Specification of unique neuronal sub-types by integration of positional and temporal cues

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Linköping University, Sweden 2010
Cover picture: Two different developmental stages of *Drosophila melanogaster* immunostained with Prospero (Red), Deadpan (Blue) and lbe(K)-Gal4 (Green). The neuroblast 5-6 lineage (green) is present in all segments, but generates different lineages in different segments throughout development. Cover image from PLoS Biology Karlsson et al 2010.

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“Unfortunately, nature seems unaware of our intellectual need for convenience and unity, and very often takes delight in complication and diversity”

Santiago Ramón y Cajal
Nobel prize laureate 1906
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List of papers

This thesis is based upon the following papers, which are referred to in the text by their roman numerals:

**Paper I**

**Paper II**

**Paper III**

**Paper IV**
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<tr>
<td>aa</td>
<td>Amino acid</td>
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<tr>
<td>A-P</td>
<td>Anterior-Posterior</td>
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<tr>
<td>bHLH</td>
<td>Basic helix-loop-helix</td>
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<td>CNS</td>
<td>Central nervous system</td>
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<tr>
<td>D-V</td>
<td>Dorsal-Ventral</td>
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<tr>
<td>FFL</td>
<td>Feed-forward loop</td>
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<td>GMC</td>
<td>Ganglion mother cell</td>
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<tr>
<td>HD</td>
<td>Homeodomain</td>
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<tr>
<td>HMC</td>
<td>Hypaxial motor column</td>
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<tr>
<td>LIM-HD</td>
<td>LIM-homeodomain</td>
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<tr>
<td>LMC</td>
<td>Lateral motor column</td>
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<tr>
<td>MMC</td>
<td>Medial motor column</td>
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<tr>
<td>MN</td>
<td>Motor neuron</td>
</tr>
<tr>
<td>NB</td>
<td>Neuroblast</td>
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<tr>
<td>PGC</td>
<td>Preganglionic column</td>
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<tr>
<td>R-C</td>
<td>Rostral-Caudal</td>
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<td>VNC</td>
<td>Ventral nerve cord</td>
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Abstract

The nervous system contains vast numbers of neuronal sub-types, generated at specific time points, in the proper location, and in proper numbers. One of the fundamental issues in neurobiology is to understand the molecular genetic mechanisms that underlie the generation of this daunting neuronal diversity.

To help shed light upon these fundamental questions, my PhD project has addressed the generation and specification of a certain group of neurons, the Ap cluster. This group of four neurons is found only in thoracic segments within the *Drosophila melanogaster* central nervous system, and consists of three different cell types. Mapping of the neuroblast (stem cell) that generates the Ap cluster neurons, neuroblast 5-6, and the highly restricted appearance of this cluster allowed me to address the following questions: How does NB 5-6 change its temporal competence over time to generate the Ap cluster neurons late in the lineage, and how is temporal competence altered to ensure diversity among the Ap neurons? What are the mechanisms that allow these Ap cluster neurons to emerge only in the thoracic segments?

My studies have helped identify a number of mechanisms acting to specify the Ap cluster neurons. One type of mechanism involves several of different feed-forward loops that play out during NB 5-6 lineage development. These are triggered within the stem cell, where the temporal gene *castor* activates a number of genes. These castor targets are subsequently involved in several regulatory feed-forward loops, that ultimately result in the unique combinatorial expression of cell fate determinants in the different Ap neurons, which in turn ultimately lead to the activation of unique terminal differentiation genes. In addition, I have identified three different mechanisms by which the NB 5-6 lineage is modulated along the neuroaxis. In the abdomen I find that an early cell cycle exit is initiated by the Bx-C gene members and Pbx/Meis cofactors, which result in the truncation of the NB 5-6 lineage, preventing the Ap cluster neurons from being generated. In thoracic segments Hox, Pbx/Meis and temporal genes act in concert to specify Ap cluster neurons, by integrating with the *castor* temporal gene. In anterior segments, improper Hox and temporal coding results in a failure to specify bona fide Ap cluster neurons, even though equivalents of Ap cluster neurons are generated.

In summary, my thesis work has helped identify a number of mechanisms acting to specify this unique neuronal sub-type, including: feed-forward combinatorial coding, opposing feed-forward loops and integrated temporal/Hox mediated specification throughout different axial levels. I suggest that these mechanisms may be widely used within the animal kingdom, hence contributing to the great cellular diversity observed within the central nervous system of most animal species.
Introduction

Early in the twentieth century, the great neuroscientists Camillo Golgi and Santiago Ramoñy Cajal, attempted to sketch maps to understand how the central nervous system is organized. They identified complicated architecture with vast cellular morphologies. This complex organ will coordinate and regulate the body in response to external and internal stimuli. To visually follow a moving object you rely on complex and highly coordinated communication between your eyes and muscles. Our heart needs to speed up when our muscles demands more oxygen. Our senses need to be alert when we touch or taste something unknown. The list of functions that our central nervous system performs each and every day, often without our knowledge, can with ease fill the pages of many theses.

A trend clearly observed within the animal kingdom is that: more neurons leads to more complex behaviors as evolution progresses (Martinez-Cerdeno et al., 2006). The ability for an animal to use a well-developed mode of communication, advanced and coordinated movements, deal with complex social patterns and have a degree of self-awareness is something rarely within the animal kingdom (Dunbar and Shultz, 2007). In humans it is estimated that the CNS is composed of $\sim10^{11}$ neurons, which further can be divided into at least 10,000 different classes, depending on their morphology, firing pattern or ion channel presentation (Muotri and Gage, 2006). So how can such a complex structure, possibly the most complex known to man, emerge and develop from nothing more than a single fertilized cell? How does a neuron pick up cues in the environment and migrate to its correct position then establishes the right number of connections, spread out a given set of receptors over its plasma membrane and release a number of neurotransmitters? What molecular mechanisms underlie this remarkable precision? How is neuronal diversity established?

Addressing these issues is important as it will contribute to our general understanding of biological processes. It is important for us to understand how living things function, how different living things use different ways to do the same things, like generating energy, eating, and moving. Knowing these things help us to find new ways to solve future problems. To know the background why a certain
medical condition emerges facilitates in the quest of eventually finding a cure. One of
today’s neurodegenerative diseases, Parkinson’s disease, is under intense research
and has been so for a long time. The condition, which early on impairs motor skills
and speech, is characterized by the fact that dopamine producing neurons die off,
resulting in a decrease, or total loss, of dopamine. With a vision to artificially
produce dopaminergic neurons \textit{in vitro}, transplant these and once again make the
patient able to produce dopamine is something that to some extent has been
successfully accomplished, but poor survival of the transplanted neurons/grafts and
tumor growth as side effects has slowed down progression. Knowledge of how to
artificially reprogram and differentiate generic cells into dopaminergic neurons,
make them more resistant to cell death and keep them from forming tumors would
save lives.

Today’s neuroscientists face different challenges from those faced by their
predecessors. Being aware of the huge cellular diversity and great precision behind a
mature functional nervous system, there has been a shift in focus towards smaller
structures and more complex processes. How does a stem cell enter or leave
quiescence? How is asymmetric division carried through? How does a neuron know
when and where to form the right synapses? Ultimately, the answers to these
questions lie within the cell itself. Through a dynamic regulation of the genomic
landscape, sophisticated gene transcription and fine tuning of the final gene products
it is only during the last two decades, as advanced molecular approaches combined
with genetically modified model systems, that these questions can finally be
answered. While most of our knowledge of the central nervous system has emerged
from studies on model systems like \textit{Caenorhabditis elegans} (roundworm), \textit{Drosophila
melanogaster} (fruit fly), \textit{Xenopus laevis} (frog), zebrafish, chick and mice, it is widely
accepted that most developmental processes are well conserved throughout the
animal kingdom, making results obtained from these studies highly relevant for
understanding our nervous system. In fact, one of the most exciting discoveries of
the past couple of decades in developmental biology has been the recognition that
similar genes make similar structures in very different organisms.
From a Nervous Net to a Nervous System

Many animals have evolved complex nervous systems throughout the course of evolution, but their architectures can differ substantially between species. The evolution of multi-cellular metazoans is thought to have begun in the seas, with small single cells that over time adapted – started working together - in order to have an efficient food uptake they had to start working together. Through a solid contact between cells, nutrients could be shared, enabling the evolution of nonfeeding cell types. A major evolutionary advantage took place in Cnidarians, which added the behavior of extensive movement in search for food. These types of animals possessed two new types of cells, which would revolutionize future animal evolution; muscle and nerve cells. The first nerve tissue was very primitive in its construction with interconnected neurons spanning around the animal in a nerve net with no clear brain structure (Fig. 1A).

The first “true” hunter was the flat worm (Planaria) that actively moved, sensed the surrounding environment and consumed pray. This was made possible through a new type of nerve tissue; a centralized nervous system with a clear brain structure, which could integrate and process sensory information coming from the periphery, make a decision and act upon it. More nerve cells and an organized structure allowed for more complex behaviors (Fig. 1B).

Figure 1
A) Cnidarians have a diffuse nerve net with interconnected neurons (sideview).
B) Planaria has a centralized nervous system. With a simple brain structure (top) it could gather information, sense its environment and make active decisions (dorsal view).
The centralized nervous system is primarily found within the bilaterian evolutionary branch of animals. Included within the subregnum bilaterian are three super-phyla; lophotrochozoa (annelids, molluscs etc), ecdysozoa (arthropods, nematodes etc), and deuterostomia (chordates etc). Bilaterian animals are defined by the fact that they have two body axes: the anteroposterior (A-P) axis that runs from the head (anterior) of the animal to the posterior (tail), and the dorsoventral (D-V) axis which runs from the back (dorsal) to the front (ventral) of the organism. Thus a D-V cleavage of a bilaterian animal will result in two mirrored body halves.

One major difference between the nervous systems of arthropods and vertebrates lies in the position of the nerve cord. In arthropods the nerve cord is located on the ventral side of the animal, while in vertebrates this structure is on the dorsal side. Due to this difference in nerve cord location between these two phyla it has been discussed that these two centralized nervous systems evolved independently of each other. However, powerful molecular- and embryological analysis of neural development has revealed remarkable similarities, implying that the common ancestor must have had a considerable degree of centralization, and that the dorsal positioning of the spinal cord must be an effect of an early dorsoventral axis inversion during chordate evolution. These findings are of great importance since they implicate that the molecular mechanisms underlying the generation of neuronal diversity and complexity might be more conserved throughout the animal kingdom than previously thought, making results gathered from different model systems relevant to all animals.

**Content of the Nervous system**

The central nervous system of many of the more advanced animal species can be divided into three different divisions: brain, spinal cord and the peripheral nervous system (PNS). The Nervous system is primarily built up of two different cell types: neurons and glial cells. These two cell types have very different functions. Nerve cells, neurons, transmit electrical signals as action potentials to other neurons, muscles, or secretory glands, and are arranged in networks (circuits). Glial cells add
physical support, insulate axons and dendrites, and ensure that the communication is carried out in an efficient and isolated manner.

**Neurons**

There are several different kinds of neurons, defined by their ability to transmit action potentials, which can be divided into three basic groups. Sensory neurons respond to outer stimuli, such as mechanical force, light or sound. Motor neurons innervate peripheral targets such as muscle or glands while the third group, the interneurons, connects one neuron to another (Fig. 2B,C).

![Neuron diagram](image)

**Figure 2**

A) Neurons have an advanced morphology with several important features needed for their complex function and behavior.

B) Three different neural cell types build up the CNS: Sensory neurons, Motoneurons and Interneurons. The red arrow illustrates the direction of the action potential.

C) The function and location of a neuron within the CNS will result in very different morphology and functions (Masland, 2004).
Branched dendrites act as contact surface for upstream neurons from which they receive electrical input (Fig. 2A). This information is passed forward through the cell body towards a clear elongated formation, the axon. The action potential moves along the pre-synaptic neuron, induces vesicles to fuse with the cell membrane, and unload their content consisting of neurotransmitters into the synaptic cleft. There are many different neurotransmitters (GABA, Acetylcholine etc) identified, and they will dictate if the interneuron is inhibitory or excitatory. These molecules will bind to an appropriate receptor located on the post-synaptic neuron, and through this interaction pass the electrical input forward. In addition, many neurons secrete peptides, neuropeptides that typically mediate slower acting effects upon other neurons.

To highlight the functional challenges that individual interneurons must cope with, the neocortex, being part of the cerebral cortex, is a striking example. Here complex information involved in higher functions such as spatial reasoning, language and sensorial input has to be processed. It is a constant challenge to keep a balance between excitation and inhibition. Both of these events can be problematic. Too much inhibition stops important information from getting through, while too much excitation could cause over-production of action potentials leading to medical conditions, like seizures. The balance between inhibition and excitation is an important way of controlling formation of action potentials allowing for rapid activation. One way to achieve this task is for the neocortex to contain a variety of interneurons, each one with a unique threshold to respond to different levels of input. Considering the amount of information and stimuli that our brain has to process each and every day this underscores the flawless precision that is governed by the great neuronal diversity found within our CNS.

Glial cells
In contrast to neurons, glial cells do not fire action potentials, instead they form myelin which surround, isolates and ensheaths neuronal circuits, making sure that action potentials are transported in a safe and undisturbed way. In mammals we find four types of glial cells: microglia, astrocytes, Schwann cells and oligodendrocytes each one with different functions within the CNS. Glial cells have been under intense research lately because they are believe to play an important role building neuronal
circuits and guiding neurons and axons during early development. Even though neurons and glial cells play two different functions within the CNS they share the same origin; both are generated from neural stem cells which ultimately produce a fully functional central nervous system wired in a highly complex three-dimensional pattern, a process that we still have limited knowledge of.

**The Drosophila Central Nervous System**

The *Drosophila* embryonic CNS develops from specific regions of the ectoderm denoted neurogenic areas. From these areas a number of multipotent stem cells, neuroblasts (NB) will delaminate. The ventral nerve cord (VNC) is formed by NBs in the ventral neurogenic region, while NB’s giving rise to the brain emerge from the procephalic neurogenic regions. Like the rest of the arthropod body, we find the CNS to be segmented in a repetitive fashion. The brain is divided into three unique segments: the tritocerebrum (Tc), deutocerebrum (Dc), and protocerebrum (Pc) which is also the largest segment (Fig. 3A&C).

The brain has been especially difficult to study due primarily to the large number of NBs, cell movement, tissue rearrangement, and the existence of more complex gene expression patterns. Recently, several groups have made attempts to decipher this highly complex structure (Urbach et al., 2003; Urbach and Technau, 2003). The suboesophageal ganglion is also divided into three areas: the mandibular (Ma), maxillary (Mx) and labial (Lb) neuromere, which is followed by three thoracic segments (T1-T3) and nine abdominal segments (A1-A9), were each segment can be divided into mirrored hemisegments (Fig. 3C). The thoracic and abdominal segments are referred to as the VNC, while the three suboesophageal neuromere is included in the brain structure.

*Drosophila* neurogenesis

Neuron and glial cells are produced by a number of neural progenitor cells, neuroblasts (NBs), delaminating from neurogenic areas. The NBs, roughly 30 in each hemisegment, will form a repetitive pattern, making each NB identifiable due to its position, size and expression of early genes (Fig. 3D). Because of their stereotypical location, each NB is named after row and column. The NB in row 2 and column 3 is
Introduction

named NB2-3, while NB in row 5, in column 6 is given the name NB5-6 (Doe, 1992) (Fig 3D). Each NB will divide asymmetrically and give rise to a smaller progeny called ganglion mother cells (GMCs, Fig. 3B), which in turn will divide once and form two neurons, two glial cells or one of each (Udolph et al., 1993). Lineage size varies between different NBs, where the largest lineage is composed of up to 40 cells and the smallest of only two cells (Bossing et al., 1996; Schmidt et al., 1997).

Figure 3
A) The embryonic Drosophila CNS consists of a brain and a ventral nerve cord (VNC). Adapted from www.sdbonline.org/fly/atlas/00atlas.htm
B) Neurogenesis starts when neural progenitor cells, neuroblasts (NBs), delaminates from the neuroectoderm (NE). Each NB will divide and produce a varied number of ganglion mother cells (GMCs) which will divide asymmetrically and produce neurons (pink) and glial cells (blue).
C) The Drosophila brain consists of three segments: Protocerebrum (Pc), Deutocerebrum (Dc) and Tritocerebrum (Tc). The suboesophageal ganglion is divided into three areas: the mandibular (Ma), maxillary (Mx) and labial (Lb) neuromere, which is followed by three thoracic segments (T1-T3) and nine abdominal segments (A1-A9). Dorsal view.
D) Each hemisegment (half a segment) contains roughly 30 NB. Each of these can be identified due to morphology, location and expression of molecular markers (to the right). Adapted from www.neuro.uoregon.edu/doelab/nbmap.html.
Due to the repetitive and relatively simple morphology of the VNC, this has been a good model for studies on cell specification of the CNS and henceforth it will also be the primary focus of this thesis. A strong interest during recent years has been on how the NB lineages progress, and upon how this progression is modified depending on spatial or temporal influences. Lineage studies of the CNS are of crucial importance if one is to take full advantage of the extensive knowledge of genetics available in flies, to in the end understand generation of specific neuronal subtypes and how they become specified throughout development.

**The vertebrate Central Nervous System**

Similar to *Drosophila*, the CNS of vertebrates can be subdivided into two main portions, the brain and the spinal cord. The vertebrate neural plate, equivalent to the invertebrate neuroectoderm, invaginates from the dorsal side forming the neural groove which will after enclosure form a hollow neural tube with a roof plate located dorsally and a floor plate located ventrally (Fig. 4A). The fluid filled center later becomes the ventricular system and spinal channel. As development proceeds different parts of the neural tube will become more specified, and can be divided into four distinct regions: Prosencephalon, Mesencephalon, Rhombencephalon and the spinal cord (Fig. 4B). As development proceeds the Prosencephalon will divide forming two structures: telencephalon and diencephalon. The Mesencephalon develops into the midbrain and the Rhombencephalon becomes the metencephalon and myelencephalon. These structures will later generate the adult vertebrate brain (Fig. 4C,D).

Prior to neural tube closure certain cells adopt a roof-plate fate. When closed, the neural tube will contain two “organizing centers” denoted the roof- and floor plates (Fig. 4A&5A). As these areas differentiate they initiate expression of specific signaling molecules. The roof plate will secrete BMP and WNT signaling molecules, while the floor plate, located on the opposite side serves as an organizer to ventralize and guide neuronal positioning and differentiation through the secretion of Sonic
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hedgehog (Shh). These cues will diffuse from these two areas and build up gradients along the D-V axis and through this specify adjacent neuronal populations (Fig. 5A).

Figure 4

- **A** The vertebrate neural plate, equivalent to the invertebrate neuroectoderm, invaginates from the dorsal side forming the neural groove which will after enclosure form a hollow neural tube with a roof plate located dorsally and a floor plate located ventrally
- **B** The neural tube will become more specified, and can be divided into four distinct regions: Prosencephalon, Mesencephalon, Rhombencephalon and the spinal cord
- **C** The Prosencephalon will divide forming two structures: telencephalon and diencephalon. The Mesencephalon develops into the midbrain and the Rhombencephalon becomes the metencephalon and myelencephalon
- **D** A simplified side view of an adult mouse brain.

