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**Genetic mechanisms controlling cell
specification and cell numbers in the
Drosophila CNS**

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Cover illustration: The Ap4 neurons enervating the dorsal neurohemal organ in the embryonic *Drosophila* CNS, visualized by FMRFa-GFP.

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“Consider the wonderful frame of the human body, this infinitely complicated engine, in which, to the due performance of the several functions and offices of life, so many strings and springs, so many receptacles and channels are necessary, and all to be in their right frame and order.”(Puckle and Longcope, 1798).

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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

Ulvklo C., Nilsson P., Bivik, C., Fransson F. and Thor S. A genetic screen for genes controlling Apterous neuron specification. *Manuscript*.

PAPER II

Benito-Sipos, J., **Ulvklo, C.**, Gabilondo, H., Baumgardt, M., Angel, A., Torroja, L., and Thor, S. (2011). Seven up acts as a temporal factor during two different stages of neuroblast 5-6 development. *Development* 138, 5311-5320.

PAPER III

Ulvklo, C., Macdonald, R., Bivik, C., Baumgardt, M., Karlsson, D., and Thor, S. (2012). Control of neuronal cell fate and number by integration of distinct daughter cell proliferation modes with temporal progression. *Development* 139, 678-689.

ABBREVIATIONS

CNS	Central nervous system
NB	Neuroblast
EGFP	Enhanced green fluorescent protein
VNC	Ventral nerve cord
GMC	Ganglion mother cell
NICD	Notch intracellular domain
NEXT	Notch extracellular truncation
ADAM	A disintegrin and metalloprotease (domain)
TACE	TNF- α - converting enzyme
bHLH	basic helix-loop-helix
HES	Hairy and enhancer of split
HESR	Hairy and enhancer of split related
BMP	Bone morphogenic protein
pMAD	phosphorylated mothers against decapentaplegic
COUP-TF	Chicken ovalbumin upstream promoter transcription factors
aPKC	atypical protein kinase C
TGF β	Transforming growth factor beta
CSL	CBF1/RBK-J κ , Su(H), Lag-1 (collective name for mammalian, <i>Drosophila</i> and <i>C. elegans</i> orthologs)
CDK	Cyclin dependent kinase
PRC	Polycomb repressive complex
NLS	Nuclear localization sequence
TAD	Transactivation domain
EGF	Epidermal growth factor

SMRT	silencing mediator of retinoic acid and thyroid hormone receptor
SKIP	Ski-interacting protein
MASH	Mammalian achaete-scute homolog
DNH	Dorsal neurohemal organ
AEL	After egg-laying

ABSTRACT

A central theme in developmental neurobiology pertains to how the diversity of different cell types is generated. In addition, it is equally important to understand how the specific numbers of each cell type is regulated. The developing *Drosophila* central nervous system (CNS) is a widely used system in which to study the genetic mechanisms underlying these events. Earlier studies have shown that a small number of progenitors produce the daunting number of cells that builds the mature CNS. This is accomplished by a series of events that in an increasingly restricted manner results in different combinatorial transcription factor codes that act to specify the different cell types in the CNS. However the factors controlling the progressive restriction in developmental potential and the ultimate fate of cells have not been completely elucidated.

My PhD project has been focused on a specific stem cell in the embryonic *Drosophila* CNS, the neuroblast 5-6 (NB 5-6), and the lineage of neural cells that is produced by that stem cell. Earlier work have provided both a lot of knowledge and a multitude of genetic tools regarding this specific stem cell, which allowed us to address these issues at single cell resolution in an identifiable lineage. In particular, a late-born group of neurons expressing the *apterous* gene, the Apterous neurons, had been extensively studied in the past. One particular Apterous neuron, Ap4, expresses the neuropeptide gene *FMRFamide (FMRFa)*, and the selective expression of this gene makes it a powerful marker for addressing many aspects of NB 5-6 development.

To identify novel genes acting to control neuronal development, a large scale forward genetic screen was performed utilizing an *FMRFa-GFP* transgenic reporter construct, thereby using a marker that reports perturbations of NB 5-6-lineage development. Flies were treated with EMS, a chemical that induces random point mutations and the progeny were screened for aberrant *FMRFa-GFP* expression. From a total of ~10,000 mutated chromosomes ~600 mutants were isolated and further characterized. One group of mutants displayed additional Apterous neurons when compared to wild type, and a number of them represented new alleles of three previously known genes: *neuralized (neur)*, *kuzbanian (kuz)*, and *seven up (svp)*. Neur and Kuz are parts of the Notch signaling pathway and Svp is the *Drosophila* COUP-TF1/2 ortholog; an orphan

Abstract

member of the steroid/thyroid receptor superfamily. These findings initiated two separate studies regarding the roles of these genes in the NB 5-6 lineage.

