse transcription was initialized with random hexamer primers. The sequences of interest were amplified via touchdown PCR and cloned into TOPO TA vector according to the manual (Invitrogen, Carlsbad, California, USA).

Lamprey *in-situ* hybridizations

Whole-mount lamprey *in-situ* hybridizations were carried out according to the protocol published by Nikitina et al. (Nikitina, Bronner-Fraser et al. 2009).

Lamprey embryos were fixed in MEMFA for 1 hour at room temperature. Afterwards the embryos were bleached with bleaching solution for 10 minutes under light. To stop the reaction the embryos were washed with PBSTw (DEPC) 3 times for 5 minutes each. Afterwards embryos were permeabilized with 20 µg/ml proteinase K in PBSTw (DEPC) for 10 minutes followed by incubation in 2 mg/ml glycine in PBSTw (DEPC) for 10 minutes.

As a next step, the embryos were postfixed with 4% paraformaldehyde in PBS for 20 minutes at room temperature. Subsequently the embryos were pre-hybridized for 3 hours in hybridization solution at 70°C and incubated in 1-10 µg/ml RNA probe labeled with digoxigenin in hybridization solution for 16 hours at 70°C. For the removal of excess probe the hybridization reaction was followed by washing 2 times for 15 minutes and 4 times for 45 minutes each with hybridization solution at 70°C. The next washing step was carried out with hybridization solution and MABT 1:1 at 70°C for 30 minutes followed by 4 washes MABT only at room temperature for 30 minutes each. Embryos were blocked for 4 hours at room temperature in blocking solution. Anti Digoxigenin-AP antibody (Hoffmann - La Roche AG, Basel, Switzerland) was used 1:2000 in blocking solution over night at 4°C. To remove excess antibody the embryos were washed with MABT for 2 times 5 minutes each, then 2 times 30 minutes each, and 6 times 1 hour each at room temperature followed by an overnight wash with MABT at 4°C.

MABT buffer was exchanged to NTMT buffer and washed 4 times 15 minutes each. BM purple substrate (Hoffmann - La Roche AG, Basel, Switzerland) was used to obtain the color reaction. Thus the embryos were incubated in BM purple, covered from light, until the desired intensity of the staining was reached. Some embryos were allowed to “over stain” to make sure that regions of low signal were not overlooked. The staining reaction was stopped by 3 PBSTw washes for 5 minutes each. Afterwards the embryos were re-fixed with 4% paraformaldehyde in PBS for 2 hours at room temperature.

Embedding and sectioning of lamprey embryos

Lamprey embryos were embedded in gelatin for cryosectioning. First, the embryos were washed 3 times with PBSTr for 15 minutes each, followed by incubation in 15% sucrose in PBS for 3 hours at room temperature. Afterwards, the lamprey embryos were incubated in a pre-warmed solution of 7.5% gelatin and 15% sucrose in PBS for 12 hours at 37°C and then in a 20% gelatin in PBS solution for 4 hours at 37°C. Subsequently, the embryos were positioned in little molds filled with 20% gelatin in PBS and shock-frozen in liquid nitrogen. The embedded embryos were equilibrated to and sectioned at -30°C with a Microm HM550 cryostat (Thermo Fisher Scientific, Waltham, Massachusetts, USA) with a thickness of 8µm – 10µm.

Lamprey injections

Lampreys were injected at late one-cell stage following the protocol given by Parker et al. (Parker, Piccinelli et al. 2011). The chicken CNEs were cloned into the cFos-I-sceI-EGFP plasmid, which consists of the mouse cFos minimal promoter and the coding sequence for EGFP, flanked by I-sceI restriction sites. The Plasmids DNA was isolated using the EndoFree Plasmid Maxi Kit (Qiagen, Hilden, Germany) according to the manual. Restriction digests were always set up freshly by mixing 400ng plasmid with 15 units I-SceI enzyme and 1x I-SceI buffer + BSA (New England Biolabs, Ipswich, Massachusetts, USA) in a 20µl reaction and allowing to digested for 40 minutes at 37°C. 2-3nl of the restriction digest were micro-injected into the late one-cell stage lamprey embryos using a Picospritzer (Parker-Hannifin Corporation, Cleveland, Ohio, USA). Embryos were screened for GFP expression on a daily basis using the Zeiss axioscope2 Plus fluorescence microscope (Zeiss, Oberkochen, Germany).