Vertebrate neurogenesis

The inner wall of the neural tube is embedded with elongated cells, which constitute the neuroepithelium and stay in contact with both the apical (towards the inside of the body cavity) and pial (towards the body cavity) surface of the neuroepithelium (Fig. 5B). In both insects and vertebrates the neural progeny is produced towards the body cavity, and this results in a multilayered structure, but the actual process of producing neurons and glial cells differ from each other. Soon after the closure of the neural tube, neuroepithelial cells down-regulate certain epithelial features giving rise to glial like properties, and become radial glial cells (RGCs).
A well-studied behavior observed in neuroepithelial cells revolves around their nuclei and their peculiar movements during neurogenesis, a phenomenon called the interkinetic nuclear migration. Going through cell cycle progression, a key feature of interkinetic nuclear migration is that the nuclear position varies in relationship to the phases of the cell cycle. The elongated cellular morphology allows for nuclei to migrate back and forth between the apical and pial layers while at the same time generating progeny. The first mode of generating progeny occurs early in development, where a symmetrical division takes place at the apical side in the ventricular zone (VZ) to generate two similar progenitor cells leading to enlargement of the neural stem cell pool (Anthony et al., 2004; Chenn and McConnell, 1995; Merkle et al., 2004; Noctor et al., 2001).

As neurogenesis proceeds many neuroepithelial cells make the switch into RGCs, that are believed to generate the majority of neuron and glial cells. In the second mode the RGCs can divide asymmetrically to generate one self-renewing progenitor (which will locate itself in the VZ) and one post mitotic neuron or one intermediate progenitor cell (IPC) which will move towards the subventricular zone (SVZ) and divide once, producing two neurons (Fig. 5B).

Figure 5
A) Illustration of a D-V cross section of the spinal cord showing the Roof- (orange) and Floor plate (blue) secreting signaling molecules which will build up gradients (left) throughout the D-V axis. The notochord (green) is believed to play an important role inducing Floor plate generation.
B) The interkinetic nuclear migration is believed to occur in a cyclic manner where the nucleus migrates along the apical and basal plates generating neurons and glial cells.
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The neurons produced populate the cortical plate (layer II-VI) in an inside out fashion where younger neurons pass older ones occupying more superficial layers. At the end of neurogenesis the RGC stops dividing and most often take the fate as astrocytes (Chenn and McConnell, 1995; Haubensak et al., 2004; Noctor et al., 2004). Recently, it was shown that the switch between neural progenitors into post mitotic neurons also include remodeling of the Swi/Snf-like chromatin remodeling complex. However this study did not clarify where during interkinetic movements this switch is vital to occur (Lessard et al., 2007; Yoo et al., 2009).

In the process of generating a fully functional CNS the neural progenitors, as mentioned above, uses a highly refined and complex way of generating different kinds of progeny. By using an efficient and dynamic switch between symmetric and asymmetric division patterns, a highly diverse and mixed progeny can be obtained. The way these differences can emerge will be discussed in the next section.

Asymmetric vs. symmetric division

The ability of neural progenitors to divide and produce neurons and at the same time remain in their proliferative state is governed by the mechanism of asymmetric division. The asymmetric division of progenitors is ultimately dependent upon the polar distribution of different complexes of cell fate determinants. By modulating the formation of the mitotic spindle the rearrangement of these products can be divided to be present in one of the daughter cells (Fig. 6A). This complex process has been best studied within the *Drosophila* NB model system due to increased resolution.

In contrast to symmetrical cell division which results in two identical cell fates, asymmetric division generates two daughter cells that are distinctly different from each other (Fig. 6A). A protein complex, consisting of the Par proteins Bazooka (Baz, Par-3/-6) and the atypical protein kinase C (aPKC), will be located on the apical side of the NB cortex. These will later recruit Inscuteable (Insc) and Partner of Inscuteable (Pins) to the apical cortex (Fig. 6B). Through the adaptor protein Discs large (Dlg) and Pins can interact with the protein Mushroom body defective (Mud) and Khc-73. Mud (vertebrate NuMA) and Khc-73 constitutes an anchor point for the microtubule and will through this play an important role orienting the microtubule in an apical
basal position (Izumi et al., 2006; Knoblich, 2008; Zhong and Chia, 2008). As mitosis is initiated, two different complexes of cell determinants become localized on the basal side (Fig 6B). Located in the center of the first complex is Miranda, which in turn interacts with Brain tumor (Brat), Prospero (Pros; vertebrate Prox1) and Staufen (Stau). Stau interact with pros mRNA. The second complex is composed of Numb and Partner of Numb (Pon). Segregating these two complexes to the basal cortex, by the action of Baz/Insc/Pins, they will be inherited by the GMC where they play different roles specifying the GMC identity and downregulating neural precursor genes (Spana and Doe, 1995; Wu et al., 2008a).

![Figure 6](image)

**A)** By modulating the formation of the mitotic spindle (green), the rearrangement of specific cell determinants (red) can be divided to finally only end up to one of the daughter cells, resulting in a difference in progeny, even though the two cells were generated from the same cell.

**B)** As mitosis is initiated Mud, on the apical side will play an important role orienting the microtubule in an apical basal position. On the basal side two different complexes of cell determinants become localized.

Even though there are differences between insects and vertebrates, a remarkable conservation of proteins involved in asymmetric cell division suggests that this is a fundamental process used in many different systems. Because of its potential to give rise to an almost endless cellular diversity this type of division can be found in many different contexts and not exclusively within the nervous system (Knoblich, 2008).
Dorsoventral patterning

The establishment of antero-posterior (A-P) and dorso-ventral (D-V) axis is critical to development of most animal species. The foundation for proper A-P and D-V axis patterning in *Drosophila* is laid down early in the egg. First, a germline stem cell divides asymmetrically to produce several cells, out of which one takes the fate to become an oocyte and locates itself to the most posterior part of the germanium. Information is exchanged between the oocyte and surrounding nurse cells, from where a delivery of *gurken* mRNA, a TGF-α homolog, will enter. *gurken* mRNA will be transcribed and the protein will bind to the *Drosophila* Egf receptor, Torpedo, present on the surrounding follicular cells (Shmueli et al., 2002). A cytoskeleton reorganization will direct components in the maternal load to distinct positions within the unfertilized egg (e.g. *bicoid* mRNA to the anterior and *oskar* mRNA and *nanos* mRNA to the posterior). As fertilization occurs, Spätzle is activated which triggers the Toll receptor (Interleukin-1 receptor [IL-1]), in the ventral part of the embryo, to activate the transcription factor dorsal (dl, vertebrate Nuclear factor kappa-light-chain-enhancer of activated B cells [NF-κB]) which together with activated Egf receptor form a ventral gradient. dl, functioning both as an activator and a repressor, activates transcription of *short gastrulation* (*sog*) and *snail* (*sna*) (Francois et al., 1994), and represses *zerknüllt* (*zen*) and *decapentaplegic* (*dpp*) expression (Huang et al., 1993; Ip et al., 1991). This will result in distinct compartments along the D-V axis, allowing the dorsal part of the embryo, which will now have low or no expression of dl, to express *dpp* and *zen*.

Due to D-V axis inversion, we find the *sog* vertebrate homologue *chordin* (*chd*) expressed dorsally and the *Dpp* vertebrate homologue *Bone morphogenetic protein 4* (*BMP4*) expressed in the ventral part of the neural tube. The ability of BMP4 (*Dpp*) to diffuse, combined with positive autoregulation, this morphogen becomes quite invasive, but as is true for Sog, Chd also has the ability to down regulate BMP4 allowing for the expression of neuroectoderm patterning genes to be misregulated in the dorsal part of the vertebrate neural tube.

When the region destined to become the neuroectoderm has been specified, this region will be further divided by longitudinal and latitudinal genes called
“patterning genes”, which further will act to specify and diversify the nervous system. These genes will be activated by previous mentioned factors, involved in establishing the early body plan and give rise to the neuroectoderm.

**Patterning of the Drosophila neuroectoderm**

In insects, Dl and the activation of the Epidermal Growth Factor Receptor (EGFR) will ventrally activate two of the three “columnar” genes, ventral nervous system defective (vnd, vertebrate homologue: nkr) and intermediate neuroblasts defective (ind, vertebrate homologue: gsh). The third columnar gene, muscle segment homeobox (msh, vertebrate homologue: msx) is expressed in a domain that has low levels of Vnd, Ind and Dpp. Since these three “columnar genes” tend to repress each other they organize themselves in longitudinal stripes. Studies have showed that over-expression of vnd or ind will repress msh expression, while dl; dpp double mutants show ectopic msh expression towards the ventral part of the embryo. The borders of msh expression are controlled by repression. So what activates msh? The answer to this question is not completely understood, but is probably due to general factors expressed early in the embryo which will initiate msh expression which later is inhibited. The expression of the three columnar genes sometimes referred to as the
“neural identity genes”, serves two purposes: First, to mark the early neuroectoderm and second establish cell fates of neuroblasts in each of the three domains (Jimenez et al., 1995; Skeath et al., 1994; von Ohlen and Doe, 2000).

**Patterning of the vertebrate neuroepithelium**

In vertebrates, the neural plate border will start signaling Wnt and FGF (fibroblast growth factor), which will induce the neuroectoderm to fold up over itself and form the neural tube. As a consequence of this, the vertebrate orthologues of *vnd* (*NK2 transcription factor related locus 2, Nkx2.2*), *ind* (*Genomic screen homeobox 1, Gsh-1*) and *msh* (*Msx-1/2/3*) are expressed in a similar fashion occupying the ventral, intermediate, and dorsal positions within the developing CNS, respectively (Fig. 7). The combined expression of *Shh*, from the floor plate and notochord, together with BMP/WNT signaling from the roof plate, will make up gradients along the D-V axis and activate several other downstream factors. These include members of the *Nkx* family (*Nkx6.1/2*), *Pax* family (*Pax6/7*), *developing brain homebox* (*Dbx*) and *Iroquois homeobox* (*Irx*) (Lupo et al., 2006). Misexpression of *Shh* has been shown to induce ectopic expression of the ventral CNS markers *nkx2.1*, *nkx2.2* and *nkx6*, while mice lacking *shh* has shown changes of gene expression in the ventral neural tube towards a dorsal fate. These results are to be expected, as this also occurs in the *Drosophila* neuroectoderm, showing the columnar genes is controlled by gradients affecting each other.

As described previously, the columnar genes not only play an early role, specifying the actual neuroectoderm, but also a post-mitotic role specifying specific cell types. *Nkx2.2*, which is a homeodomain TF, plays an essential role in the specification the serotonin (5-HT) neurons (Briscoe et al., 1999; Cheng et al., 2003)
Anteroposterior patterning

Segmentation within the Drosophila embryo

In the early Drosophila embryo, mRNA of nanos (nos) and oskar (osk) will localize to the posterior pole, while bicoid (bcd) mRNA is transported to the anterior part of the embryo. These three mRNAs will be translated and the protein products will build up gradients throughout the embryo (Fig. 8&9). Through a complex network they will start to activate the expression of the Gap gene family (Fig. 8&9). The Gap gene family belongs to a larger family of genes: the segmentation genes, where the Gap genes are the first to be expressed in order to set up a clearly defined A-P axis in the developing embryo. One Gap gene that plays an important role here is hunchback (hb), that will prevent the expression of posterior Gap genes in the anterior regions. hb mRNA is first expressed in a broad anterior domain controlled by the bcd morphogen, which over time will be further modulated. The early repressive gradients are critical for the establishment of future expression pattern of downstream target genes, including other Gap genes such as: Krüppel (Kr), knirps (kni), giant (gt), and tailless (tll) that will further contribute to specific patterning functions during early embryogenesis (Fig. 8).

The Gap genes will directly activate the Pair-rule genes. Nine members constitute this family: even-skipped (eve), hairy (h), odd-skipped (odd), paired (prd), runt (run), fushi tarazu (ftz), odd-paired (odd), sloppy paired (Slp1/2), and tenuis chaetae (ten). Each Pair-rule protein is expressed in seven stripes determined by the Gap gene expression profile. The seven stripes of Pair rule gene expression identifies either all the odd-numbered para-segments (like eve) or the even-numbered segments (like ftz). Two of
the Pair-rule genes, *eve* and *h*, are called primary Pair-rule genes because of their early expression will influence the expression of the other Pair-rule genes. The borders of these stripes will later start to express the segment-polarity genes, which are the final class of segmentation genes. This family constitutes *wingless* (*wg*), *engrailed* (*en*), *inverted* (*inv*), *fused* (*fu*), *armadillo* (*arm*), *pangolin* (*pan*), *cubitus interruptus* (*ci*), *patched* (*ptc*), *gooseberry* (*gsb*) and *hedgehog* (*hh*). These genes not only fine tune the segmentation process, but they also have additional roles in providing positional information to NB’s and controlling cell fate specification during neurogenesis. Their expression pattern is characterized by the fact that it is row-specific, e.g. *wg* and *gsb* in row 5 and 6 (Fig. 3D & 9).

In summary, the developmental process from the maternal load, through the gap- and pair rule genes, all the way to the activation of the segment polarity genes plays a critical role in dividing the fertilized egg to a clearly segmented embryo. These gene families will in the end activate the Homeotic genes which will give each segment its spatial identity.

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**Figure 9**

Early in the *Drosophila* egg a maternal load consisting of mRNA from *bicoid* and *nanos* will be translated. This will lead to a protein gradient throughout the embryo making a first step to build up an anteroposterior axis. The maternal load will activate the gap genes, which together with the pair rule genes and the segment-polarity genes divide the embryo in more specific compartments. Finally this will activate the homeotic genes which will specify and generate cellular diversity throughout the adult body plan.
Initiation of Hox expression within vertebrate CNS

The initiation of Hox gene expression looks a bit different when you compare vertebrates with insects. Vertebrates to a greater extent rely on extrinsic factors which will be discussed below.

Retinoic acid

In the early 1980 it was shown that retinoic acid (RA), a vitamin A derivate, functions as a ligand for several nuclear receptors. Mice fed on a diet low in vitamin A showed severe abnormalities, e.g. in the retina, endothelial structures and developmental failure of the CNS. Later it was discovered that this morphogen regulates embryonic A-P patterning by controlling expression of specific Hox genes, and by regulating growth and patterning of the developing CNS (Marklund et al., 2004). RA thus has two main roles in the developing CNS: patterning and neuronal differentiation.

Retinoic acid is synthesized by retinaldehyde dehydrogenases (e.g. RALDH2) and diffuses from its production source, e.g. somites, into adjacent tissues. RA enters nearby cells and binds to different isoforms of nuclear retinoic acid receptors (RAR) and retinoic X receptor (RXR) (Dolle et al., 1989). The receptor enters the nucleus as a heterodimeric complex and binds to specific sites, retinoic acid response elements (RARE’s), located adjacent to a promoter where they will induce transcription. Knock-out of any of the receptors has only minor effects, probably do to redundancy, whereas compound mutants are more severely affected (Fig. 10) (Marletaz et al., 2006).

![Figure 10](image_url)

After converting Retinal to Retinoic Acid (RA), through the enzyme RALDH2, RA will enter the cell membrane and bind to the RAR (green) which will dimerize with a RXR (blue) and enter the nucleus. Once inside it will bind to Retinoic Acid Response Elements (RARE) which will activate expression of Hox genes.
In vertebrates, Hox genes are direct targets of RA signaling and are involved in regulation of the collinear Hox expression along the A-P axis in the developing embryo (Kessel and Gruss, 1991). Together with other factors discussed below, RA is responsible for patterning and organization of the posterior hindbrain and the anterior spinal cord (Fig. 11A). It has also been shown that the actual timing of induction of RA activity plays an important role in embryonic development, showing that RA acts as a temporal mechanism.

The role of RA in neuronal differentiation can best be exemplified by studies performed on motor neuron (MN) specification. The spinal cord has been shown to receive extrinsic RA from the paraxial mesoderm where RALDH2 synthesize RA, which will diffuse and enter the spinal cord. Studies in the chick spinal cord have shown that a reduction of RA results in a reduced number of islet-1-positive MNs and failure to innervate the target muscle. Further, it has been shown that moving brachial somites and placing them at the thoracic level will lead to a switch in motor columnar identity, by going from a preganglionic column (PGC) to a lumbar motor column (LMC) fate (Ensini et al., 1998). Later in development MNs up-regulate RALDH2 themselves and start to express RA, making them independent of somatic RA synthesis.

An important question to ask is why RA signaling is used in vertebrates, but not as a way to control Hox expression in Drosophila. In vertebrates several of the proneural genes (Gli3, Zic2 and Xiro2) lie downstream of RA indicating that they must have come under the control of RA as vertebrates evolved. Recruiting RA to act as a transcriptional regulator perhaps a higher degree of cellular diversity could be achieved. Retinoic acid, as stated above, is important during early stages of development, but studies have shown that a lack of Vitamin A during adulthood could result in severe conditions, such as schizophrenia and motor neuron disease. (Maden, 2002)
Fibroblast growth factors regulate patterning

Another regulator of A-P patterning has been shown to be the Fibroblast growth factor (FGF). FGFs are a well conserved gene family, where FGF ligands bind to the Fibroblast growth factor receptors (FGFRs), and have been shown to be very diverse in their function. The FGF ligand is prone to splicing and post-translational modifications such as glycosylation, resulting in a variable manner in which this ligand conveys the signal through the plasma membrane. Because of their diversity, FGFs are multifunctional proteins with a wide variety of effects such as: A-P patterning, limb development and wound healing, and because of this are often referred to as “pluripotent” growth factors.

The primary source of FGF signals comes from an organizing center at the caudal tip of the embryo called Hensen’s node, which together with the presomitic mesoderm controls the elongation of the spinal cord (Fig. 11A). Here, FGF signaling has been shown to be implicated in the induction of Hox gene expression in vivo (Bel-Vialar et al., 2002; Liu et al., 2001). In the chick spinal cord, MNs throughout different R-C levels, display different Hoxc-5, -6 , -8, -9 and -10 expression depending on their position. Studies on how different Hox genes are expressed at different axial levels show that the Hox-c expression varies with the FGF concentration, inducing a more caudal appearance with a higher concentration (Dasen et al., 2003; Liu et al., 2001).

Interestingly, FGF is not the only secreted factor inducing a caudal profile. Gdf11, a TGFβ family member, is expressed during development in the tail bud region of the mouse embryo. Gdf11 loss-of-function studies have shown severe defects of vertebral morphogenesis with elongated thoracic segments, suggesting that a posterior signal is missing. Interestingly the cervical region appeared normal and only minor defects in the lumbar region. Thus, the mutant phenotype can be considered to interpret homeotic transformations of the vertebrae to more anterior developmental fates (McPherron et al., 1999). Studies have shown that neither Gdf11, nor Gdf8, have much intrinsic capacity to evoke Hox-c expression. Instead it seems that Gdf11 enhances the ability of FGF to induce Hox-c expression (Liu et al., 2001). Currently, it is not known how Gdf members interact intracellularly or how these factors regulate transcription. Additionally, FGF has been found to be secreted from the midbrain-
rhomobomere (r) 1 boundary and promote r1 identity regulating the production of neuronal populations, including the midbrain dopaminergic neurons. Further to this, two other regulators, Krox-20 and Kreisler, have been found to be expressed within discrete compartments in the rhombomeres. Two conserved Krox-20 binding sites have been found upstream of Hoxb-2 gene in mice (Sham et al., 1993) and in chick (Nonchev et al., 1996), and they are believed to help specify rhombomere (r) 3 and 5. Mutation of these sites leads to alterations of both r3 and r5 rhombomeres (Schneider-Maunoury et al., 1993). Another study by Manzanares et al, identified binding sites for Kreisler (Kmr11), a Maf/b-Zip protein, upstream of Hoxb-3 which is important for correct expression in r5 (Manzanares et al., 1997). Recently PIASxβ, belonging to the PIAS family, was found to function as an activator of Krox-20, which adds new complexity to how Hox genes can be regulated and specify certain cell fates (Garcia-Dominguez et al., 2006).