Mutants in the Notch pathway i.e., *neur* and *kuz* displayed an excess number of Apterous neurons, born from NB 5-6. We initiated detailed studies regarding the origin of these ectopic neurons and could show that Notch signaling is critical for controlling a switch in proliferation mode in the latter part of the NB 5-6 lineage. With this new mechanism we could independently and simultaneously manipulate cell proliferation and temporal progression, and thereby predictable control cell fate and cell numbers born from the NB 5-6.

The screen further identified additional mechanisms acting to specify the Ap cluster neurons. During NB 5-6 lineage development several temporal transitions acts to specify neurons born in different time windows. The temporal gene *castor* is expressed in a fairly large temporal window and the Ap neurons are sub-specified during that window by several combinatorial feed forward loops of transcription factors. In the screen, we identified a novel allele of the *syp* gene. We found that *syp* acts as a sub-temporal factor, fine-tuning the *castor* window into three different temporal parts. Previous studies have shown a role for *syp* earlier in the temporal cascade and we could confirm this in the NB 5-6 lineage. Together these data for the first time identify dual temporal roles of the same gene in a single NB lineage.

In summary, my thesis has helped identify novel genetic mechanisms controlling neuron subtype specification and numbers.

INTRODUCTION

Developmental neurobiology – insight into neural stem cell biology.

Developmental biology is the study of how a single cell, the fertilized egg develops into an adult organism. At the turn of the twentieth century cytological studies established the fundamental concept of genomic equivalence. In other words, in a multicellular organism each somatic cell has the same set of genes as all the other cells. Given this concept, one of the major questions facing biologists was how nuclear genes could direct development when these genes are the same in every cell type? Based on embryological studies and bacterial models of gene regulation, a consensus emerged in the 1960s that the answer to this question lies in differential gene expression. Massive genetic screens in *Drosophila melanogaster* (*Drosophila*) resulted in the identification of a high proportion of the genes that control development. In the molecular era it has been proven that most of these regulatory mechanisms are highly conserved throughout the animal kingdom (Rubin et al., 2000).

Selective gene expression controls four essential processes in development: cell proliferation, producing the correct number of cells from one precursor cell; cell specification, creating cells with different functions at different positions; cell interactions, coordinating connections between cells; cell movements, rearranging the cells to form structured tissues (Carroll et al., 2005; Gilbert et al., 1997).

In order to study regulatory mechanisms at high resolution it is advantageous to focus attention to the development of a single tissue or organ in a system that allows genetic manipulations. For these reasons the *Drosophila* central nervous system(CNS) has been a central model system that has provided much insight in embryonic development in general, but also in neural stem cell biology in particular (Lawrence, 1992; Rubin et al., 2000).

With a deeper understanding of developmental mechanisms follows the understanding of the underlying mechanisms behind several major human diseases as cancers,

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arthritis and neurodegenerative diseases, like Parkinson's disease and Alzheimer's disease. Understanding growth and regeneration processes is also an important issue for future therapy of neural injuries (Kandel et al., 2000; Sanes et al., 2011; Sanes and Lichtman, 2001).

The Nervous System

Nerve cells are among the most ancient of all animal cell types and they display an enormous variety in identity i.e., in their morphology, types of neurotransmitters used, function and connectivity. Estimates hold that there are at least 10,000 different types of neural cells in the human CNS. Neurons are almost always produced in association with glial cells which provide a supporting framework. In vertebrates, the neurons and glia of the CNS, including the spinal cord, the brain and the retina derive from the ectoderm that forms the neural tube. Signal proteins secreted from the ventral and dorsal sides of the neural tube act as morphogens, causing neurons born at different dorsoventral positions to express different gene regulatory proteins. The same principle applies to the anterior-posterior axis, where differences in homeotic Hox gene expression control the positional information. Moreover, neurons born at different time points acquire different identities due to temporal transitions in transcription factor expression (Sanes et al., 2011).