CNE chicken reporter vector

Sequences for the CNE were obtained using the UCSC genome browser (http://genome.ucsc.edu/cgi-bin/hgGateway) "vertebrate multi-way alignment" function. Blocks of highly conserved sequence, lying between the two neighboring genes and outside of the coding sequence of Phox2b were downloaded from the annotated chicken genomic sequence. The Primer3 software (http://frodo.wi.mit.edu/primer3/) (Rozen and Skaletsky 2000) was used for the primer design. For later cloning steps the sequence for the restrictionsite of Asp718 or Bgl II (both Invitrogen, Carlsbad, California, USA) were added to the forward and reverse primer sequence respectively and three additional thymine nucleotides were added to enhance the restriction.

The CNEs were amplified from a chicken Phox2b BAC (BACPAC Resource Center, Oakland, California, USA; BU464222; chEST716a20; 261-89F23) using the "expand long template" PCR system (Hoffmann - La Roche AG, Basel, Switzerland) according to the manual.

Gradient PCR Program:		
	1. 94°C	4 minutes
	2. 94°C	30 seconds
	3. 55°C - 65°C	2 minutes
	4. 68°C	3 minutes
	5. 68°C	10 minutes
	40 repeats of step 2 - 4.	

The amplified sequences were loaded onto an agarose gel and the bands of the right size were excised. DNA was isolated from the agarose gel piece using the Promega Wizard SV Gelextraction kit (Promega, Madison, Wisconsin, USA) according to the manual. Afterwards the amplified CNE DNA and the ptkEGFP vector were digested with the restriction enzymes

Asp718 and Bgl II according to the manual. The ligation of the single CNEs into the digested ptkEGFP vector was carried out using the NEB T4 DNA ligase (New England Biolabs, Ipswich, Massachusetts, USA) following the directions of the manual. The DNA was precipitated using NF pellet paint (Merck KGaA, Darmstadt, Germany) according to the manual. Resuspended DNA was electroporated into electrocompetent One Shot Top 10 cells (Invitrogen, Carlsbad, California, USA) following the directions of the manual. 10 colonies were picked with a pipet tip for each CNE. The tip was dipped into 100µl ddH_2O for subsequent colony PCR and then dipped into 2ml LB to expand the colony overnight at 37°C. For the colony PCR 1µl of the ddH_2O were used in the PCR reaction aforementioned for the amplification of the CNEs. The QIAprep spin Miniprep Kit (Qiagen, Hilden, Germany) was used to isolate the DNA of the clones from which a DNA fragment of the right size could be amplified. A portion of the eluted DNA was adjusted to 100ng/µl and sent for sequencing with the ptk fwd and ptk rev primers (Laragen, Culver City, California, USA). The obtained sequence was blasted using the UCSC blat program (http://genome.ucsc.edu/cgi-bin/hgBlat) and sequences matching the desired CNE were used for subsequent injections into chicken embryos.

Abreviations

TH	Thyroxin Hydroxylase
DBH	Dopamine-β-hydroxylase
CNS	Central nervous system
PNS	Peripheral nervous system
GRN	Gene regulatory network
E	Embryonic day

CNE	Conserved non-coding element
pTK	Promoter Thymidine kinase
eGFP	Enhanced green fluorescent protein
ddH_2O	Double distilled water
NBT/BCIP	nitro blue tetrazolium chloride/5-Bromo-4-chloro-3-indolyl phosphate

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