Cdx

It has been suggested that the vertebrate Cdx genes (Cdx1/2/4, Drosophila: caudal (cad)) play an important role in the development of the posterior embryo (Fig. 11A). Loss-of-function studies of Cdx has indicated that Cdx lies upstream of Hox expression, in contrast to Drosophila were cad seems to work in parallel to the Hox genes. Being transcription factors binding to cis-acting regulatory elements (CRE), several of these sites has been found in a number of Hox loci, where mutation of these sites negatively affects Hox gene expression. This supports the notion that the regulation probably occurs in a direct manner (Subramanian et al., 1995). Work conducted in chick and Xenopus supports the role of Cdx (in frog Xcad3) in patterning of the A-P axis through regulation of the Hox genes. Over expressing of a dominant negative form of Xcad3 resulted in a loss of posterior Hox gene expression. Conversely, over expression of Cdx and Xcad3 results in ectopic expression of the same set of Hox genes (Bel-Vialar et al., 2002; Isaacs et al., 1998).

The expression of cdx has been shown to be sensitive to RA levels. Over-expression of RA led to early expression of cdx1 in the primitive stream and forelimb bud mesenchyme, while reduced levels of cdx1 was seen in a RARα1/γ double knock out. Furthermore, binding sites for RAR and RXR have been found upstream of cdx1
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suggesting that cdx1 is a direct target of RA (Houle et al., 2000). In addition, RA has been proposed to act as an inducer of cdx1 early within the primitive streak, but that the actual maintenance of cdx1 is dependent upon another factor. Members of the Wnt family have been proposed to provide such a mechanism. Indeed, recent results have shown that Wnt signaling is implicated in regulating cdx expression. In the hematopoietic system Wnt binds together with BMP on a cdx1 regulatory element and will activate the Cdx-Hox pathway (Lengerke et al., 2008). Furthermore, it has been shown that Wnt signaling provides positional cues that later allow FGF and RA to activate cdx and Hox profiles in hindbrain and spinal cord allowing for proper motor neuron specification (Nordstrom et al., 2006).

Figure 11

A) The expression of Hox genes will be controlled by extrinsic cues delivered from nearby tissue. RA is responsible for patterning and organization of the posterior hindbrain and the anterior spinal cord, while the FGF and Cdx signals is expressed in the caudal part of the spinal cord.

B) A combinatorial network between RA, FGF, Wnt/BMP, and Cdx is believed to induce a correct Hox expression. Out of these, RA has been the only one found to regulate Hox expression in a direct fashion.

Specification and patterning of the developing vertebrate CNS is much more dependent upon a number of extrinsic cues. However, with the exception of RA, the links between extrinsic signals and the actual initiation of Hox expression still remains elusive. Several studies has shown an intricate combinatorial network between above mentioned factors; RA, FGF, Wnt/BMP and Cdx that act to induce a correct Hox expression pattern in larger tissues, as well as within individual cells (Fig. 11B).
Introduction

Organization and expression of the Hox cluster

All bilateral animals possess a common genetic mechanism that regulates development towards a clearly defined A-P-axis. Within the animal kingdom this is, partially controlled by the Homeobox genes, the Hox genes. Despite their evolutionary and developmental significance, the origin of the Hox gene cluster is obscure. It is generally thought that the Hox clusters emerge from an ancient paralogue: The ParaHox gene cluster (Brooke et al., 1998). Throughout evolution the Hox cluster has changed dramatically, both through duplication of individual genes as well as whole clusters, resulting in major differences between animals. The apparent advancement in regulation of the Hox genes may have contributed to the increased morphological complexity of vertebrates. Hox genes in different phyla have been given different names which has led confusion about the nomenclature. Simply put: Hox genes present in Ecdysozoa (arthropods, nematodes) are referred to as the Homeotic Complex (HOM-C), while the homeotic genes in deuterostomes (echinoderms, chordates) are referred to as Hox genes.

In Drosophila the homeotic complex (HOM-C) controls key developmental programs giving rise to vast number of morphologically different structures found throughout the A-P axis. Peculiarly, the Hox gene family tend to be organized in a collinear fashion located on one single chromosome and are expressed in a sequential manner corresponding to their position within the HOM-C cluster. Individual Hox genes are expressed in such a way that they never trespass the Hox gene expressed in front of them, although some coexpression can be observed.

In Drosophila, the eight HOM-C genes are divided into two complexes: the Antennapedia- and the Bithorax complex. Five Hox genes belong to the Antennapedia complex (ANT-C): labial (lab), proboscipedia (pb), Deformed (Dfd), Sex combs reduced (Scr) and Antennapedia (Antp). These genes are expressed in the anterior part of the CNS and are responsible for patterning of the three brain-, three suboesophageal- and three thoracic segments (Pc, Dc, Tc, Ma, Mx, Lb, T1-T3, Fig. 3C&12). The lab gene is expressed in the Tc neuromere, while pb is only expressed in a few cells belonging to the Dc neuromere as well as in the brain, making this an exception to the “trespassing-rule”. Dfd is expressed in the Md and anterior half of
the Mx neuromere, while Scr starts its expression pattern in the posterior part of Mx into the anterior half of the labial neuromere. Antp, on the other hand, is expressed broadly from the posterior part of the labial neuromere anteriorly towards the VNC (Fig. 3C&12).

The bithorax complex (BX-C) consists of three genes: Ultrabithorax (Ubx), abdominal-A (abd-A) and Abdominal-B (Abd-B). Being expressed in the posterior part of the embryo they are responsible for specification of the nine abdominal segments (A1-A9, Fig. 3C & 12). Ubx is expressed from the posterior part of T2 down to A7, showing its highest expression in the A1-2 segments. Abd-A is expressed from A2 down to A7, while Abd-B can be seen in the most posterior parts of the VNS, A8-9.

In mouse (and human) 38 Hox genes are localized in four different clusters: HoxA, B, C and D, with each cluster spanning more than 100 kilobases (kb) on chromosome 6, 11, 15 and 2, respectively. The Hox genes are numbered anteriorly from 1 to 13, and also associated with the cluster (e.g. Hoxb-2, Hoxd-11)(Fig. 13). Following the nomenclature conventions, these genes are named HOXB-2 and HOXD-11 in humans. In spite of major differences in organization between the Drosophila HOM-C and the vertebrate Hox clusters, a high similarity in sequence and structure can be seen between each Hox homolog. For example, genetic experiments have demonstrated that the mouse orthologue of Dfd, Hoxb-1, can perfectly rescue a Drosophila lab mutant background (Popperl et al., 1995).
Activators, Selectors and Realizators

Recently it has been established that transcription and regulation of Hox genes is controlled in a complex and intricate hierarchal system where a maternal load will activate basic genetic programs and various classes of segmentation genes. Furthermore, downstream it will regulate the spatial and the temporal expression of the Hox genes. Once activated the Hox genes will select specific batteries of genes that will translate these signals into developmental programs, e.g. transcription of specific receptors or cell death genes (Jones et al., 1992). Due to this, depending on where in the hierarchal system a gene is thought to work, they are named Activators, Selectors or Realizators (Pradel and White, 1998). However, this does not exclude the possibility that the Hox genes can work both horizontally, at the same level, or downstream together with other realizators (Fig. 14) (Veraksa et al., 2000).
Two models of regulation

Mutations in Hox genes were first observed in Drosophila which frequently resulted in “homeotic transformations”, and refers to the fact that the effect most often transforms one structure to resemble another present on the body (e.g. the haltere becoming an extra pair of wings). Hox genes do not always give rise to such dramatic phenotypes, which is often the case in vertebrates. In trying to understand how these transformations can occur, two models have been proposed; One model favors Posterior dominance or Posterior prevalence, where a posterior Hox expression dominates over the more anterior one (Duboule and Morata, 1994). By removing a posteriorly located Hox gene, e.g. Ubx, the dominance over Antp would be lost, enabling Antp to move more posteriorly. However, even though this concept has been shown to hold true to some extent also in vertebrate model systems, it has not been able to explain all phenotypes observed (Lufkin et al., 1992). In vertebrates, single Hox gene mutations are quite rarely observed due to three main reasons. Firstly, a more complicated regulation of the Hox genes at a transcriptional, as well as on a global level, is seen. Secondly, a higher degree of redundancy exists where one Hox gene phenorescues another one. Finally, a much higher overlap between Hox genes makes it quite difficult to decipher a true effect of only one single Hox gene mutation. Taking this into account an additional model has been proposed where quantitative differences in Hox gene expression within an individual cell builds up a
“Hox code”, which will determine the outcome and specification of a particular cell fate (Kessel et al., 1990). One can envision a “Hox code” taking effect instantaneously or over a longer time period, by differences in the initial levels of expression, onset of Hox expression, or by asymmetries in the strength of different Hox transcription factors to repress each other. Obviously more research is needed for a deeper molecular understanding to predict Hox patterning along A-P-axis.

**Homeotic transformations in Drosophila**

When a Hox gene is inappropriately expressed, due to failure in the regulatory machinery, it may cause alterations in body patterning. In flies, a mutation in the Ubx gene can cause the haltere to develop into an extra pair of wings, and misexpression of Antp can cause the antennae to become extra legs. Because the appearance of mutations, or misexpression of the Hox genes, often phenocopies other structures present on the organism they are called “homeotic transformations”. These mutations were first discovered in flies, when a spontaneous mutation caused a transformation of the haltere, the balance organ of the fly, into an extra set of wings. How does this remarkable transformation take place? Below classic homeotic transformations will be discussed.

**Wing versus haltere**

Wings and halters are homologous structures derived from imaginal discs. Imaginal discs are contained in pairs within the body of the larva and will give rise to structures seen outside of the fly, e.g. wings, legs and antennae (Fig. 15A). At first they all appear undifferentiated, but throughout development they become more specified giving rise to a specific structure e.g. wing or a haltere. Imaginal discs are easy to identify and dissect, and because of their great developmental capacity, these discs has come to serve as an isolated “model system” on its own.

The wing and haltere discs, showing great difference in size and pattern are located in the second and third thoracic segments, respectively. Looking at the Hox expression, Ubx is only expressed within the haltere discs and mutating Ubx, reprograms the developmental program of the haltere disc into a wing, this serves as
an excellent model for studying how homeotic genes control and execute different developmental programs within different tissues.

Two studies illustrate how Ubx controls this by regulating the presence of the type1 Dpp receptor, Thickveins (Tkv). In both wing and haltere Decapentaplegic (Dpp), belonging to the bone morphogenetic protein (BMP) family and a well documented tissue growth inducer, is produced and secreted from a specialized stripe of cells called the AP organizer (Nellen et al., 1996). Expression of P-Mad, the activated form of the Dpp pathway transcription factor Mothers against Dpp (Mad), reveals that in the wing activation of the Dpp pathway is scattered in a broad and even pattern from the AP organizer. The haltere on the other hand shows a distinct P-Mad stain overlapping with the Dpp producing cells located in the AP organizer. By regulating the diffusion of Dpp, tissue size can be controlled. One way to modulate the activation of the Dpp pathway is to regulate its receptor Tkv. In contrast to the wing, where a weak tkv expression is seen within and in proximity to the Dpp expression, the haltere shows a strong expression profile overlapping the Dpp source. Based on data found in the labs of Mann and Sánchez-Herrero it is suggested that the expression of Ubx in the haltere promotes the expression of tkv, making the Dpp ligand less diffusible and hence resulting in an alteration of tissue size. These findings illustrate how a selector gene modifies organ growth by regulating the receptor, and consequently the distribution of the ligand, Dpp (Fig. 15B) (Crickmore and Mann, 2006; de Navas et al., 2006). Downstream of Ubx lies other developmental programs as well, giving the haltere its pronounced appearance. The mechanism by which Dpp controls proliferation is not fully understood. However results could suggest that the medial wing disc cells and the lateral cells respond to Dpp levels differently (Rogulja and Irvine, 2005).
Antennae versus leg

Two other homologous structures found in *Drosophila* are the antennae and leg structures developing from the antennal and leg imaginal discs. Misexpression of *Antp* in the antennal imaginal disc results in a transformation of the antennae into a leg structure. Conversely, removing *Antp* from the thoracic region transforms the leg into an antenna. This indicates that *Antp* does not promote antennal structure formation, but instead promotes leg formation by repressing the antennal formation pathway. Casares and Mann show that *Antp* induce leg transformation in the antennal imaginal disc by inhibiting the homeobox gene *hth*. In an *exd* or *hth* mutant background the antennae turn into legs, and misexpression of *Antp* leads to the repression of *hth* expression. Further proving this point is the finding that the misexpression of the murine *hth* homologue *Meis1* will transform other body structures to antennal structures, indicating not only a high structural conservation but that *hth* act as an antenna selector gene (Casares and Mann, 1998).

Changes in body axis patterning do not always go hand in hand with differences in *Hox* expression, but with changes within the protein structure itself. Comparing two
Ubx homologs from *Drosophila* and the crustacean *Artemia franciscana* studies have revealed differences in binding properties to the upstream enhancer fragment *Dll304*. *Distal-less* (*Dll*) is an important limb promoting gene in most arthropods, and in *Drosophila* *Dll* transcription is directly repressed in the abdominal segments by Ubx acting directly on the *Dll304* fragment (Vachon et al., 1992). The difference in binding properties rests within the serine/threonine amino acid motif located in the *Artemia* C-terminus which makes Ubx unable to bind to the *Dll304* enhancer, allowing development of leg structures. (Ronshaugen et al., 2002).

**Homeotic transformation seen in vertebrates**

Due to a much higher redundancy of *Hox* gene function in vertebrates, homeotic mutations have been difficult to interpret and have only resulted in minor malformations and no clear case illustrating homeotic transformations such as those seen in *Drosophila*. Because of this, the mutations affecting clear anatomical boundaries, such as cervical versus rib bearing thoracic segments or lumbar and sacral vertebrae, are most commonly studied. Important work done in 2003 by Wellik and Cappecchi showed clear evidence that Hox activity also controls regional patterning along the A-P axis in vertebrates. Using *Hox10* and *Hox11* compound *Hox* mutant mice (Fig. 13&16A-O), they showed severe skeleton alterations. In *Hoxa/c/d*-10 triple mutants, skeletons completely lack lumbar vertebrae which are replaced by rib bearing thoracic vertebrae (Fig. 16A-E). Similar results is seen in the *Hoxa/c/d*-11 triple mutant, where the last sacral segments assume a lumbar morphology (Fig. 16K-Q) (Wellik and Cappecchi, 2003). Another example showing a clear homeotic transformation can be observed by mutating the *Hoxb-4* gene, a member of the *HoxB* cluster (*Dfd*-paralog). In this mutant the second cervical vertebra (axis, C2) is transformed into the shape of the more anterior vertebrae (atlas, C1, Fig. 16P,Q) (Ramirez-Solis et al., 1993). Similar results were found in *Hoxa*-4 mutant mice where defects in the vertebrae C3 and C7 were found (Horan et al., 1994). In these cases the A-P transformations clearly follow the rule of posterior dominance, where a posteriorly located *Hox* gene represses an anterior one.

Modification of *Hox* gene expression may also give rise to different malformations observed in humans. For instance, the classic hand-foot-genitalia (HFG) syndrome is
associated with a nonsense mutation caused in the homeodomain of HOXA13 (Mortlock and Innis, 1997).

The thought of how the Hox genes, especially the AbdB group, have been able to modify and contribute to the expansion of body shapes during the evolution of vertebrates is quite remarkable. It is important to underscore that differential Hox gene function is the result of both changes in protein structure and gene expression (Di-Poi et al., 2010).

**Hox functionality and proposed binding models**

Hox transcription factors give rise to many different shapes and appearances seen throughout the animal kingdom. In spite of this, there have been surprisingly few Hox target genes identified. A potential reason for this “HoxParadox” is that Hox proteins, as monomers, recognize similar and rather unspecific DNA sequences in vitro. On the other hand, their activity must be highly controlled, since a failure in their activity can have disastrous consequences. So how do Hox proteins find their specific targets in vivo? The ambiguous nature of Hox proteins to perform different tasks depends upon the context within which Hox proteins act, as well as within the protein structure itself, making sure that the level of accuracy is flawless.
Basically three different regulatory levels control the transcription of the *Hox* genes and the expression of the translated Hox protein itself. Firstly, a global regulation on chromatin level governed by the Polycomb and Trithorax proteins either represses or activates gene transcription. Secondly, a post-translational regulation consisting of the DNA-binding homeodomain itself and cofactors will regulate Hox proteins activity on downstream realizator genes or regulate its own transcription through autoregulation. Additionally, *Hox* gene transcription or translation can also be regulated by non-coding RNA. I will discuss these different levels of control below.

Global regulation governed by Polycomb and Trithorax

The expression pattern of homeotic genes must be tightly regulated throughout development, but it must also be sustained at the proper level. How is this controlled? In *Drosophila* the maintenance of this system is overseen by the Polycomb (PcG) and the trithorax (trxG) group genes. These genes were discovered in *Drosophila* when it was observed that removing them caused inappropriate homeobox gene expression. These factors act directly on the chromatin and are recruited to specific chromosomal elements termed PcG or trxG response elements (PRE/TRE), several of which have been found in the *Drosophila* Bithorax complex. Proteins, some of which ATP-dependant, associated with the PcG and the trxG families are recruited to maintain a repressed or active state of gene transcription, acting on *Hox* gene transcription as well as many other genes. They perform these actions by modifying the N-terminal tails of the histones, by adding or removing chemical groups, according to the “Histone code”. This will modify the genomic landscape, either hiding or exposing DNA.

The trxG protein Brahma (Brm) complex is closely related to the well-known SWI/SNF ATP-dependant chromatin remodeling complex that is conserved from yeast to humans. TrxG associated proteins have been under intense interest recently due to a *Drosophila* RNAi screen where several interesting targets were identified affecting neural morphology (Parrish et al., 2006). Among these, *Brahma* (Brg1/Brm) together with two Brahma associated proteins/factors, *Bap55* and *Bap60* (*BAF53b, BAF60*), were found to affect dendrite outgrowth. It has become clear that PcG and
Trithorax associated proteins show a tremendous diversity and flexibility, and perform an array of tasks by acting in large protein complexes consisting of different, probably several hundred subunits (Fig. 17). This constant exchange of subunits was recently shown to play an important role during neurogenesis in the vertebrate spinal cord. Neural progenitors exchange BAF53a and BAF45a subunits within the SWI/SNF-like neural progenitor BAF complex for the BAF53b and BAF45b subunits within the neuron-specific BAF (nBAF) complex in post-mitotic neurons, suggesting that the BAF complex serve an important role regulating the progenitor/neuron switch (Fig. 17) (Lessard et al., 2007; Yoo et al., 2009). In addition, a study in zebra fish highlighted the importance of performing histone demetylation in order for proper A-P axis formation (Lan et al., 2007). Considering the fact that the Polycomb (PcG) and Trithorax (TrxG) group of proteins regulate gene transcription throughout the whole genome, and acting in a combinatorial manner to regulate gene transcription, one can envision a scenario with almost endless possibilities leading to tremendous cellular diversity. From an evolutionary perspective, the expansion of the gene families encoding the subunits participating in the chromatin remodeling complexes may have been an important step to take towards the extraordinary diversity of neural subtypes seen within the vertebrate nervous system today.