The differences in gene expression modulate the characters of the neurons and govern the connections they will form. The components of the nervous system – the various classes of neurons and glial cells and their axonal targets, originate in separate locations in the embryo and to connect, axons and dendrites grow out along specific routes, setting up a functional neuronal network. The complexity of this is illustrated by the fact that the human brain contain approximately 10^{11} cells and that each cell interconnects with thousands of others (Sanes et al., 2011; Sanes and Lichtman, 2001).

Neural stem cells are the precursors that generate this astonishing cell diversity. They are multipotent and undergo multiple self-renewing divisions. These divisions can be either symmetric, whereby two equal neural stem cells are born, or asymmetric, which generates one neural stem cell and one daughter cell with more restricted mitotic potential (Gilbert et al., 1997; Kandel et al., 2000; Sanes et al., 2011).

Drosophila CNS Development

The developing *Drosophila* CNS is sub-divided into the brain and the ventral nerve cord (VNC). The VNC develops from the neuroectodermal sheets on the ventral side of the embryo, while the brain develops from the anterior procephalic regions (Fig 1). Early in development, the neuroectodermal sheet is subdivided into a checkerboard pattern of neural equivalence groups by patterning genes that act in gradients along the dorso-ventral and anterior-posterior axes. As a result the cells in each equivalence group will express a unique combination of regulatory genes. Initially all cells in the equivalence group have the same potential to become neuroblasts by the expression of the *ac/sc* genes, but lateral inhibition, mediated by Notch signaling, eventually sorts out one cell, which acquires neuronal fate, delaminates and moves basally into the embryo. After delamination, each neuroblast begins to divide asymmetrically along the apical-basal axis (reviewed by Skeath and Thor, 2003).

Each NB has a unique identity and can be identified by its gene expression profile, the size of its lineage and the types of neurons and glia generated. In the embryo, each hemi segment of the VNC contains 30 NBs that have delaminated into seven distinct rows and six columns. Given the unique identity and position, each neuroblast has been assigned a name corresponding to the row and column in which it resides, hence the neuroblast positioned in row 1, and column 1 is named NB1-1, and so forth (Doe, 1992). The lineage size produced by the different NBs within a hemi segment varies between two cells and 40 cells with an average of 12 cells. Together, the 30 NBs generate ~400 cells during embryonic development.

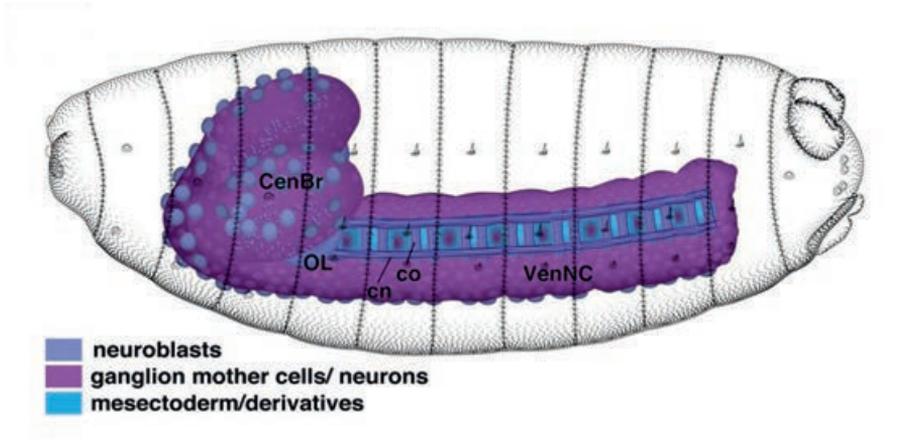


Figure 1. The central nervous system of a late Drosophila embryo. Anterior with the central brain is to the left. The ventral nerve cord resembles the vertebrate spinal cord. (Adapted from Bate and Arias, 1993).

Neuroblast specification

Genetic and cell transplantation experiments indicate that NB identity is determined by the equivalence group from which it segregates (Chu-LaGraff and Doe, 1993; Skeath et al., 1995; Udolph et al., 1995). The different transcription factors expressed by different NBs are called ‘NB identity genes’; however none of them determines a specific NB fate. Instead it is the specific and unique combinations of genes expressed in each NB that comprise a combinatorial code to specify the distinct identity of each neuroblast. Although the identical set of 30 NBs delaminates in every embryonic hemi segment, there are broader segmental differences in anterior-posterior axis regarding the size of the clones produced by the NBs. The clones produced by several thoracic NBs are larger than the corresponding NBs in the abdominal segments. This anterior posterior difference in lineage size is a result of influence of the homeotic gene network working in the combinatorial transcription factor codes (Karlsson et al., 2010).

Maintenance and differentiation

Symmetric versus asymmetric divisions

Neural stem cells divide either symmetrically, producing two identical daughter cells, or asymmetrically, producing two different daughter cells. Neural stem cells can also divide in a symmetric differentiative division mode, where none of the daughter cells retain the stem cell fate. This will deplete the stem cell pool and produce cells with more restricted mitotic potential. Vertebrate neuro-epithelial cells initially divide symmetrically, repeated times, in order to increase the stem cell pool. During neurogenesis in the ventricular zone of the developing cortex the neuro-epithelial cells instead divide asymmetrically, and while self-renewing also directly produce post mitotic neurons. Recently it has been shown that symmetric differentiative divisions occur in the sub ventricular zone of the developing cortex where intermediate progenitors then produce two post mitotic cells (reviewed by Gotz and Huttner, 2005). All these division modes can be seen in *Drosophila* neuroblasts, where for example proliferative symmetric divisions occur in the optic lobes. During the third instar larval stage, these progenitors switch into asymmetric neurogenic divisions (Egger et al., 2007; Hofbauer and Campos-Ortega, 1990; White and Kankel, 1978). There are also cases where neuroblasts divide asymmetrically to self-renew and directly produce post mitotic neurons without an intermediate precursor, for example NB 5-6 (Baumgardt et al., 2007). Differentiative divisions occur in the specialized progenitor cells (MP2 precursors) (Doe, 1992; Spana et al., 1995). However, most *Drosophila* neuroblasts in the VNC divide asymmetrically, by self-renewing and budding off smaller intermediate precursor cells called ganglion mother cells (GMCs) with lower mitotic potential. The tuning between symmetric and asymmetric division coordinates proliferation and differentiation, enable stem cells to create different lineage trees and by this control the number of nerve cells produced (Fig. 2).

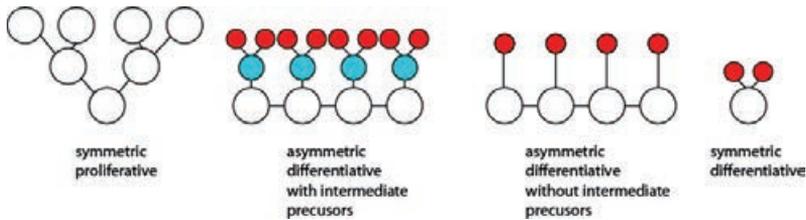


Figure 2. Neural stem cell divisions. Neural stem cells (white) can divide in different modes and thereby control the number of proliferative (white or blue) versus differentiated (red) cells produced.

Asymmetric segregation of cell-fate determinants

The main mechanism by which a cell division results in two daughter cells that are different from each other is by portioning cell fate determinants unequally between the new cells. In vitro experiments show that mainly intrinsic factors regulate the machinery that enables stem cells to divide asymmetrically (Broadus and Doe, 1997). Asymmetric division relies on the establishment of an apical-basal polarity in the stem cell. In *Drosophila* neuroblasts, this polarity is directed by cues expressed in the neuroectodermal epithelium and are inherited by the neuroblast when it delaminates. Neuroectodermal cells undergo horizontally oriented divisions within the epithelial plane which ensures symmetric divisions. After de-lamination the mitotic spindle of the neuroblast rotates 90°, which in turn results in divisions along the apical basal axis (Kaltschmidt et al., 2000). A protein complex located in apical cortex of the neuroblast is critical for spindle rotation and also directs cell fate determinants to the basal cortex. The apical complex consist of the Par proteins Bazooka (Baz, Par-3/-6) and the atypical protein kinase C (aPKC). These bind to Inscutable (Insc), Partner of Inscutable (Pins) and Locomotion Defective (Loco) (reviewed by Chia and Yang, 2002; Doe et al., 1998; Jan and Jan, 2000). When mitosis is initiated, the apical complex will direct cell fate determinants to the basal cortex by excluding the anchor protein Miranda (Mira) from the apical side. Mira binds to the basal cortex and interacts with Brain tumor (Brat), Prospero (Pros) and Staufen (Stau). Stau interact with Pros mRNA. After cytokinesis, Mira is degraded and Pros is released from the basal cortex (Schuldt et al., 1998; Shen et al., 1998). A second complex is also located