Figure 17
A model of BAF complex activity in embryonic stem cells (esBAF), neural progenitors (npBAF) and in post-mitotic neurons (nBAF). Going from an undifferentiated state (esBAF) to a differentiated neuron (nBAF) subunits within the BAF complex is exchanged, providing new ways to regulate gene transcription. Ultimately this will lead to inhibiting proliferation in neural progenitors (bend arrows) and promote cell differentiating genes in post-mitotic neurons. Adapted from (Yoo and Crabtree, 2009)
Molecular structure of the Homeodomain

Hox proteins come in a variety of different lengths, e.g. Dfd with 586 amino acid (aa) and Abd-A with 350 aa, but they all include the homeobox, characterized by a conserved 183 base pair DNA sequence coding for the 61 aa homeodomain (HD) DNA-binding motif. Many *Drosophila* homeotic and segmentation genes code for transcription factors that use a homeodomain to bind DNA. Homeodomains are found in important regulators of development in a wide range of organisms.

The homeodomain consists of three α-helices, connected by rather short loop regions. These helices all have different functions, where helix three, known as the recognition helix, interacts with DNA in the major groove (Billeter et al., 1993; Kissinger et al., 1990). Here, two amino acids are more conserved than others, Glutamine 50 (Q50) and Asparagine 51 (N51), which make either direct or water-mediated contacts with their consensus site 5’-TAAT-3’ (Affolter et al., 1990; Dorn et al., 1992). Even though all Hox proteins bind the same TAAT-site they do this with some differences, and their affinity is matched to their location on the Hox cluster, with Lab having the highest and Abd-B the lowest affinity (Pellerin et al., 1994). Other interaction sites, especially within the N-terminus, have been shown to make important interactions in the minor groove of the DNA possibly adding selectivity to each Hox monomer (Ekker et al., 1994; Furukubo-Tokunaga et al., 1993; Otting et al., 1990). The way these TF’s recognize appropriate sites are probably due to, as studies have suggested, an intricate collaboration between these helices. First an initial contact is made in the major groove by the recognition helix. Later the N-terminal arm and the linker residues select among these sites by reading the structure and the electrostatic potential of the minor groove (Joshi et al., 2007). In this way several interaction points become important and not only the preferred TAAT-site, but the ability to bind to a particular DNA target site does not account entirely for the properties found within the HD. In the next section conserved motifs outside of the HD will be discussed.
Cofactors and proposed binding models

Studies of Hox protein interactions in vertebrates as well as in invertebrates have shown that Hox proteins most often use cofactors to achieve a higher DNA binding specificity. In Drosophila, two cofactors have been identified: Extradenticle (Rauskob et al., 1993) and Homothorax (Pai et al., 1998), each having two distinct functions. Both of these cofactors belong to the Three Amino Acid Loop Extension (TALE) homeodomain proteins which are divided into two groups: the PBC family, including the vertebrate Pbx proteins, fly Extradenticle and worm Ceh-20, and the MEIS family, including vertebrate Meis and Prep, fly Homothorax (Hth) and worm Unc-62. In vertebrates, both Pbx and Meis, has been indentified to be involved in several diseases e.g leukemia and neuroblastoma (Geerts et al., 2003; Nakamura et al., 1996; Rauskolb et al., 1993; Van Dijk et al., 1993).

Exd is located cytoplasmically and requires Hth for its translocation into the nucleus (Waskiewicz et al., 2001). Due to the flexible translocation pattern between the cytoplasm and the nucleus, the focus has been turned towards a region close to the N-terminal, which is thought to play an important role in the translocation process. This region contains several potential phosphorylation sites and is also present in other homeodomain proteins (Aspland and White, 1997). Once in the nucleus, Exd will bind a Hox protein through a well conserved amino acid sequence, the YPWM (HX) motif located on the N-terminal side of the HD (Johnson et al., 1995). Other studies have however revealed that Hox proteins can recruit Exd through the UbdA motif, located on the C-terminal side of the HD (Fig. 18) (Merabet et al., 2007). The actual interaction between Pbx and Meis has been shown to stabilize the Pbx protein, which also favor the formation of a Hox-Pbx-Meis trimeric complex (Jacobs et al., 1999; Waskiewicz et al., 2001). Furthermore, this complex will bind preferable sites within the genome and affect transcription, either in an inhibitory or an activating manner (Ebner et al., 2005; Kobayashi et al., 2003; Piper et al., 1999; Rauskolb and Wieschaus, 1994). Although cofactor binding clearly affects Hox-DNA-binding specificity, the effect on DNA-binding affinity differs for the different Hox proteins (LaRonde-LeBlanc and Wolberger, 2003).
By using the translocation mechanism, Hox proteins can also regulate their own activity by regulating the expression of their cofactor. In an interesting study, a fork-head (frk) regulatory fragment was tested for its activation by the Hox gene Sex combs reduced (Scr). It was shown that the Scr/Exd complex were only able to regulate the frk expression during early stages of embryogenesis since Scr negatively regulates hth expression, and thus nuclear translocation of Exd later in development. In this way Scr regulates its own activity by excluding Exd from entering the nucleus and through this making it impossible to form the dimer needed for initiation of frk transcription (Ryoo and Mann, 1999).

**Proposed Binding Models**

Studies on how Hox proteins interact and collaborate with their cofactors has suggested three different models: In the first model, The Cooperative Hox binding model (Fig. 19A), Exd binds to a preferable motif. This will increase specificity and allow for the dimer to bind to a Hox responsive element (HRE) on the DNA. In this model Hox proteins are not capable to bind DNA on their own. Second, the “Widespread binding model” states that Hox proteins can bind to their HRE’s without the assistance of cofactors (Fig. 19B). This model also implies that Exd can change a Hox protein from a transcriptional repressor into a transcriptional activator (Pinsonneault et al., 1997), but since it has never been reported that a Hox protein can switch from an active state to a repressed one, it has been postulated that the recruitment of cofactors to HRE’s perhaps enables other TF’s to be recruited. One
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striking example of this is where two segmentation proteins, Engrailed (En) and Sloppy paired 1 (Slp1) is needed for the repression of \textit{distalless} (\textit{Dll}) (Gebelein et al., 2004). The third model, the “Hox collaboration model”, states that Hox proteins are not dependent upon traditional cofactors (Fig. 19C). To exemplify, the Hox protein Ubx works together, in a co-factor independent manner, with two TF’s downstream of the Dpp/TGFβ-pathway, Mothers against Dpp (Mad) and Medea (Med), to repress the Hox target gene \textit{spalt major} (\textit{sal}) in the haltere (Walsh and Carroll, 2007). It was further shown that Mad and Med recruit the co-repressor Schurri (Shn) which allows for efficient repression of \textit{sal}. In this case Mad and Med control the regulatory activity of the Hox protein to work as a repressor or an activator. This model proposes that Hox proteins, in contrast to previous views, can act outside the “cofactor box” and work with TF’s in a more context-specific manner. Considering the fact that every cell expresses a unique combination of transcription factors, the combinatorial interactions for the broadly expressed Hox proteins would be almost limitless. A similar mode of action could be proposed for the cofactors as well. Recently Kobayashi and colleagues showed that Exd and Hth can interact with Engrailed, a non Hox-homeodomain protein, to repress \textit{slp} and \textit{wingless} (\textit{wg}) (Kobayashi et al., 2003).

Although Hox proteins can modify and transcribe genes throughout the genome with the help of two cofactors and other environmental factors and perform an array of different functions, it has to some extent not been enough to explain observed phenotypes. Therefore, an important goal of studying the Hox genes is the identification of other protein-interaction domains, which has led to a broader perspective on how Hox proteins can utilize its whole structure, and not only the HD and HX/UbdA motifs. Lately, several initiatives has been made finding new cofactors and motifs (Prince et al., 2008, Paper IV). Furthermore, another reason why Hox downstream targets have been so difficult to identify stem from the realization that Hox proteins do not only bind in a traditional “Hox/Hth/Exd manner”. Future initiative including genome-wide approaches such as microarray, ChIP-on-chip, and ChIP-seq together with advanced \textit{in silico} binding modeling studies will hopefully reveal new leads to finally solve the “HoxParadox”.

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Autoregulation

In order to sustain an active, or repressed, transcriptional state, Hox genes may rely on their own gene product to positively or negatively regulate their own transcription in a process called autoregulation. This has been shown in Drosophila, where Ubx negatively autoregulates its own expression (Irvine et al., 1993). In another study it was shown that Ubx, instead of inhibiting its own expression directly, used a PcG dependant negative autoregulatory mechanism (Garault et al., 2008). Additionally, Hoxb-1 expression in rhomobomere 4 in mouse hindbrain uses both a positive and direct Pbx dependant autoregulatory feedback mechanism to regulate its own expression. Extrapolating from this, Popperl and colleagues, showed that the same enhancer fragment reports lacZ expression also in the Drosophila head. Additionally, looking in a Drosophila lab mutant, which is the Drosophila homologue of Hoxb-1, the expression is abolished (Kuziora and McGinnis, 1988; Popperl et al., 1995), emphasizing that this mechanism may be conserved in vertebrates and invertebrates (Pinsonneault et al., 1997).

Figure 19

A) A Hox protein (Hox) and cofactor (CoF) colocalize and interact, increased binding specificity for HRE’s will occur and enable the complex to activate or repress transcription.

B) The “Widespread binding model” Hox proteins can occupy the HRE’s without the aid of cooperative interactions with cofactors. Cofactors, as well as other context specific transcription factors (TF) will be recruited and modulate transcription in a repressive or activating way.

C) The “Hox Collaboration model” Hox proteins and many other context-specific transcription factors bind to HREs. Other transcription factors may later recruit additional factors (X) which will work in a repressive or activating way. Important to notice is that in this model the Hox protein does not necessarily dictate the outcome by itself, but in collaboration with others.
Post-transcriptional control by Non coding RNA

Noncoding RNAs (ncRNA) are functional RNA molecules that are not translated into proteins. It has become obvious that ncRNA, which includes many different variants, participate in complex epigenetic and transcriptional behaviors that so far are poorly understood.

In *Drosophila*, upstream of *Ubx*, in a region called *bxd* there are three TREs (trithorax response elements), which are thought to play an important role controlling *Ubx* expression. A recent study revealed that ncRNA transcribed from the three TREs, recruits “absent small and homeotic disc” (ash1) which will initiate *Ubx* transcription. By either reducing or ectopically misexpressing TRE ncRNA they found that it plays a vital role controlling *Ubx* transcription (Sanchez-Elsner et al., 2006). A similar mechanism has been proposed to regulate the human HOXA complex (Guenther et al., 2005). In addition it has been shown that the Gap gene *Hb* acts directly upon another *Ubx* regulatory region, *bx*, mediating anterior suppression of *Ubx* (Qian et al., 1991), illustrating that these, and other, control elements within the Hox cluster play important functions regulating *Hox* transcription temporally as well as spatially.

Non-coding transcripts also encode microRNAs (miRNAs). They are processed by the nuclear RNase Drosha to a hairpin precursor and are transported to the cytoplasm where they will be cleaved by the cytoplasmic RNase Dicer into a mature miRNA, resulting in a length of ~22nt. Being complementary to other mRNA transcripts, miRNAs will complement with target mRNA and inhibit translation, reducing the amount of protein product of the target gene. A relatively large number of miRNAs have been identified, but there are only few validated *in vivo* targets presently known. However there are indications that *Hox* genes might be an important class of miRNA targets. Having identified Hoxb-8 to be a target for miR-196, several studies has been made deciphering its role throughout development (Yekta et al., 2004). In the chick caudal neural tube, it was shown that Hoxb-8 plays an important role down-regulating the Olig2 TF, a marker for motor neurons precursor (pMN). Olig2 further activates MNR2 and Lim3 which are important MN determinants. Hoxb-8 appears to impact the late phase of pMNs resulting in
prolonged expression of Olig2 status in pMN, which in turn leads to a failure of postmitotic differentiation; hence the timing of down-regulating Hoxb-8 is critical. Noting that miR-196 knockdown phenocopies Hoxb-8 misexpression, the authors present a miRNA-based mechanism that controls Hoxb-8 activity within a spatio-temporal domain. In this way miRNA is used late to quickly down-regulate Hoxb-8 expression in the pMN and promote of MN differentiation (Asli and Kessel, 2010).

In summary, one can envision miRNA to play two different roles throughout development. First, with its dynamic expression pattern within different organ systems and tissues, miRNAs play important roles in specifying different tissue types and cell fates. Second, miRNA can be expressed as a precaution to safeguard against inappropriate gene expression.

**Generation of Neural diversity along the anteroposterior axis by Hox genes**

Most animals appear to display different types of neurons at different anteroposterior (A-P) positions. Studies have shown that there are several mechanisms used to achieve cellular diversity along the A-P axis. Firstly, this could be controlled by regulating the cell cycle machinery, since differences in proliferation pattern will ultimately lead to differences in the number of cells being produced at different segmental levels. Secondly, an equal number of cells may be generated at all levels, but subsequently, some cells will be programmed to enter a cell death program, eventually leading to differences in cell pattern. Thirdly, and equal number of cells may be generated at all axial levels, but further cellular diversity could emerge by their location, thus being exposed to certain external as well as internal stimuli. Below, these examples will be discussed and not surprisingly, Hox genes are involved in all of them.

**Modulation of Cell generation throughout the Neuroaxis**

In the *Drosophila* VNC there are segmental differences in the numbers of neurons and glial cells that arrive from an initially equivalent sheet of 30 neuroblasts in each hemisegment. In a number of studies trying to decipher how these differences in NB lineage size can emerge, three different methods have mainly been used; DiI
labelling, transplantation techniques and injection of horse-radish-peroxidase (HRP) into individual NBs. These studies revealed that ~12 out of 30 NBs display a difference in thoracic vs. abdominal lineage size (Bossing et al., 1996; Schmid et al., 1999; Schmidt et al., 1997; Udolph et al., 1993). This difference is, in part, due to the action of Hox genes modulating cell survival (see below) and cell generation.

One might wonder if the NB “knows” prior to delamination where throughout the A-P axis it is located, or if a particular A-P fate is given later when it has already delaminated. Results from transplantation studies of the neuroblast NB 1-1 have revealed that the commitment to segmental specificity occurs already within the neuroectoderm prior to delamination. The given fate is later maintained during post-embryonic development (Prokop et al., 1998; Prokop and Technau, 1994).

Another NB showing A-P difference is NB6-4. The thoracic NB6-4 (NB6-4T) produces ~five neurons and three glial cells, while the progeny from the abdominal NB6-4 (NB6-4A) only consist of two glial cells (Schmidt et al., 1997). How is this difference achieved? Berger and colleagues noticed that the wt NB6-4T distribute the glial fate determining glial cell missing (gcm) mRNA in both daughter cells during the first division, but subsequently gets rapidly removed in the cell that functions as the neuronal precursor. Pros is transferred asymmetrically into the glial precursor where it is needed to maintain and enhance expression of gcm. In CycE mutants, gcm is distributed equally, promoting solely glial cell fate. NB6-4A was not affected in a CycE mutant. Interestingly, misexpression of CycE within NB6-4A resulted in an increase of lineage size, and some of the extra cells did not express the glial cell marker Repo, indicating neuronal identity. Performing cell transplantation techniques they were able to show that CycE confers neuronal identity in a cell-autonomous manner. Furthermore, comparing the expression pattern of CycE between NB6-4T and NB6-4A it was shown that CycE is expressed prior to the first division of NB6-4T, which later only can be detected in the neuronal precursor. CycE was never detected in NB 6-4A. Additionally, in an abd-A mutant CycE was up regulated in NB6-4A which resulted in a homeotic transformation of NB6-4A to NB6-4T.
To summarize, the NB 6-4T lineage consist of both neuron and glial cells, compared to the NB 6-4A which only consist of two glial cells. NB 6-4T up-regulate the expression of CycE which result in glia and neurons being generated. In the abdomen, the expression of Abd-A will repress CycE expression, which result in glia generation only. Finding binding sites for Abd-A, Exd, Hth and En upstream of the CycE promoter, the inhibition of NB 6-4A lineage progression would probably work according to the “Widespread binding model” (Fig. 19B) (Berger et al., 2005a, b; Kannan et al.). Through this mechanism, NB 6-4T is differentiated into become a neuroglioblast, in contrast to NB 6-4A where gcm mRNA will be distributed equally in the progeny because of lack of CycE expression (Fig. 20).

Programmed Cell Death induced by Hox activity

An important mechanism to remove unwanted cells is to fine-tune the system by letting cells undergo programmed cell death (PCD). This is a powerful mechanism to allow for differences in cellular diversity along A-P axis. The cell death machinery can be activated in several ways, extrinsically as well as intrinsically, and often involves one of the three, well conserved members of the Bcl-2 family (Bak, Bax, Bid), which will activate downstream target genes usually ending up being members of the Cysteine-dependent ASPartate-directed proteASES (Caspase) family. Once activated, caspases cleave, and thereby activate other procaspases, resulting in an amplifying proteolytic cascade. Activation of PCD can be compared to entering into a new phase of the cell cycle: once triggered you are beyond rescue in this irreversible process. In Drosophila, programmed cell death is dependent upon the IAP (Inhibitors
of the Apoptosis) family, which in turn are inhibited by the RHG-motif genes \textit{reaper (rpr)}, \textit{grim} and \textit{head involution defective (hid)}. In \textit{Drosophila} a commonly used chromosomal deletion \textit{Df(3L)H99 (H99)} lack these three genes and show an apparently complete absence of apoptosis. Programmed cell death, being a common seen phenomenon throughout the animal kingdom, the IAP binding motif is also shared by the mammalian apoptotic proteins SMAC/Diablo and HtrA2/Omi. Rpr, Hid and Grim independently or combinatorially activate caspase-dependant pathways by binding to and inactivating inhibitors of PCD proteins, IAPs.

\textit{Hox} genes have been demonstrated to induce PCD modulating cell survival along A-P axis. Indeed, studying the maxillary/mandibular segments, Lohmann and colleagues showed that the \textit{Drosophila} \textit{Hox} gene \textit{Dfd} is responsible for the formation of the dorsal ridge located between the maxillary and mandibular segments. Wanting to know if the regulation of \textit{rpr} was direct or indirect they searched for potential binding sites upstream of the \textit{rpr} promoter. Finding four binding sites and performing site-specific mutagenesis they could draw the conclusion that this is a direct activation of the \textit{rpr} gene, and also that this binding probably is cofactor-independent (Lohmann et al., 2002).