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at the basal cortex and consists of Numb and Partner of Numb (Pon). When Pros is released from the basal cortex it can enter the nucleus of the newly born GMC and promote expression of GMC-specific genes. Numb in turn plays out its role after the GMC division, where it discriminates between the two sibling neurons (reviewed by Betschinger and Knoblich, 2004). Hence establishment and maintenance of an apical basal polarity together with asymmetric distributions of cell fate determinants is the basis for controlled asymmetric cell divisions.

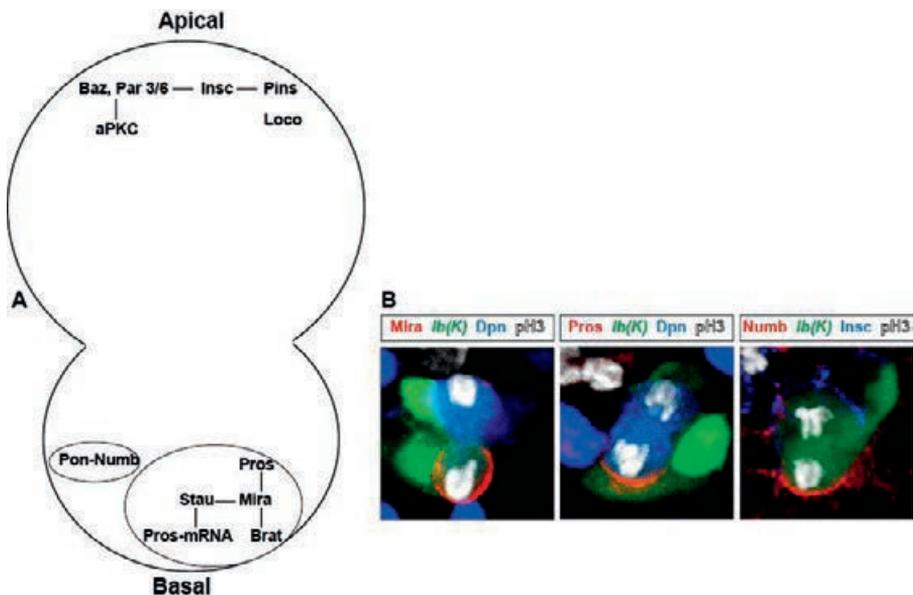


Figure 3. Asymmetric stem cell divisions are governed by the polar distribution of different protein complexes. (A) The apical complexes include factors that govern cell polarity, cell size and spindle alignment. The basal complexes include cell fate determinants that also limit the proliferative potential of the daughter cell.

(B) Confocal images of dividing *Drosophila* neuroblasts reveal asymmetric distribution of cell fate determinants into one of the daughter cells (from paper III).

Control of daughter cell proliferation by Prospero

Stem cell proliferation and daughter cell differentiation must be precisely regulated to produce the proper formation of the different neural lineages that form a functional CNS. In the embryonic *Drosophila* VNC, neuronal stem cells undergo multiple asymmetric divisions, producing a new NB and one intermediate progenitor, the GMC. The GMC divides only once to produce two post mitotic cells, that in turn differentiate into neurons or glial cells (reviewed by Gotz and Huttner, 2005). One of the key factors that regulate the restricted mitotic capacity of GMCs is the transcription factor Prospero (Pros) (Fig. 3). After cytokinesis Pros is released from the basal GMC when its adaptor protein Mira is degraded. Pros can now enter the nucleus and repress cell cycle genes, such as *Cyclin A*, *Cyclin E* and the *Drosophila cdc25* homologue, *string*. Moreover, Pros is also required to activate the expression of *dacapo*, a cyclin-dependent kinase inhibitor (Li and Vaessin, 2000; Liu et al., 2002). By these actions the asymmetric distribution of Pros into the GMC leads to repression of a number of cell cycle regulators and a subsequent shut-down of the cell cycle. It has been proposed that the single terminal division that is completed by the GMC is a result of previously transcribed and/or translated gene products (Li and Vaessin, 2000).

Specification of Temporal Identity

A relatively small number of neural stem cells give rise to not just a vast number of cells, but also to a multitude of different cell types. This is accomplished by stem cell multipotency, which enables the stem cells to generate complex lineages in a fixed temporal order. The anterior-posterior and dorsal-ventral patterning gives every delaminating neuroblast in a hemi-segment a unique identity. Moreover, temporal transitions in the neuroblast, by a now classical transcription factor series, give every GMC a distinct fate. Taken together, these mechanisms enable most if not all GMCs in each hemi segment to acquire unique fates, which in turn give them the possibility to produce a multitude of different post mitotic cells.

The temporal cascade consists of the sequential expression of five transcription factors: Hunchback (Hb - Kruppel (Kr) – Pdm2/Nubbin (collectively referred to as Pdm) – Castor (Cas) – Grainyhead (Grh). The GMC that is born within the temporal expression window of one of these genes will retain the expression of that factor and so will the post mitotic cells produced by the GMC (Brody and Odenwald, 2000; Isshiki et al., 2001; Kambadur et al., 1998; Novotny et al., 2002). The mechanisms that regulate the transitions between the genes are not fully understood. Cross-regulatory interactions between Hb, Kr, Pdm and Cas led to a model where each gene activates the next in the cascade and repress the ‘next plus one’ gene (Isshiki et al., 2001).

It has been shown that neuroblast cytokinesis is necessary for down regulation of Hb, and thus for the Hb to Kr transition (Grosskortenhaus et al., 2005). The requirement of mitosis for the first temporal transition is coupled to the expression of the orphan nuclear receptor Seven up (Svp) (Kanai et al., 2005), which is expressed in most neuroblasts during early embryogenesis (Mettler et al., 2006). Cytokinesis and cell cycle progression is however not needed for the subsequent Kr – Pdm – Cas cascade. Moreover, recent experiments show that the temporal genes are not necessary for expression of the next gene in the cascade (Grosskortenhaus et al., 2005; Tran and Doe, 2008), with the exception of Cas, which is necessary for the activation of Grh (Baumgardt et al., 2009). These data indicate that most temporal factors are not critical for the temporal progression; rather they are modulating the timing of the

transitions. Other underlying, yet unidentified, factors could shed more light on these mechanisms.

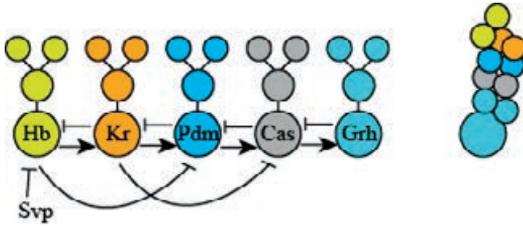


Figure 4. Temporal neuroblast progression. Neuroblasts sequentially express the transcription factors *Hb*, *Kr*, *Pdm*, *Cas* and *Grh*, but their progeny will retain the expression pattern present at the time of its birth which generate intra-lineage diversity. Arrows summarize known regulatory relationships between the temporal genes. Large circles represent the neuroblast dividing asymmetrically to self renew and bud off a GMC (intermediate circles). Small circles represent post mitotic progeny.

Populations of multipotent progenitors are found in the vertebrate cortex, retina and spinal cord (reviewed by Pearson and Doe, 2004). Similar to *Drosophila* NBs, vertebrate neural progenitors generate different subtypes of cells at different time points. The temporal order of neurogenesis in these structures is largely cell-autonomous, and can be recapitulated in vitro (Shen et al., 1997). The details in the genetic control of the distinct temporal order of vertebrate neurogenesis are largely unknown. However, the Ikaros transcription factor has been shown to control early born neuron fates in mouse retinal progenitors (Elliott et al., 2008). Ikaros is a orthologue of the *Drosophila* Hunchback temporal factor, which is the first transcription factor in the temporal cascade. Intriguingly, the vertebrate *Svp* orthologue COUP-TF1/2 has been found to have a role in regulating the temporal transition between neurons and glia in the mouse brain (Naka et al., 2008), which is in line with the role of *syp* in *Drosophila*.