Within the \textit{Drosophila} CNS, \textit{Hox} genes have been shown to regulate PCD in neural progenitors. One mechanism to regulate the amount of progeny emerging from postembryonic NBs (pNB) has been shown to occur through induction of the \textit{Hox} gene \textit{abd-A}. Bello and colleagues did not clarify which, out of the three, RHG-motif genes that are responsible for PCD, or if this mechanism was acting in a direct mode of fashion, but either way, this Hox-induced PCD in pNB is responsible for the segmental differences observed when comparing the thoracic and abdominal region in the adult fly (Bello et al., 2003). Furthermore, it was shown that \textit{abd-A} lies downstream of the temporal gene \textit{grainy-head (grh, see below)}, and that they together induce PCD (Cenci and Gould, 2005; Maurange et al., 2008). This is not to be considered a general way to undergo PCD in the abdomen, since many NB lineages do not go through the \textit{grh} temporal window (see below).

However, Hox-induced PCD can also act later throughout development modulating the survival of post-mitotic neurons. This has been shown to occur in almost all NB
lineages (Rogulja-Ortmann et al., 2007). A recent study showed that this could also be done in a coordinated segment-specific fashion in only a small subset of neurons. The dMP2 neurons, generated within the MP2 lineage, are generated in all VNC segments, but at later embryonic stages are only present in the abdominal segments A6-A8. The posterior survival of these cells mediated by the Hox gene Abd-B, inhibits the RHG-motif genes rpr and grim from activating the PCD program. Intriguingly, in more anterior segments dMP2 neurons are actually generated and allowed to differentiate and fulfill their role of guiding non-pioneering neurons to their targets before they later, due to lack of Abd-B expression, are removed by PCD (Miguel-Aliaga and Thor, 2004). This is a striking example of that Hox genes can not only remove, but also save cells from PCD. Additionally, studies made so far does not show any preference of RHG-motif used in post-mitotic neurons versus neuroblasts (Peterson et al., 2002)

Due to technical challenges, examples showing single cell induced PCD in vertebrates is rare in general, and Hox induced PCD in particular. However, in a broader context, work from the lab of Capecchi has shown that a Hoxa-13 mutant mouse revealed a loss of normal apoptosis in the interdigital regions of the autopod. Even though this study did not reveal if this is a direct target it shows that Hoxa-13 plays a part in inducing PCD (Stadler et al., 2001). Furthermore, human breast cancer biopsies have revealed a correlation between HOXA-5 and the tumor suppressor gene p53. Noticing a reduced HOXA-5 mRNA expression levels, Raman and colleagues showed that HOXA-5 play an important role up regulating p53 expression which stimulates the cells of entering PCD. Finding HOXA-5 binding sites upstream of the p53 promoter, they conclude that loss of expression of p53 in human breast cancer may be primarily due to lack of HOXA-5 expression (Raman et al., 2000).

Cell specification mediated by Hox activity

A more recently discovered mechanism for how Hox genes control segment-specific cell fate revolves around postmitotic instructive roles of Hox genes. This involves activation of a highly coordinated battery of terminal differentiation genes ultimately leading to differences in gene expression profiles, axodentric morphology or neurotransmitter receptors.
That Hox genes, when mutated, cause homeotic transformations is by now a familiar scenario, but cannot be considered a respecification of a post-mitotic cell since, for instance, the actual re-specification from a A1 to a T3 segment within the *Drosophila* VNC takes its beginning long before that cell has even been born. So how important is Hox expression post-mitotically?

In contrast to the *Drosophila* system, work from the labs of Capecchi, Jessell and Krumlauf has made ground breaking studies in the vertebrate system, trying to decipher the role that Hox gene expression plays late in post-mitotic neurons. Studies on post-mitotic differentiation have by tradition been focused on motor neurons (MNs), mostly because of their morphology, size, obvious locomotion defects but also due to that their different projection patterns makes them a perfect readout in the study of cell specification.

**Motor neuron specification**

Among the most sophisticated motor programs are those executed by the limbs, and since each movement activates only a specific set of MNs, this must demand a high degree of specificity. The repetitive and stereotypic pattern of MNs innervating specific muscle targets, led to the idea that MN possess subtype identities, that are somehow programmed to extend its axon along a determined migratory rout, form a specific branch pattern and in the end innervate a specific muscle groups (Landmesser, 2001). So how is this remarkable specificity achieved? As we shall see, the vertebrate locomotor system is laid down by an underlying Hox-based transcriptional network that together with combinatorial transcription factor codes result in an extraordinary specificity.

**Induction of a Generic motor neuron identity**

During development, motor neurons are organized along the A-P axis or rostral-caudal axis (R-C) of the spinal cord into distinct longitudinal columns, which will innervate different muscular targets. Throughout the spinal cord four columnar classes have been described: Lateral motor column (LMC), Median motor column (MMC), Preganglinoic column (PGC), and the Hypaxial motor column (HMC). Since they innervate different muscle targets they are only present at certain axial levels (Fig. 21B). Motor columns can further be divided into divisions, which will project their axons to a specific limb area. Each division can further be divided into motor
pools, where each MN within that group will acquire an early identity that instructs their axons to grow along highly specific trajectories to their distinct and stereotypic muscle targets (Fig. 22). The molecular control of neuronal subtype specification has been investigated in detail within the spinal cord, hence most examples below will be taken from that context.

MN progenitors within the spinal cord, are generated and specified due to graded extrinsic signals along the D-V axis consisting of RA and FGF from the paraxial mesoderm together with secretion of Shh from the floor plate. Differences in concentrations along the D-V axis will induce a broad response in the progenitors which will start to express an array of TFs. Through an intricate network of D-V cross-repressive interactions between TFs, progenitor domains will be set up, each with a unique cellular identity. The expression of RA and FGF8 will result in Hox gradients (Hox6/9/10) which will be expressed within specific R-C position. The Hox expression will decide the position of each motor column present within the spinal cord. Throughout the spinal cord four motor columnar classes have been described: Lateral motor column (LMC); Median motor column (MMC); Preganglionic column (PGC) and the Hypaxial motor column (HMC). Each one of these columns will project MNs to distinct muscle targets.

Figure 21

A) Motorneuron progenitors will be influenced by extrinsic signals along the D-V axis, consisting of RA and FGF from the paraxial mesoderm together with secretion of Shh from the floor plate. Differences in concentrations along the D-V axis will induce a broad response in the progenitors which will start to express an array of TFs. Through an intricate network of D-V cross-repressive interactions between TFs, progenitor domains will be set up, each with a unique cellular identity.

B) The expression of RA and FGF8 will result in Hox gradients (Hox6/9/10) which will be expressed within specific R-C position. The Hox expression will decide the position of each motor column present within the spinal cord. Throughout the spinal cord four motor columnar classes have been described: Lateral motor column (LMC); Median motor column (MMC); Preganglionic column (PGC) and the Hypaxial motor column (HMC). Each one of these columns will project MNs to distinct muscle targets.

MN progenitors within the spinal cord, are generated and specified due to graded extrinsic signals along the D-V axis consisting of RA and FGF from the paraxial mesoderm together with secretion of Shh from the floor plate (Fig. 5A & 21A). Differences in concentrations along the D-V axis will induce a broad response in neural progenitors which will start to express an array of TFs, typically belonging to the paired-box transcription factors (Pax3/6/7), bHLH TF Olig2 and the homeodomain factors Nkx2.2 and Nkx6.1/2 (Fig. 21A). Out of the three Pax genes, in vitro analysis has shown that Pax6 and Nkx2.2, with its dorsolateral expression pattern, is vital for proper MN specification, both in the spinal cord as well as in the hindbrain. Pax6 mutation results in a loss of MNs (Osumi et al., 1997; Pituello et al., 1995). Mutating Nkx2.2 does not affect Pax6 expression, but cells tend to undergo a ventral to dorsal switch in that they generate MNs instead of interneurons (Briscoe et al., 1999).
Through an intricate network of D-V cross-repressive interactions between TFs, progenitor domains will be set up, each with a unique cellular identity. But how are these MN progenitors specified along the R-C axis? There are basically three reasons to suspect why Hox genes should play a role arranging motor columns along the R-C axis. First, Hox expression has been documented within motor neurons innervating limb muscle (Liu et al., 2001). Second, Hox mutants have revealed difficulty with muscle movement and defects in motor neuron projection (Dasen et al., 2005;
Goddard et al., 1996) Third, the organization of motor columns depend upon Hox function (Dasen et al., 2003).

After a D-V specification the progenitors will also acquire positional information. Important studies have clarified that positional identities are regulated by signals derived from adjacent structures, such as node or paraxial mesoderm (Dasen et al., 2003; Ensini et al., 1998). In an attempt to decipher the role that Hox genes play during motor neuron specification the expression of 21 Hox genes and Meis1, Meis2, Pbx1 and Pbx3 were detected in MNs at brachial, thoracic and lumbar levels of stage 29-31 spinal cord. Studies in the brachial, thoracic and lumbar segments reveals an intricate Hox expression pattern, with a broad Hoxc-6, Hoxc-9 and Hoxc/d-10 expression (Fig. 21B) (Wu et al., 2008b). Recently, Jung and colleagues showed that Hoxc-9 plays an important role specifying the thoracic area by repressing Hoxc-6 expressed within the brachial segment. By doing electroporation of Hoxc-9 dsRNA, Hoxc-6 is allowed to be expressed within the thoracic region, transforming PCG into LMC columns, as seen by the expression of RALDH2 followed by a reduction in the PCG marker, pSmad. This shows that a single Hox gene acts as a global organizer of motor neurons and their connections (Jung et al., 2010). Furthermore, misexpression of the cofactor Meis1 displayed an axial specific function, where an exclusion of Meis1 appears to be necessary in brachial LMC MNs. Interestingly, lumbar levels were not affected (Dasen et al., 2003).

Focusing on brachial levels two Hox-dependant programs seems to be at play. One decides R-C motor pool position, while the other program will divide this pool into smaller intrasegmental identities. The first program will be induced by FGF and RA signaling which will initiate the expression of the Hox-5 and Hox-8 expression (Fig. 23B). This seems to resemble the same way as columnar identities are established (Dasen et al., 2003; Dasen et al., 2005). When FGF8 is misexpressed differences in Hox expression patterns can be observed where the thoracic level of the spinal cord elongates to involve the brachial level. As a result of ectopically expressed FGF8, the thoracic expression of Hoxc-9 will extend into the brachial area which will lead to a reprogramming of LMC into PCG MN cell fate. It seems that FGF8 act on neural progenitors, and that manipulation of FGF signaling can switch Hox profiles early during development. Furthermore, it is interesting to notice that even though this
seems to follow the rule of posterior prevalence, cross-repressive interactions seems to take place, suggesting that asymmetric differences in strength of different Hox transcription factors repress each other. The second Hox program seems to fine tune the system where minor fluctuations of Hoxc-3, Hoxc-4, Hoxa-7 and Hoxb-7 in the end will result in unique “Hox codes” (Fig. 23B) (Dasen et al., 2005). Not only are they important for proper motor pool organization, but also for activating proper differentiation programs as MN axons start to migrate for proper muscle targets (Fig. 23D).

The early establishment of Hox-6 and Hox-10 expression within LMC specification is important for directing axon toward the limb. However, even though many aspects of motor pool identity seem to be programmed through Hox intrinsic networks, expression of some TFs seems to rely on extrinsic signals as well. The EST TF Pea3 seems to be one such factor since it needs the expression of Hoxc8 to be up regulated within the MN once it has innervated an appropriate muscle (Fig 23D). Misexpression of Hoxc8 has further been shown to broaden the Pea3 expressing domain (Dasen et al., 2005), while Hoxc-8 mutant fail to fully activate the Pea3 expression. Therefore, it appears that Hox proteins make MNs able to respond to specific cues within the environment, perhaps by initiate the expression of specific receptors. Manipulating Hox expression leads to respecification of motor pool and its typical TF expression, which ultimately leads to failure in finding its proper peripheral target (Dasen et al., 2005; De Marco Garcia and Jessell, 2008; Livet et al., 2002). Recent studies have shown that specification of motor columns can also occur in a Hox independent fashion. Since MMC is present at all levels (brachial, thoracic and lumbar) it does not need Hox gradients to divide it along the R-C axis, but instead use a Wnt mediated program (Wnt4/5a/5b) to specify the MMC fate (Agalliu et al., 2009)

To summarize, the columnar identity is determined by broad Hox expression (Hox6/9/10) along the R-C axis. Later, a second Hox program fine-tunes the columns into motor pools where they together with extrinsic cues regulate MN identities (Fig. 23B).
Refinement of Motor Neuron Differentiation

Although Hox genes are critical for MN subtype specification, several other factors has been shown to be important to specify MNs as well. Since Hox proteins is broadly expressed within the CNS, not exclusively within MNs, and the fact that the same Hox protein can be expressed by multiple columnar subtypes this would argue for that several other factors might be working together with Hox proteins to in the end specify specific tissues and cells. Several of these TFs have been found to belong to the well conserved LIM homeobox gene family (Fig 24). While LIM and Hox proteins contain conserved protein interaction motifs outside of the HD, most homeodomain proteins do not, and in those cases were conserved domains actually do exist, they often provide for DNA-binding. This might have added a unique potential for LIM proteins to combinatorially interact with other TFs to participate in a wide range of developmental programs.
Introduction

Chick Islet1, Islet2, Lhx1 and Lhx3 were found to be expressed in different combinations that correlate with both the location of the cell body and the axonal projection. Further, similar results have been shown in zebrafish spinal cord where primary motor neurons express unique combinations of LIM-HD genes. The expression of these codes has also been shown to be quite plastic. When transplanted, their LIM-HD expression profile, morphology and axonal projection change and resemble those neurons at the implant site (Appel et al., 1995; Tsuchida et al., 1994). These observations suggest that MN identity and axonal projection is controlled by combinatorial expression of different LIM-HD proteins (Thor et al., 1999). However, LIM-HD proteins are found in interneurons of the spinal cord where there is evidence that they control the specification of distinct interneurons in a cell autonomous fashion (Thaler et al., 2002).

Dissecting individual functions of the LIM-HD proteins, Islet1, which is expressed within all MNs has been shown to be necessary for MN generation since Islet1 mutant mice show total absence of MNs (Ericson et al., 1992; Pfaff et al., 1996). Islet2 mutant mice generate MNs in the spinal cord, even though subclasses of visceral MNs show axon projection failure (Tsuchida et al., 1994). Two other LIM-HD genes, Lhx3 and Lhx4, have also been studied and are coexpressed in all MNs that extend their axons ventrally from the neural tube. Mutant mice of both Lhx3 and Lhx4 switch their identity and instead project their axons dorsally. Misexpression of Lhx3 in neurons that normally extend their axons dorsally now reroute their axons to instead enter ventrally (Sharma et al., 1998). This result show a striking resemblance to the case in Drosophila were Lim3 directs axon projection.
Recently, it was shown that *Drosophila* Hox proteins cooperate with a Forkhead domain protein, Sloppy paried1, and Engrailed to directly control gene expression and downstream targets (Gebelein et al., 2004). Their vertebrate counterparts, the Engrailed and Fox proteins, are expressed by subsets of MNs. In addition, LIM-HD expression has been shown to be regulated by the Forkhead domain TF FoxP1, which displays an abnormal motor projection pattern where MNs no longer project into the limb in an organized way. FoxP1, being expressed within LMC and PGC columns, has been shown to lie upstream of RALDH2 and Lhx1, important for proper LMC specification (Rousso et al., 2008). Further, it has been shown, by misexpressing a dominant-negative version of *Hoxc-6* or *Hoxc-9* using chick electroporation, that MNs generated after branchial expression failed to express FoxP1, illustrating that FoxP1 lies under Hox control (Dasen et al., 2008). Additionally, the levels of FoxP1 have been suggested to be important; LMC have high levels whereas PCG have low levels of FoxP1, a difference controlled by Hox expression. In similarity with Hoxc-6 and Hoxd-10, FoxP1 seems to be able to re-specify PGC and HMC neurons into LMC neurons through misexpression. Interestingly, FoxP1 seems to be dependent upon a continuous expression of Hox proteins in order to perform this task, suggesting that this “accessory factor” appears to act in parallel, rather than in a linear intermediary fashion in order to specify neuronal cell fate (Dasen et al., 2008).

Because most studies concerning post-mitotic Hox specification has focused on motor neurons this has led to a gap in knowledge since MN function depends upon interneurons to provide excitatory and inhibitory commands in order for proper function (Zagoraiou et al., 2009). To fully understand the function of motor neurons one must also understand the formation and specification of interneuron, which has proven to be problematic because of difficulty identifying them. However, during recent years this area has been given much more attention. By identifying transcription factors Pitx2, Dbx1, Hb9, and Chx10 as expressed within interneuron progenitors, as well as in post-mitotic interneurons, this has facilitated in the aim of trying to decipher how interneurons are specified. In the *Drosophila* CNS, Hox function in post-mitotic cell specification has been surprisingly understudied. Even though *Hox* genes has been implicated in several important developmental process in the periphery e.g. Antp regulating the LIM-HD gene *apterous* (*ap*, vertebrate: *Lhx2*)
and collier/knot (col, COE/Ebf family member) (Capovilla et al., 2001; Mandal et al., 2007), and Ubx which directly regulates dectapentapleigeic (dpp) and Distal-less (Dll) (Capovilla et al., 1994; Vachon et al., 1992), none of these studies clearly explain how Hox genes work in the CNS, be it in MN, interneurons or glia.

In summary, differences in neuronal architecture along A-P axis can emerge by different mechanisms. Firstly, Hox genes may act in neural progenitors or in post-mitotic neurons to either induce or inhibit cells from entering the cell death program. Secondly, Hox genes may control cell cycle progression in neural progenitors and through this modify the actual presence of cells at specific A-P levels. Additionally, Hox genes may work through different Hox programs to specify post mitotic cells differently, depending on where along the A-P axis they are present.

**Temporal axis**

How diverse types of neurons and glia are generated by stem and progenitor cells during CNS development is a very engaging biological question. As mentioned above, both spatial and temporal cues must act upon neural progenitor cells in order to produce the great variety of neurons and glia that build up the nervous system. While significant progress has been made in understanding regional patterning, many fundamental questions still remain regarding how temporal cues act, and in extension how temporal cues are integrated with spatial cues.

**Temporal transitions within the Drosophila system**

Neural progenitors in both insects and vertebrates have been found to undergo temporal changes in their competence to produce different types of neurons and glial cells at different time points. These temporal changes have been extensively studied in the *Drosophila* system, where a “temporal cascade” acting within the NB has been revealed - consisting of the following transcription factors: *hunchback* (hb; vertebrate *ikaros*), *Krüppel* (Kr; vertebrate B-cell leukemia/lymphoma 6 [Bcl6], *pdm1/2* (henceforth *pdm*; vertebrate *oct1/2*), *castor* (cas; human *casz1*) and *grainy-head* (grh; mammalian *grainyhead* [mgr]). These TFs are transiently expressed within the NB in a sequential manner, Hb/Kr→Kr→Pdm→Cas→Grh, and have been found to be necessary and sufficient for the specification of neural and glial fates generated within their
respective expression windows (Fig. 25A). The temporal program will play out in the NB and the temporal identity is inherited within the GMC and its progeny. As discussed above, NBs will differ from each other depending upon their time of birth, lineage size and neuron/glia production. In the Drosophila embryo, most of the 30 NBs in each hemisegment, including the earliest NBs to form (e.g. 7-1 and 7-4) as well as some of the last NBs to form (e.g. 2-4 and 7-3), progress through the cascade in the same order. However, depending upon lineage size, some NBs may reach later temporal stages than others. It should also be emphasized that the temporal windows are not synchronized on a global level (Isshiki et al., 2001), and will in fact often progress normally in cultured NBs (Brody and Odenwald, 2000; Grosskortenhaus et al., 2005). Thus, in Drosophila the temporal cascade appears to progress primarily via NB intrinsic and/or lineage-intrinsic cues.

The temporal cascade is believed to be regulated by different mechanisms. Certain steps in the temporal progression are mediated by the fact that each temporal gene positively regulates the next gene, and suppresses the next plus one. To exemplify, a Kr mutant background delays the onset of pdm, and a pdm mutant delays the onset of
Both Hb and Cas has been found to bind to, and negatively regulate, cis-regulatory elements within the pdm1/2 enhancers (Kambadur et al., 1998). Conversely, misexpression often results in premature onset of the gene next after the one misexpressed (Fig. 25B).

Moreover, temporal transitions have been found to be coupled to cell cycle progression. Specifically, in string mutants, where the cell cycle is arrested before the first NB division, there is a failure in the NB to down-regulate Hb (Grosskortenhaus et al., 2005). However, Hb expression is also maintained in pebble mutants, in which NBs progress through the cell cycle but fail to divide. Thus, the Hb→Kr transition is dependant not upon cell cycle, but on cytokinesis. In contrast, if Hb is removed in string mutants the temporal gene cascade progresses through the Kr→Pdm→Cas window without mitosis occurring, indicating that these transitions are regulated by forward regulatory mechanisms.

Seven-up (Svp, vertebrate chicken ovalbumin upstream promoter-transcription factor I/II, COUP-TFI/II) has been shown to play an important role in allowing the temporal gene cascade to enter the Kr window, and is referred to as a “switching factor”. Mutation of svp causes an increase in the number of Hb+ cells within several NB lineages, whereas misexpression of svp leads to a loss of early generated neurons. So how does Svp regulate temporal progression? It turns out that the svp mRNA is already expressed within the NB before the generation of the first GMC but remains untranslated until the NB enters mitosis. This triggers svp mRNA translation and Svp down-regulation of Hb, which promotes the temporal cascade to proceed. These observations suggest that down-regulation of Hb is controlled by cytokinesis which, by an unknown mechanism, allows the svp mRNA to be transcribed (Kanai et al., 2005) (Mettler et al., 2006).

Not only do sequential temporal switches regulate cell fate specification during lineage progression, but it has also been shown that temporal genes play key roles regulating and inducing cell cycle exit and programmed cell death (PCD). Maurange and colleagues identify two different types of postembryonic, Type1 and Type2, and propose that these different types use different temporal dependant programs to either leave the cell cycle or enter PCD (Maurange et al., 2008). This shows that
temporal cascades within lineages do not only regulate specification of the neurons and glia born, but also neural proliferation itself. Additionally, Tsuji and colleagues noticed that temporal switches also regulate NBs quiescence, and that these static states can be induced by a combined temporal and spatial code (Tsuji et al., 2008). Studying the NB3-3T they noticed that in both cas and Antp mutant backgrounds the NB retained a proliferate state long after the wt NB had entered quiescence. However, it is unclear how these two axes are integrated on the molecular level, and whether or not this is a general mechanism to enter quiescence.

In spite of progress in this field, a number of questions pertaining to the temporal cascade and temporal transitions in NBs remain. What initiates, regulates and terminates the temporal gene cascade within each NB? How are the often quite large temporal windows sub-divided? How many steps away from terminal differentiation genes are the temporal genes? These were outstanding issues at the onset of my thesis work.

Temporal transitions within the vertebrate system

Compared to the temporal transitions in Drosophila NBs, similar temporal phenomena have been found in the vertebrate nervous system, where specific cell types are generated at specific time points. These processes have been found to occur in several different regions of the CNS. In the retina, multipotent retinal progenitor cells (RPCs) give rise to different retinal cell types in a reproducible order during development. At an early embryonic stage, retinal ganglion cell and horizontal cells are generated. As development progresses, a switch in competence within the RPCs occurs resulting in the generation of amacrine cells and rod photoreceptors (Livesey and Cepko, 2001). Similar phenomena can be found within the spinal cord where MN progenitors (pMN) sequentially generate MNs early and later glial cells (oligodendrocytes, astrocytes) (Kessaris et al., 2001). Further, through transplantation experiments, it was shown that the neurogenic potential was lost once pMN cells reached the gliogenic window (Mukouyama et al., 2006). In the cortex, transplantation studies have shown that cortical progenitors become progressively restricted in their developmental potential during corticogenesis, such that progenitors at early stages of cortical neurogenesis are multipotent and can generate
projection neurons of most layers, whereas the later-stage progenitors are restricted to forming only the upper layer (Desai and McConnell, 2000; Frantz and McConnell, 1996). A conclusion drawn from these studies is that different progenitors become restricted in their potential as development progress. However, it should be emphasized that, due to the complexity of the mammalian CNS and the lack of markers, it is in many cases unclear if the temporally ordered generation of different cell types in a certain CNS region is really due to temporal changes within the same neural progenitor, or if different sets of progenitors are active during different time points.

Moreover, in the cases where it seems clear that there are temporal progression in the same neural progenitor, such as in cultured cortical progenitors, the extent to which these competence windows are controlled by intrinsic versus extrinsic cues, what these cues actually are, and how they are regulated remains poorly understood.

Even though no temporal sequence equivalent to the one seen in insects has been identified so far, studies in the mouse retina has revealed that Ikaros, a mouse ortholog of *hb*, is both necessary and sufficient to confer early temporal competence. When misexpressed it was sufficient to generate early-born neurons without increasing clonal size. Interestingly, late-born cells can still be generated even though previous temporal states has not been down regulated properly, which clearly lies in contrast with findings found in *Drosophila* (Elliott et al., 2008). Studies have further revealed a double nature of Ikaros being able to recruit Swi/Snf chromatin-remodeling components and modify the histone code, either allowing or repressing transcription (Harker et al., 2002; Koipally et al., 2002; Koipally et al., 1999).

*In vitro* studies suggest that an intrinsic mechanisms is incorporated within cerebral cortical progenitors which will maintain switches generating stereotyped lineages with a birth order similar to the one found *in vivo* (Cayouette et al., 2003; Gaspard et al., 2008; Shen et al., 2006) One example of how lineage cues may be provided by the lineage itself, in an “intrinsic-extrinsic” way stems from studies on the DNA – binding transcriptional repressor Sip1. Being expressed at high levels in post-mitotic neocortical neurons, it has been found to regulate progenitor fate in a non-cell autonomous way. In *Sip1* mutants it was shown that Sip1 affects the proportions of
neural cell types generated, having an expanded upper layer generated at the expense of deep layers. In the loss of Sip1 signaling a premature end of neurogenesis paves the way for an enhanced proliferation of glial precursors and gliogenesis. Additionally, Fgf9 was found to be possible direct target gene of Sip1. To decipher whether Fgf9 is important during gliogenesis it was precociously up regulated, which resulted in a premature specification of glial precursors. Further it was found that the primary source of Fgf9 is within the cortical plate, believed to be controlled by the expression of Sip1 through a feedback signal (Seuntjens et al., 2009).

The switch from a neurogenesis to a gliogenic state has been proposed to occur in different ways i.e. either through reduced levels of proneural genes, such as Neurogs (Sun et al., 2001), or by activation of proglial genes, such as NFIs (Deneen et al., 2006). In addition, it was recently found that the vertebrate svp orthologue Coup-tfl/II, seems to regulate the neurogenic\rightarrow gliogenic switch, where down-regulation of Coup-tfl/II within cortical progenitors allow these to respond to proglial cues and promote the expression of the astrocytes specific gene GFAP. These results seem to be in line with results found in Drosophila where svp mutants show an increase in the number of early born cells (Kanai et al., 2005; Naka et al., 2008). Furthermore, Cardiotrophin1 and BMP2/4, use similar mechanisms to induce gliogenesis. However, since Cardiotrophin1 is released already during early phases of neurogenesis a major question has been why these cues do not induce gliogenesis earlier, but somehow they seem restricted in their competence to respond to proglial cues. An important study revealed that many of astrocytic genes are kept epigenetically silenced, illustrating that histone modifications are important mechanism to restrict stem cell competence (Okano and Temple, 2009; Takizawa et al., 2001).

A number of studies demonstrate that vertebrate neural progenitors, in similarity to Drosophila NBs, change their competence intrinsically to generate different types of neurons and glia over time. Importantly, a given temporal window does not specify a given fate, but rather integrates with other cues to further activate downstream cell fate programs. Studies suggesting that late-born neurons can be specified before the previous temporal window has been down-regulated could suggest that temporal progression are more dynamic and flexible in vertebrates, and that potentially parallel temporal programs act together to specify neuronal sub-types. Since only a
few temporal genes has been indentified in *Drosophila*, the presence and function of similar cues within vertebrates remains elusive. However, already two mammalian orthologs of the *Drosophila* temporal genes (ikaros and COUP-TF1/2) have been found to play temporal roles. In addition, there are several candidate genes that may play such roles, such as members of the SRY-box containing family (SOX2/5/6), which have been proposed to play temporal roles, as well as other Hunchback orthologs (*aiolos, helios, eos* and *Pegasus*). Thus, it is likely that temporal cascades are utilized in mammals as well, but that they may indeed be more plastic and dependent upon extrinsic cues.

**Neuroblast 5-6 and the Ap cluster model**

In the *Drosophila* VNC, expression of the LIM-HD protein Apterous (Ap, vertebrate Lhx2/9) is restricted to only 3 cells within each hemisegment. One cell located dorsally (dAp), and two located ventrally (vAps). In addition, there are four Ap expressing cells (5 in T1), Ap1/NpLp1, Ap2, Ap3 and Ap4/FMRFa, located ventrally and laterally in each thoracic hemisegment (Fig. 26). Because of their Ap expression, this cluster is referred to as the Ap-cluster. Each Ap cluster is derived from a specific progenitor cell, the thoracic neuroblasts 5-6 (NB 5-6T) Studies presented in paper I-III have all been revolving around the NB 5-6T and the Ap cluster neurons within the *Drosophila* model system.

Most Ap neurons are interneurons and project their axons toward midline where they will fasciculate with their homologues, ending up forming medial axon bundles. In contrast, one of the Ap cluster neurons, the Ap4 neuron, will exit the CNS and innervate a gland, the dorsal neurohemal organ (DNH) which is believed to serve as the release site of the neuropeptide FMRFa (Benveniste et al., 1998). The diverse features seen within this peculiar cluster, e.g. the morphology and axon projection, unique expression of certain TFs and neuropeptides and the fact that these four cluster cells emerge from the same NB makes this model highly interesting to use when studying neuronal differentiation and specification of specific cell fates.
Noticing a highly restricted expression pattern of Ap, studies followed trying to decipher the role of the LIM-HD proteins as cell fate determinants. The work took off in the lab of John Thomas, when they noticed that \( ap \) mutants, although the Ap neurons are generated and present, display aberrant axon pathfinding, indicating that Ap play a late role in the process of specifying these cells (Lundgren et al., 1995). This launched a series of continuing studies that identified several other regulators each one playing unique and important roles specifying the Ap neurons. These include Eyes absent (Eya; vertebrate Eya1-4), Dachshund (Dac; vertebrate Dach1-2), Chip (vertebrate NLI), the zinc finger TF Squeeze (Sqz; vertebrate CIZ), the bHLH TF Dimmed (Dimm; vertebrate Mist1) and TGFβ/BMP signaling (Allan et al., 2005; Allan et al., 2003; Hewes et al., 2003; Marques et al., 2003; Miguel-Aliaga et al., 2004). Additionally, two of the four Ap cluster neurons are peptidergic. The Ap4 neuron express the myomodulator FMRFamide (Schneider et al., 1993), while the Ap1 and dAp neurons express the neuropeptide Nplp1 and the dopamine D1 receptor (DopR) (Park et al., 2004, PaperI). With an aim to clarify each role of above mentioned factors specifying these neurons they will be discussed separately in the following sections.

**Apterous**

Having show that \( ap \) itself is not required for the actual generation or survival of the Ap cluster cells, it did affect dAp to fasciculate proper axon pathfinding. However, \( ap \) mutant does not affect the axon pathfinding projecting from the Ap4 neuron (Allan et al., 2003). Further, in \( ap \) mutants a loss of the peptidergic determinant Dimm can be observed, especially within the Ap1/Nplp1, dAps and T1 Ap cluster.
Also, a difference in expression of FMRFa could be seen where it was commonly lost in the T1 cluster, while less affected in T2/T3 cluster (Allan et al., 2005). Having identified the Tv neuron specific enhancer for FMRFa, and found three Ap binding sites, this argues for a direct regulation FMRFa. Furthermore, having all these three sites mutated and still observing ~50% of the lacZ reporter, the idea arose that other transcription factors must be at play (Benveniste et al., 1998). Ap, being a LIM-HD protein, has been show to interact physically with the co-factor Chip, also proven to play a role activating the Ap4/FMRFa neuron-specific enhancer (van Meyel et al., 2000).

**Squeeze**

Having studied the expression of the zinc-finger gene *squeeze* (*sqz*, mammalian *CIZ*) and seen that it was only restricted to the Ap cluster, within the Ap neuron compartment, an interesting follow up to the previous work became to investigate the role of *sqz* and *ap* in relation to each other and on their affect upon the Ap4/FMRFa neuron. In *sqz* mutants expression of FMRFa is reduced, especially within T1 clusters, probably due to a frequent axon pathfinding failure. Being able to restore FMRFa expression rescue experiments with *apGAL4* clearly suggest a late post-mitotic role of *sqz*. Additionally, the *ap*;*sqz* double mutants reduce the number FMRFa expressing cells, as well as the actual level of the FMRFa expression. The fact that the double mutant showed a much more reduced expression level compared to single mutant backgrounds, combined with the fact that they ectopically can activate FMRFa expression suggest that *ap* and *sqz* can work together combinatorially. Additionally, the fact that *sqz* alone can activate FMRFa when misexpressed suggest a direct function upon the FMRFa enhancer (Allan et al., 2003).

Finding that *sqz* and *ap* function post-mitotically in a combinatorial and cell-autonomous fashion to trigger ectopic FMRFa expression from three different drivers (*apGAL4*, *VaGAL4* and *VapGAL4*) expressed in neuronal subsets that all exit the VNC led Allan and colleagues to explore the idea regarding extrinsic target-derived signaling. Noticing that in a *tinman* (*tin*) mutant the development of the DNHs fails, resulting in failure of Ap4 axon projection and as a consequence display a sever loss of FMRFa which suggests that an extrinsic signal is vital for proper FMRFa expression.
Noticing an expression of the phosphorylated Receptor Smad protein Mad (pMad), a downstream effector of Drosophila BMP signaling, Allan and colleagues clarified the importance of a target derived BMP signal showing the importance of a proper axon pathfinding and axonal transport. Misexpressing roundabout (robo), a receptor that mediates repulsion from the VNC midline, and a dominant-activated form of rac which stall axon progression, led to an almost complete loss of FMRFa expression. Clarifying the importance of proper axonal transport, a dominant-negative version of the P150/Glued dynactin motor component was expressed, shown to specifically interfere with retrograde axonal transport. The misexpression resulted in a complete loss of FMRFa-lacZ expression. Furthermore, they found this target-derived signal, mediated by the Glass bottom boat (gbb) BMP ligand acting upon the Wishful thinking (Wit; the type-II BMP receptor), to phosphorylate, and result in nuclear translocation, of pMad, which further is important for the activation of the terminal differentiation gene FMRFa. This was the first case ever demonstrating that an extrinsic cue is integrated with an intrinsic TF setup to specify a final cell fate (Allan et al., 2003).

Previous results led to interesting questions: How complex are these combinatorial codes? How many regulators are required and how important is each component individually to in the end contribute to the final neuronal identity? In a study conducted by Miguel-Aliaga and colleges they identified additional regulators expressed within the Ap cluster; the two co-factors: Dachshund (Dac) and Eyes absent (Eya).

Dachshund

In the Ap cluster neurons Dac is first seen within all four Ap cluster neurons, but is quickly down-regulated in one neuron and ends up being expressed in three out of four Ap cluster neurons at stage 16. Using the c929-GAL4 line, which is restricted to peptidergic neurons, in combination with FMRFa-lacZ it could be concluded that Dac is down regulated within the Ap1/Nplp1 and dAp neurons. dac encodes a transcriptional co-factor that has been intimately linked with the transcriptional co-factor Eyes absent (Eya), which was found solely to be expressed within Ap neurons after stage 16. Dac and Eya were found to overlap within the Ap2, Ap3 and
Ap4/FMRFa neurons. Noticing a reduction in the actual levels of FMRFa expression, which further could be rescued reintroducing dac from \textit{ap\textsuperscript{GAL4}}, supports a cell-autonomous role for \textit{dac} in controlling expression of FMRFa in the Ap4 neuron (Miguel-Aliaga et al., 2004).

Eyes absent

By contrast, in \textit{eya} mutants, FMRFa expression was severely reduced and also showed a reduction in Dac expression in either A2 or Ap3. In addition, noticing a more severe reduction of FMRFa expression in an \textit{eya} mutant combined with the \textit{ap\textsuperscript{GAL4}} allele, this indicated a genetic interaction. Additionally, \textit{eya} mutants display a severe pathfinding phenotype and as a consequence also a nearly complete loss of pMad in the Ap4/FMRFa neurons. Doing rescue experiments, using \textit{ap\textsuperscript{GAL4}}, gave a nearly perfect rescue of FMRFa expression. This indicates that \textit{eya} plays a cell autonomous function in controlling axon pathfinding. Trying to reintroduce active BMP signaling in the Ap4/FMRFa neuron in an \textit{eya} mutant, the expression of pMad was only partly restored, indicating that Eya plays a function between the BMP receptor Wit and pMad, perhaps by regulating the expression of the receptor itself. Additionally, expressing an activated BMP type 1 receptor, even though pMad is restored, the expression of FMRFa still remains inhibited suggesting a downstream role of Eya either by acting direct down on the FMRFa enhancer or acting through an intermediate (Fig. 27) (Miguel-Aliaga et al., 2004).

Figure 27

Eya play an important role controlling axon pathfinding. However, studies suggest that Eya plays several roles. Either a function between the BMP receptor Wit and pMad, or in a more direct fashion, on the FMRFa enhancer.
Dimm

A transcription factor that has been shown to act as a master regulator of the peptidergic fate is the bHLH TF Dimm. Mapping the expression of Dimm in larval CNS, it was found to be expressed in 306 cells. Furthermore, a substantial correlation was seen where most, if not all, peptidergic phenotypes also expressed the Dimm TF (Park et al., 2008). Dimm is not vital for the survival of the peptidergic neurons; instead a recent study revealed that Dimm coordinates a gene expression program which induces several essential features, all important for storage, cleavage, and exocytosis within peptidergic neurons (Hamanaka et al., 2010). In fact, dimm mutants, display a reduced level of mRNA levels of a number of the neuropeptide processing enzymes, such as peptidylglycine-α-hydroxylating mono-oxygenase (PHM) and Furin 1 (Fur1). These results show that dimm lie upstream of several factors necessary for the peptidergic cell fate. Studies in dimm mutants reveal that in the addition to PHM and Fur1, several neuropeptides, such as FMRFa and Leucokinin is also severely reduced (Hewes et al., 2003). In addition, studies by Allan and colleagues showed that by misexpressing dimm pan-neuronally using an elavGAL4 driver they could ectopically activate PHM expression in most, if not all VNC neurons (Allan et al., 2005). Showing that expression of dimm is vital for proper expression of FMRFa led to further studies incorporating already known factors involved in Ap cluster specification: dac, eya, ap, sqz and BMP signaling (Allan et al., 2005).

This study addresses the issues of: How complex can combinatorial codes be, and how important are each component individually to the final neuronal identity? Findings outlined above focusing of the specification of the Ap cluster shows that each and every one play distinct roles when it comes to specify the Ap cluster neurons. However when conducting gain-of-function experiments it is clearly pointed out that they individually has a restricted capacity to activate ectopic FMRFa. Importantly, even though they are expressed individually in a broader context they are not able to specify the FMRFa producing cell fate. Rather it seems that these combinatorial codes expressed in combinations of two, or preferably, three that can only do their work and achieve the task to respecify neurons into FMRFa expression neurons when the proper intersection between them is fulfilled (Allan et al., 2005).
Having found potent combinatorial codes, questions emerged regarding the nature of this combinatorial code versus each individual regulator. Can factors within the combinatorial code play independent roles outside the code, within the same neuron? Focusing on \textit{ap}, \textit{sqz} and \textit{dimm}, Allan and colleagues dissected this question. While \textit{dimm} acts independently to dictate the peptidergic fate through the activation of PHM, \textit{sqz} alone acts to control the cell number of \textit{Ap} cluster generate in TI segment. Additionally, \textit{ap} controls axon pathfinding independently of \textit{dimm}, clearly showing that these factors serve several functions outside the code activating the expression of FMRFa.

So how is all this reflected at a molecular level? Within the FMRFa enhancer a 446 bp enhancer fragment has been identified (Benveniste and Taghert, 1999). This enhancer fragment carries putative binding sites for Dimm and Sqz, in addition to the Ap binding sites mentioned previously. Furthermore, Dimm and Sqz have both been shown to interact strongly with Ap, however not with each other (Allan et al., 2005). This data supports a model in which Ap, Chip, Sqz, and Dimm physically interact in a combinatorial transcriptional complex, binding directly to a particular fragment of the \textit{FMRFa} gene enhancer.

In summary, the important findings being highlighted above enables clarification of concepts like intrinsic vs extrinsic cues and master regulator vs combinatorial coding. Additionally having been able to identify several TFs, \textit{eya}, \textit{ap}, \textit{dac}, \textit{dimm} and how they are affected in relation to each other a regulatory genetic cascade between these can now be outlined (Fig. 28).

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{Figure28.png}
\caption{The regulatory relationship between the identified Ap neuron determinants. See above for details.}
\end{figure}
Aims

My PhD project has solely revolved around the NB5-6 lineage and the Ap cluster, with the aim of gaining deeper knowledge in neuronal subtype specification. How do these four Ap cluster neurons emerge throughout development? Why are they only present within the three thoracic segments? The questions regarding the generation and specification of these Ap neurons made us focus mainly on the following major questions: First, how are neural stem cells spatially programmed to generate a varied lineage size throughout the CNS?, and second: How do neural stem cells change their competence to in the end develop a diverse progeny throughout development?

In order to find an answer to the questions mentioned above we needed to determine the lineage that generates the Ap cluster. This neuroblast turned out to be NB5-6, and it turned out to be a superior model lineage due to a number of characteristics. First of all, NB5-6 generates a rather large and diverse lineage which enables us to study how the “competence” is changed throughout development in order to generate different types of progeny. Second, the NB5-6 generates a difference in lineage size depending on where throughout the CNS it is located, having a larger lineage in the thoracic region compared to the abdominal area. Additionally, applying the knowledge already gathered revolving the Ap cluster neurons, together with the intense studies around Hox genes and the temporal gene cascade have resulted in applicable antibodies, transgenic lines and fly stocks which in the end make NB5-6 a highly interesting target for our studies.
Material & Methods

The results described in paper I-III have focused on the *Drosophila* embryonic development of the central nervous system, where different genotypes has been dissected and analyzed. Immunohistochemistry, different reporter constructs (GFP, RFP, lacZ) or *in situ* were used in combination with confocal microscopy to analyze developmental effects.

In loss-of-function (LOF) experiments, the effect of losing one or several genes has been analyzed. Depending on what kind of mutation the gene has, these mutations can vary in severity. Using amorphic alleles, which we did where possible, these alleles display a complete loss of gene function where the mutation can cause loss of protein function by affecting the actual transcription (no RNA) or the translation (no protein). In addition with an amorphic allele we routinely used it together with a “deletion” thus creating a hemizygous genetic background assuring that no gene product was expressed. Deletions, in which larger regions covering not only the gene in question but also nearby areas, were often used when available and appropriate. LOF experiments can also be caused by RNA interference, often creating a number of ingenious applications, or through the presence of dominant negative genes, i.e. antimorphs.

In gain-of-function (GOF) experiments, a gene of choice is expressed in a context where it is normally not found. The purpose of such experiment is most often to serve as a complement and work as a proof-of-principle in addition to results gained in LOF experiments. GOF experiments are done using the binary Gal4/UAS system. Gal4 is a transcription factor which will start to be transcribed when its enhancer or promoter will be activated within a given context. The Gal4 product will diffuse away and bind to an upstream activating sequence (UAS), which will drive the expression of a gene of choice. This opens up an array of possibilities. Depending on where, when or how the promoter is activated a given gene can be expressed in a wide variety of spatial and temporal compartments.

One last type of experiment that I would like to emphasize is “rescue” and “cross-rescue” experiments. In short, this is a LOF and GOF experiment in one, where a
gene is ectopically expressed in a mutant background. This type of experiment can be used in many different ways and are very informative, especially when studying genetic cascades. This is the ultimate test to know if the certain mutant phenotype is indeed caused by the LOF of a gene of interest. If this is the case, reintroducing this gene will rescue the phenotype. Additionally, it becomes possible to nail point when a specific gene plays out a specific function. For example, a gene might play several functions, one early and one late, within the same cell. By introducing the gene at a late time point, by using a Gal4-line activated late, the late function might be rescued. Furthermore, envision having found several genes which are believed to work in a genetic cascade, e.g. $a \rightarrow b \rightarrow c$. Is gene $a$ only important in activating gene $b$, or does gene $a$ work in a feed-forward kind of manner in a combined effort with gene $b$ to activate gene $c$? Questions like these can be sorted out performing so called “cross-rescue” experiments. By driving gene $b$ in an $a$ mutant background and successfully activate gene $c$, tells us that in order to activate gene $c$, gene $a$ is not necessary in this decision which play out its function early, only activating gene $b$. Above mentioned scenarios make rescue experiments very informative.
Results & Discussion

Paper I

Background
Having identified several regulators important for proper Ap cluster, and to some extent also for dAp specification, these findings however opened up new unresolved questions. One intriguing question revolved around how these Ap clusters neurons are generated. The proximity, expression of similar TFs and the fact that they appear during the same time period suggested that they should all belong to the same lineage. One important question thus remained: which NB lies behind the generation of the Ap cluster? Having noticed a sequential expression of ap and sqz, later followed by dimm ultimately leading to correct specification of the Ap4/FMRFa and Ap1/Nplp1, which also turned out to be peptidergic— even though the neuropeptide expressed by this neuron at this time was unknown – presented us with a unique opportunity to decipher how this specification process is executed. How can it be that four related neurons within the same lineage are generated, share several differentiation factors, but in the end display very different terminal identity? The additional factors found, (Miguel-Aliaga et al., 2004), presented us with the rare opportunity to investigate the potency of these additional factors in activating FMRFa, but also as it turned out, the activation of the second neuropeptide Nplp1. This would also bring important knowledge to what extent these different codes are combined and separated in activating their target genes.

Principal findings
The completion of the Drosophila genome sequencing led to the prediction of several additional neuropeptide genes. Expression of one of these turned out to lie in close proximity to the Ap cluster. In situ could indeed confirm that the neuropeptide gene Nplp1 were expressed within the Ap1/Nplp1 and the dAp neurons. Furthermore, we also identified the dopamine receptor (DopR) to be expressed within the Ap1/Nplp1 neuron. These results now confirm that the Ap cluster consists of two peptidergic neurons that both express the TF dimm and that the Ap1 and Ap4 can be separated by the fact that they express different neuropeptides: FMRFa and Nplp1. Analyzing
ap, eya and dimm mutants showed that these factors are not important for Ap4/FMRFa specification, but also play important roles specifying the Ap1/Nplp1 neuron. So what factors separate these two neurons from each other allowing them to adopt different peptidergic fates? The involvement of the conserved COE family genes plays important roles during CNS development. The only member found in Drosophila, collier (col) and its expression within the developing embryonic CNS, prompted us to investigate the possible role of col during Ap cluster specification. Indeed, analyzing col mutants, we found a complete loss of ap^{lacZ}, eya and dimm, and hence also a total loss of the expression of Nplp1 and FMRFa. Importantly, the Ap cluster neurons were still being generated and also expressed dac and sqz^{lacZ}.

Analyzing the expression of Col within Ap cluster neurons revealed a dynamic expression pattern were Col at stage 15 is expressed within all Ap cluster neurons, but later down regulated to only be expressed within the Ap1/Nplp1 neuron at stage 16-17. This made us hope that Col perhaps is expressed already within the progenitor, allowing us to track a potential NB that give rise to these clusters. Taking advantage of mapped NB morphologies and gene expression patterns (see section The Drosophila Central Nervous System) we were able to identify NB5-6 and map the expression of Col to emerge, maintain and be down regulated in Ap2, Ap3 and Ap4/FMRFa between stage 13-16.

Having identified several factors, Col, Eya, Ap, Dimm and being able to order these in a simple genetic cascade we wanted to decipher how each and every one fit within this order and if they work together. Performing a series of loss- and gain-of-function studies it became clear that Col must have several roles to play out since a cross-rescue experiment, driving eya/ap within a col mutant, even though Ap cluster neurons were rescued back, they only expressed FMRFa and dimm, while the expression of Nplp1 was missing. This indicated that ap/eya can partially rescue the Ap cluster neuron fate, but in lack of col these neurons do not activate the neuropeptide Nplp1. We were able to show that col acts on three different levels. First, col acts within all four Ap neurons to activate the expression of eya/ap. col here after works in a feed-forward loop (FFL) to activate the expression of dimm. Finally, Col is maintained in the Ap1/Nplp1 neuron, and we here showed that the maintenance actually plays a late role in activating the Nplp1 neuropeptide gene
through a final FFL. For instance, knocking down Col late in the larvae, using RNAi, the expression of Nplp1 was lost.

Having found that *col, ap, eya* and *dimm* work in a collaborative, rather than in a linear way to activate Nplp1, suggested that these regulators build up a core to specify the Ap1/Nplp1 cell fate. Additionally, having found that *ap, eya, dimm, sqz* and *dac* in combination with the TGFβ/BMP signaling is important for the Ap4/FMRFa cell fate led us to the idea of combing these factors in new combinations and performing GOF experiments. What we found was that these codes seem to act in a very instructive and specific way. By adding the factor Col or Dac to the general Ap/Dimm code, each one being important in specifying the Ap1/Nplp1 and Ap4/FMRFa neuron, a dramatic increase in neuropeptide expression was seen. Not only was there a robust ectopic expression of the terminal differentiation genes, but to our surprise they also showed very little cross-reactivity, indicating that these codes are not only instructive, but also very specific. Interestingly, noticing strong ectopic expression of FMRFa and Nplp1 throughout the whole anteroposterior-dorsoventral- and mediolateral axis reflects the potency in activating these terminal differentiation genes without any boundary restrictions.

**Conclusion & Discussion**

Within this paper we made some important findings. First we identified the neuropeptide Nplp1 to be expressed within the Ap1/Nplp1 neuron. Additionally, we identified a new regulator, col, to be expressed early with all four neurons, but later to be found only within the Ap1/Nplp1. Importantly we found that col act on several different levels in the specification of these lineage related neurons. Firstly, Col is found to activate the expression of the Ap cluster generic TFs Ap and Eya. Secondly, Col together with ap/eya works to activate the expression of *dimm* in a FFL. An interesting notion here is that col is needed a final time to activate the expression of the neuropeptide Nplp1. This shows how a single regulator can work at several different hierarchical levels during the specification of a number of lineage related neurons. Additionally, due to the early expression of col we were able to identify that the Ap cluster neurons emerge within the lineage of the thoracic NB5-6.
The finding that the combinatorial codes, just by switching one factor, Ap/Dimm/Dac and Ap/Dimm/Col, become highly specific as well as very potent illustrates that only three factors is, in a way, enough to secure a certain level of specificity. However, this finding also suggests that, because of their potency, the factors taking part in a code probably are restricted in their expression pattern. Indeed, this seems to be the case with Col since it is quickly down regulated, while others are added to provide specificity to the generic code. Is this a common way in vertebrates to achieve a high cellular diversity? This is very likely, since the number of cell types greatly surmounts the number of transcription factors, making combinatorial codes a natural step in achieving cell type diversity. Do vertebrate combinatorial codes involve more factors? Perhaps, but in addition, having a much more refined way of utilizing non coding DNA and modulate global chromatin structure the expansion of the gene families encoding the subunits participating in the chromatin remodeling complexes, and their combinatorial way of regulating gene transcription, may have been another important step to take towards the extraordinary diversity of cellular subtypes seen within the vertebrate nervous system today. When considering two different contexts where combinatorial codes are used, a chromatin remodeling complex and a transcriptional combinatorial code, an almost endless cellular diversity can be envisioned.

**Paper II**

**Background**

Important advancements were taken in paper I where the finding that the conserved COE family gene *collier* was found to be necessary for the Ap cluster specification. The expression of *col* also made it possible to identify the thoracic NB 5-6 giving rise to these four Ap cluster neurons. Further, *col* was shown to be necessary for the expression of Ap/Eya in all Ap cluster neurons, which together with *col* activate the peptidergic fate determining gene *dimm* in the Ap1/Nplp1 and Ap4/FMRFa. A down regulation of *col* proved to be necessary in order to specify the Ap4/FMRFa fate, while it had to be maintained within the Ap1/Nplp1 neuron in order to participate in a second FFL together with Ap/Eya/Dimm to activate the *Nplp1* gene. These results boiled down to one important question: How is the expression of Col down regulated in time in order for a proper Ap cluster specification? In addition to
these studies, we became interested in the temporal genes and how they are expressed within NB5-6 lineage. Utilizing a NB5-6-specific transgenic marker (*lbe(K)-lacZ*, *lbe(K)-Gal4*) (De Graeve et al., 2004) this presented us with a unique opportunity to for the first time study temporal transitions within a relatively large lineage. While most lineage mapping studies so far had only been focused on smaller lineages, often with narrow temporal windows, this together with a now deep understanding of a parallel expression pattern of specific TFs and terminal differentiation genes opened up an opportunity studying the importance of temporal transitions throughout lineage progression.

**Principal findings**

An important initial step was taken mapping the NB5-6 lineage, using the NB5-6 specific lineage markers, which resulted in a detailed overview of how the lineage progressed and the temporal transitions seen within it. The NB5-6 delaminates at stage 8 and generates a lineage consisting of 20 cells. The temporal progression follows the canonical temporal gene cascade where the NB5-6 ends with a large Cas+ window consisting of 10 cells, where the four last cells also express Grh. Going through a number of GMC producing cycles, the NB suddenly stops producing GMCs at stage 13, and instead starts generating neurons directly, subsequently producing the four Ap cluster neurons, where Ap1/Nplp1 is generated first followed by the Ap2, Ap3, and Ap4/FMRFa neurons. These four cells are also the last cells born, before the NB enters apoptosis at stage 16.

*Cas* mutants showed a total loss of several Ap cluster regulators, such as Col, Dimm, *apnlacZ*, Dac, *sqz lacZ* and as a consequence also a loss of the terminal differentiation genes FMRFa and Nplp1. Importantly, the lineage progressed, and even though Eya were still seen within the lineage is was slightly deregulated. In addition, *grh* mutants displayed a reduced expression of Dimm, pMad and FMRFa, all typical signatures of the Ap4/FMRFa cell fate. This suggested that *grh* plays a more specific role within the Ap4/FMRFa neuron, which considered being an enigma, since Grh is expressed in all four neurons. As it turned out, the ability for *grh* to play out it function lies in the actual levels of Grh, from a weak expression in the first born Ap1/Nplp1 neuron with an increasing expression towards the last cell being born,
which is the Ap4/FMRFa neuron. Misexpression of *grh* led to expression of FMRFa in all four Ap cluster neurons.

The interesting finding that misexpression of Col in a *cas* mutant background caused a severe Nplp1, but no FMRFa expression suggested that Col in an important regulator of the Ap1/Nplp1 cell fate. The fact that Col is being quickly down regulated within the NB soon after the Ap1/Nplp1 neuron has been born suggests, combined with misexpression of *col*, that *col* plays an important function regulating the Ap1/Nplp1 cell fate. So what mechanisms act within the NB to quickly down regulate Col before Ap4/FMRFa neuron is generated? The answer to this question came when studying the expression of Col in *sqz* mutants where a prolonged expression of Col is seen. This suggested that *sqz* plays an important role down regulating Col, but how is this achieved? Since Sqz is expressed within all four Ap cluster neurons, why is not Col down regulated before the Ap1/Nplp1 neuron is even generated? The answer to this question relied within the previously studied co-factor *Nab* (Clements et al., 2003) which is expressed within the NB after the first neuron, Ap1/Nplp1, has been generated. Together Sqz and Nab, now allowed to form a complex, act to repress the expression of Col. In this way Col is able to perform its function within the Ap1/Nplp1 neuron, but down regulated in time for the other neurons to be specified in an appropriate way.

**Conclusion & Discussion**

Several findings from this paper need to be highlighted. Firstly, mapping and performing LOF analysis of the temporal genes it became clear that temporal genes play an important part specifying these Ap cluster neurons. Secondly, an interesting finding was that the lineage progression seemed to continue beyond the point where it normally would undergo apoptosis which suggests that the final temporal genes play a role making the decision to leave the cell cycle and undergo apoptosis or enter quiescence.

The finding that the thoracic NB5-6 lineage contains wide temporal windows tempted us to study how these large windows are subdivided, promoting neuronal progenitors to achieve a higher neuronal diversity. This study illustrated how a single temporal gene, *cas*, activates two different feed-forward loop. First, *cas*...
activates the FFL col→ap/eya→dimm→Nplp1. When activating Col expression, cas at the same time activates sqz. When the Ap1/Nplp1 buds of the NB, this will activate the expression of Nab, which together with Sqz probably forms a dimer, and repress the expression of Col. Since Col in the end will be down-regulated anyway, before the specification of the Ap4/FMRFa neuron, this implies that other Col-repressive mechanisms must be at play as well, the first initial down regulation is probably meant to inhibit the expression of Col in the Ap2 neuron, which would be re-specified into a Ap1/Nplp1 neuron if col was allowed to be expressed.

So what is the purpose of using these FFLs throughout lineage progression? For a temporal gene to divide its sometimes large temporal window, we have proposed that they can activate so called “sub-temporal” genes which we define by the fact that they act downstream of, and do not regulate temporal genes. Additionally, they act to divide larger temporal windows and act to specify very restricted subtypes of neurons. Do these temporal mechanisms also take place in other neural stem cells, in other organisms? Considering that emerging evidence points to that temporal specification of neurons is a widespread phenomenon in many regions of the developing mammalian CNS, and considering the fact that vertebrate neural progenitor lineages tend to be quite large only adds to the possibility that sub-temporal mechanisms, similar to those found in this paper, will be found in a near future in vertebrates as well.

**Paper III**

**Background**

Many neural progenitor change their potential to generate specific progeny over time, in addition they are also topographically organized in response to the expression of distinct combinations of Hox genes, which also give rise to specific cell types throughout the neuroaxis. Having found that the lineage of the thoracic NB 5-6 is larger than the one found in the abdomen, together with the fact that NB5-6 equivalents also had been found in the three suboesophageal and the three brain segments, we became interested in how lineage progression display itself throughout the A-P axis, knowing that the Ap cluster neurons only are present in the three thoracic segments.
As outlined before, we envisioned three different scenarios giving rise to this segmental difference. (I) The NB5-6 generates the same number of progeny throughout the whole neuroaxis, but a portion of the lineage – including the Ap neurons – are removed through PCD. (II) The NB5-6 generates the same number of neurons in all segments, but the neurons tend to be specified differently. (III) Furthermore, the NB5-6 could remain or leave the cell cycle earlier or later depending on where it is located. Hence the lineage will end up being different in size depending on if it is located in the brain, thorax or abdomen.

The newly gained temporal knowledge, the valuable lineage specific marker together with already found differentiation genes presented a unique opportunity to address the question of how progeny from a specific neural progenitor is modified along the whole A-P axis.

**Principal findings**

Because of previous studies, showing that the abdominal lineage is smaller (Schmid et al., 1999; Schmidt et al., 1997), we conducted a detailed mapping comparing early expression patterns of Hox and temporal genes in both the thoracic and abdominal NB5-6 lineage (NB5-6T, NB5-6A). Initially the NB5-6A follows the same temporal expression pattern as NB5-6T, however during stage 12, when reached the pdm temporal window, NB5-6A stops dividing. Examining the H99 mutant background (see section: Programmed Cell Death induced by Hox activity) we noticed no excess lineage progression, suggesting that the smaller lineage seen in NB5-6A, when compared to NB5-6T, is not the result of apoptosis, but rather an earlier cell cycle exit. Hence, the Ap cluster neurons are never generated from the NB5-6A. Mapping the expression of Hox genes, we find Antp and the Bx-C members (Ubx, abd-A and Abd-B) to be expressed at stage 12 within each respective expression domains, and are seen within the lineage throughout development. The two co-factors start to be expressed prior to Hox gene expression and found within the NB5-6 lineage at stage 11. Interestingly, a sharp increase in Hth levels suggests that it could play dual roles depending on levels present.
Results & Discussion

Focusing on the thoracic area it was shown that in Antp, hth and exd mutants a complete loss is seen of the following Ap determinants: Col, ap lacZ, Eya, Dac and Dimm, as well as the terminal identity markers; the neuropeptides Nplp1 and FMRFa. Importantly, the lineage was still generated. Due to the known fact that Hox proteins and co-factors often work together we saw this as a unique opportunity to see if their primary role is to activate col expression. Cross-rescue experiments showed that while misexpression of col within a hth mutant background could rescue hth mutants. This was not the case in an Antp mutant background, suggesting that while the primary role of hth is to activate Col, Antp plays additional functions specifying the Ap neurons. Having found a sharp increase of Hth expression prior to Col expression, this prompted us to misexpress hth, examining a potential instructive role. Strikingly, when misexpressed using the early lbe(K)-Gal4, a robust premature expression of Col was seen, consequently leading to additional Ap cluster neurons being specified.

Since the Bx-C and Pbx/Meis cofactors are expressed during a critical time during NB5-6A lineage progression, we studied Ubx, hth and exd mutant backgrounds and focused on the A1 segment, utilizing the lineage marker lbe(K)-Gal4. In contrast to wild type, where cas and col are not expressed within abdominal NB5-6 lineages, in Ubx, hth and exd mutants, the NB 5-6A lineage continues to progress, generating a larger cell number and expression of Col and Cas. This clearly shows that the lineage continues going through cell cycles, enters an additional temporal window, and starts to expresses the NB5-6T gene col.

Even though Bx-C, hth and exd mutants display an increase in NB5-6A lineage cell numbers, approaching the number normally seen within the NB5-6T, as well as ectopic expression of Cas and Col, they still display very different phenotype when analyzing these mutant backgrounds with late Ap differentiation markers. Results suggesting that hth may play dual roles; acting early in the abdomen to inactivate lineage progression, and a late specifying role, promoted us to misexpressing hth late in a hth mutant background. As expected, the neurons now being generated in the mutant background, entering the Ap cluster window, is also specified to differentiated Ap neurons due to the late role of hth. Doing the opposite, rescuing hth back at an early stage, hth was allowed to play out its other role in shutting of the
cell cycle. Hence, the NB5-6A lineage will never generate the Ap cluster neurons, which will therefore never be specified.

Interestingly, analyzing the NB5-6 lineage in anterior segments we found that in contrast to abdominal lineages, anterior ones actually do enter the cas window. The reason why these cells become specified into Ap neuron turned out to be due to the lack of Antp. Misexpression of Antp led to a respecification of these anterior NB5-6 lineage cells, now starting to express \( \text{ap}^{\text{proZ}} \), Eya and Nplp1. A failure in activating FMRFa, combined with the finding of low expression level of the temporal factor Grh, led us to perform a co-misexpression of \( \text{Antp} \) and \( \text{grh} \). Indeed, this triggered the specification of the anterior Ap4/FMRFa neuron to express the neuropeptide FMRFa. Importantly, these misexpression experiments did not change the number of cells generated within the anterior NB5-6 lineages.

These results decipher how three axial levels use three different mechanisms to modify NB5-6 lineage progression. Firstly, an early cell cycle exit, mediated by the Bx-C and Pbx/Meis cofactors, restricts lineage progression in the abdomen. Secondly, positive input from Antp and Pbx/Meis cofactors specify the Ap neuron within the thoracic NB5-6 lineage. Thirdly, the lack of correct Hox and temporal expression allow for these cells to adopt another cell fate. As an ultimate proof-of-principle we were able to generate a “thoracic CNS” by misexpressing \( \text{Antp} \) and \( \text{grh} \) in a Bx-C mutant background.

**Conclusion & Discussion**

This study allowed us to see how development of a given neural progenitor along the A-P axis is controlled by different mechanisms at different axial levels. Additionally, it also revealed several interesting features of how the Ap-cluster is specified at the thoracic level.

At the thoracic level, the NB5-6 generates the Ap cluster neurons and they later become properly specified due to expression Antp and Pbx/Meis cofactors. At abdominal levels, the NB5-6 enters an early cell cycle exit, mediated by the Bx-C member and the Pbx/Meis cofactors. In contrast to the abdomen, at more anterior levels we found large lineages, but due to improper Hox and temporal coding these
neurons never become specified into typical Ap cluster neurons. Co-misexpressing *Antp* and *grh* “reprograms” these neurons to adopt a more complete Ap cluster identity, with the expression of typical markers for the Ap cluster neurons.

The finding that *hth* mutants can be cross-rescued by misexpression of *col*, in contrast to the *Antp* mutant, shows that *Antp* plays several functions downstream of *col*. Acting within the *col*→*ap/eya*→*dimm*→*Nplp1/FMRFa* pathway, *Antp* may be involved at several different levels. The fact that several *Antp* bindings sites has been found in the enhancers of both *col* and *ap*, suggest that *Antp* first activates *col*, and then works together with Col to activate *ap*.

In addition, genetic analysis of the cofactors *Hth/Exd* suggested that they both play important roles, acting together with the Bx-C members to induce cell cycle exit within the NB5-6A. As Hth levels increase, between stage 12 and 13, prior to the expression of *col* within NB5-6T, Hth seems to function as a timing mechanism to divide the large *cas* window and induce a late Ap cluster fate. This would explain why Col is only activated late, even though the *cas* is turned on much earlier. In addition, it can be envisioned that since *hth* is expressed in broad thoracic and anterior brain segments one can envision that *hth* might play similar functions in other lineages as well. Even though this might be an often used timing mechanism, they can unfortunately be quite difficult to decipher without a clear readout assay.

Different levels of a homeodomain protein causing differences in specification process has been found once before where different levels of Cut in the da peripheral sensory neurons cause differences in dendrite pattern (Grueber et al., 2003).

Interestingly, this study allowed us to analyze anterior NB5-6 lineages, which showed that even though they are generated they are not specified into bona fide Ap cluster neurons. However, an interesting peculiarity is seen within the S3 NB5-6 lineage, where neurons are specified into expressing both *aplacZ* and Eya. Since these neurons probably are specified by the homeotic gene Scr, it illustrates that *Antp* and Scr share specific qualities specifying some but not all Ap cluster features. This observation allows for future interesting studies.
Results & Discussion

Paper IV

Background

While Hox proteins encode highly related DNA-binding factors, the function of each Hox protein in vivo is quite significant. As mentioned previously, proteins have specific domains/motifs that will interact with other co-factors proteins. These interactions will modulate the function of the protein to change its properties in different ways. How separate domains/motifs within a protein contribute to specific protein activities remains to be an important question to answer, because it could, especially for the Hox proteins, be one of several solutions to the “HoxParadox”. In this study we focused on two Bx-C members, Ubx (containing the HX and UbdA domain) and abd-A (containing the HX, TD and UbdA domain, Fig. 29). Taking advantage of different biological readouts, both phenotypic traits and direct target genes, a misexpression series of different mutated variants of abd-A, were conducted which would bring knowledge to what extent these different motifs are used. Additionally, to gain further insight into the function of the UbdA motif, the crystal structure of a Ubx-Exd-DNA complex was solved, containing the UbdA motif, a structure that had not been crystallized before. Ubx was chosen to allow for comparison with a previous studied structure, where the Ubx peptide included the HX and HD domains, but lacked the UbdA domain.

Principal findings

This study is an initial step to decipher the role that each domain play with a protein to conduct a specific function. Studying the expression of dll and Antp, seen within the thoracic segments, misexpression of mutated forms of abdA (HX, TD and UbdA, Fig. 29) lost their ability to repress. Interestingly, mutating all three of the domains did not totally restore a full repressive function, indicating that mutated abd-A
protein can still practice an inhibitory role, either by interacting through other domains or as a monomer. The fact that the repressive activity diminishes when all three domains are mutated would also indicate that they perform a repressive function in a cooperative manner. Additionally, when studying the expression pattern of wingless and dpp, mutating specific domains turned the abd-A protein, from being an activator into a repressor or vice versa.

Analyzing phenotypic traits, one of the domains, the UbdA domain, seemed to be more important than others. The repressive effect that abd-A has on the formation of the tracheal cerebral branch, seen by the expression of a Breathless (Btl) GFP reporter, was studied. Misexpression of mutated abd-A constructs revealed that the cerebral branch was impaired by the combined TD/UbdA and HX/TD/UbdA transgenic lines. However, since HX/UbdA did not show the same pattern, this would suggest a functional redundancy between the TD and UbdA domains, where only one of these has to function in order for induced impairment. In contrast, when studying the NB5-6T lineage it has been shown that by misexpressing abd-A, as well as Ubx, using the lbe(K)-Gal4 driver this is sufficient to suppress thoracic lineage progression, resulting in a smaller lineage, resembling the NB5-6A lineage (Karlsson et al., 2010). Misexpression of single, double or triple mutants, where the UbdA is included, nearly restores the lineage size to wild type. These results suggest a strict dependence upon the UbdA motif in order to suppress thoracic lineage progression.

Wanting to know how the molecular interaction surfaces differs between the Ubx/Exd/DNA complex without the UbdA motif (Passner et al., 1999), and the Ubx/Exd/DNA complex which includes the UbdA motif (UbxU/Exd/DNA) (Paper IV), the crystal structure of the UbxU/Exd/DNA complex was solved. Even though the UbdA motif has been suggested to interact with Exd (Merabet et al., 2007), this had never been shown to actually occur physically. This study suggests that the UbdA motif may interact with the cofactor Exd. Furthermore, finding that the UbxU/Exd/DNA can adopt two different conformations, which result in that the protein:DNA interactions ends up being different, proposes that this variability could provide functional diversity.
Conclusion & Discussion

This study allowed us to explore the importance of different motifs found within two abdominal Hox proteins; abd-A and Ubx. Generating a number of different transgenic lines expressing mutated forms of the abd-A protein, we found several interesting mutant features which could suggest important roles regulating gene expression, phenotypic traits as well as complex behavior.

Results suggest that specific domains encoded within a protein structure in a combinatorial way can regulate a specific feature, e.g. in the regulation of the tracheal cerebral branch and dorsal visceral musculature were a redundancy between specific domains was found. This illustrates that specific motifs within a protein can work together to regulate a specific outcome. However, not surprisingly, a strict motif dependence within specific contexts were also seen, e.g. within the NB5-6, where the UbdA motif proved to be vital for abd-A’s function to suppress the NB5-6 lineage progression within the thoracic area. Such a clear dependence upon the UbdA motif find support in the fact that an abdominal-sized thoracic NB5-6 lineage is also seen when misexpressing Ubx from lbe(K)-Gal4 (Karlsson et al., 2010). Knowing that the Ubx protein lacks the TD motif it would have been surprising to find that two Bx-C proteins use different motifs in regulating NB5-6 suppression. Another interesting aspect of how these motifs work, came from studying the expression of wg and dpp. By mutating specific motifs the protein turned either into a repressor or an activator. Because of these results one can envision that a protein either expose or hide different surfaces depending on context in order to perform certain gene regulatory activities.

Conducting studies like these, using the binary Gal4/UAS system, can turn out to be disappointing since depending on where each of the two Gal4 or UAS segment is located the efficacy in activating the UAS sequence will be influenced. Since constructs used in this study were not incorporated within the genome at the same place, e.g. by using integrase-mediated transformation, the efficacy by which these UAS constructs will be transcribed will probably fluctuate.

The UbxU/Exd/DNA complex was crystallized, which confirmed that the proposed Exd interacting motif UbdA indeed may interact with the cofactor Exd due to the
proposed proximity. However, the nature of this interaction is obscure. Do they interact through hydrogen bonds or weak van der Waal interactions? In addition, the two ways proposed for the UbxU/Exd/DNA complex to achieve conformational variations could simply be due to the way the complex crystallized. Further investigation needs to be done before a physical interaction between the UbdA motif and the cofactor Exd can be considered to occur.
Future challenges

With a clear focus, trying to sort out how a specific cluster of interneurons, the Ap cluster neurons, are specified, several important mechanisms specifying the final neuronal fate has been identified acting both in actual progenitor as well as in post-mitotic neurons. However, these results opened up new interesting angles and questions that would provide further insight into how neural progenitors regulate their progeny, both when it comes to actually specify post mitotic neurons, as well as generating them.

An interesting study to pursue would be to clarify at which levels Antp act. Having identified Antp as playing an important role initiating the expression of col, combined with the finding that misexpression of Col failed in rescuing an Antp mutant clearly suggests that Antp plays several roles downstream of col. Initially it would be interesting to find, at the molecular level, how Antp regulates col. Clearly Antp does not regulate col by itself, since hth, exd and cas mutants all display a total loss of Col as well, making col an important point of integration between spatial and temporal cues. In addition, the actual levels of Hth showed to play an instructive role activating the expression of col. A search for potential binding sites, focusing on conserved upstream regions of col, would open up to interesting results how spatial and temporal genes bind down in a common enhancer target.

In the S3 segment a cluster of neurons emerging within the NB5-6 lineage differentiate and express Eya and Ap. Why do these neurons not differentiate into becoming bona fide Ap cluster neurons, i.e. expressing Dimm and the neuropeptides? Obviously they lack proper Hox coding for bona fide Ap cluster neurons. Studies focused on the expression pattern of Col and on structural differences between the Scr and Antp proteins should lead to interesting findings. Additionally, performing domain swapping experiments would clarify the importance of specific domains/motifs for proper Ap cluster specification, hence it would be interesting to dissect and compare genetic mechanisms within a clearly defined neural lineage in the S3 and thoracic segments.
Furthermore, another interesting question deals with temporal progression and its dependence upon the cell cycle machinery and cytokinesis. Having noticed that Hb seems to be dependant of both cell cycle and cytokinesis to be down regulated, a question emerges regarding if Hb is the only temporal gene to be regulated in such a way. If not, cell cycle progression together with cytokinesis could serve as effective check points in order to control lineage progression and specification. To in the future identify and map contexts where process like these could be studied would add knowledge this enigmatic problem which could explain the difficulty producing certain cell types \textit{in vitro}. It has become clear that it is not just enough to expose stem cells to certain TFs and essential growth factors and expect neurons, muscle or skin cells of a specific nature to differentiate. Promising results, gathered both from nematodes, insects and vertebrate systems, where dissection of molecular mechanisms behind temporal switches now start to appear can only be considered to be in its prime, however knowhow to achieve certain temporal windows and reverse progressive restriction is believed to be of great significance in order to fully take advantage of tissue repair and stem cell therapy.

Another important question is how these genetic regulatory mechanisms actually are built up and allowed to be initiated, executed and, importantly, down regulated within specific progenitors and post-mitotic neurons. What allows these events to occur? When studying the \textit{Drosophila} VNC it is quite remarkable that only 6 out of 10,000 cells express the neuropeptide FMRFa. How this specificity is achieved has in part been explained by studies highlighted within this book. However, \textit{Antp, lth, exd} and \textit{cas} are all expressed in other cells, which do not express the Ap cluster determinants or the neuropeptides. This implies that the Hox and temporal cues are somehow integrated with other progenitor identity cues found within the NB 5-6, but how this occurs is unknown. Future studies regarding the upstream transcriptional regulators such as the segment polarity- and columnar genes, and how these factors act together to perhaps pave the way for a suitable chromatin landscapes would end up giving a final answer why NB5-6 generates a large lineage, specifying the unique Ap cluster neurons only within the three thoracic segments.

During recent years, even though major achievements within the field of neuroscience have been made, the need for new knowledge seems endless in order to
cure future disease or injury. Today’s neuroscientists face different challenges from those faced their predecessors. In order to understand how stem cells enter quiescence or generate a specific type of cell, an important future challenge is to continuously improve within fields such as microscopy, molecular probes and labeling techniques. Looking back throughout history the bars to what has been scientifically possible has always been set by the technology available. Indeed, being able to use the latest confocal microscopy can be compared to the first achromatic lens used by Santiago Ramón y Cajal which allowed him to, late in the 19th hundred, make significant contributions to the neuron doctrine. Ultimately, technological advances must always go hand in hand with hypothesis-driven research to make the most of the scientific process.
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