Terminal cell fate specification

After each postmitotic neuron or glial cell has been born, a process of terminal differentiation is activated. In this process a cascade of distinct genes are expressed that give each cell subtype unique properties regarding morphology, axon pathfinding and expression of neurotransmitters, neuropeptides and ion channels. Recent work shows that the final identity of neuronal cells is specified both by intrinsic transcription factor codes and extrinsic cues, received from target tissues (Allan et al., 2003). To study the underlying mechanisms of terminal cell fate specification, peptidergic neurons in the *Drosophila* VNC have often been used as a model because of their restricted expression patterns that give the required resolution for these studies (Hokfelt et al., 2000; Nassel, 1996). The main conclusions from these studies are that both “master regulators” and combinatorial codes of transcription factors, as well as Hox homeodomain genes, act to specify the identity of individual neurons and glia (Allan et al., 2005; Allan et al., 2003; Baumgardt et al., 2009; Baumgardt et al., 2007; Karlsson et al., 2010; Miguel-Aliaga et al., 2004). The papers presented in this thesis are based upon previous detailed analysis regarding the mechanisms underlying terminal differentiation in a special subset of neurons; the Ap neurons. The Ap neurons and the model system that they comprise will be described in a later chapter.

Notch signaling

Metazoans use a relatively small number of highly conserved signaling pathways to control development, diversity of cell fate and tissue renewal. Among these, the Notch signaling pathway enables short range signaling between cells, because both the signal sending ligand and the signal receiving receptor are large transmembrane molecules. Notch signaling acts in a multitude of developmental mechanisms and the signals are often used to select between preexisting developmental programs. Depending on context, Notch signaling can activate or suppress cell proliferation, cell differentiation or cell death. In *Drosophila*, separation of sibling cell fates, boundary formation and early neuroblast selection, are also known to depend upon Notch signaling. During organogenesis, Notch signaling can be active both in binary cell fate choices and adopting an inductive role, and by this dual role generate complicated patterns of differentiated cell types. Given the multitude of fundamental roles in most, if not all tissues, dysfunctions in the Notch signaling pathway has been directly linked to many human disorders, both with respect to developmental syndromes and cancers. Because Notch pathway activation depends upon direct contact between the interacting cells, the spatial effect of the Notch signal is restricted, which allows for localized fine tuning of different fate choices. Along with this, the simplicity of the signal transduction pathway, without second messengers, allows for a rapid activation of downstream effectors, and the proteolysis of the receptor after activation, in turn allows for rapid down regulation and subsequent reactivation of the signal, which also gives high temporal sensitivity (reviewed by Andersson et al., 2011; Bray, 2006).

The canonical Notch signaling pathway and its core components

The highly conserved Notch signaling pathway has a surprisingly simple core signal transduction pathway that is used in most Notch dependent processes. However the duration and amplitude of the signal is regulated at various levels of the pathway, including at the level of ligand and receptor maturation, receptor proteolysis and downstream target selection (reviewed by Fortini, 2009; Kopan and Ilagan, 2009).

Notch receptors are single-pass type I transmembrane proteins with large extracellular domains, consisting of 29-36 tandem epidermal growth factor (EGF) like repeats. Whereas *Drosophila* has only one Notch receptor, mammals have four Notch receptors, which have both redundant and unique functions. The Notch receptor is synthesized as a single transmembrane receptor, which in vertebrates is cleaved by Furin proteases to yield a bipartite heterodimeric Notch receptor. The intracellular domain of the Notch receptor contains multiple cleavage sites, followed by a high affinity binding module with a conserved WxP motif, seven ankyrin repeats (ANK domain) and a nuclear localization sequence (NLS). C-terminal to these domains is an evolutionary divergent transactivation domain (TAD) and a highly conserved degradation signal (Artavanis-Tsakonas et al., 1995; Fleming, 1998).

Notch ligands are themselves type I transmembrane proteins with varying numbers of EGF like repeats (reviewed by D'Souza et al., 2008). Based upon the presence or absence of a cysteine rich (CR) domain, the ligands are subdivided into two classes: Delta or Delta like and Serrate (Jagged in mammals). Receptor-ligand interactions involve direct binding of an N-terminal ligand domain to the EGF-like repeat 11-12 region of the Notch receptor. Recent atomic force microscopy studies reveal that this interaction is extremely strong when compared to other ligand-receptor interactions (Ahimou et al., 2004), which suggests that the activation mechanism includes physical force needed for the successive proteolysis of the receptor, by exposure of the cleavage sites. Ligand binding promotes two proteolytic cleavage events of the Notch receptor. The first cleavage is catalyzed by extracellular located ADAM-family metalloproteases: ADAM 10 or TACE (TNF- α - converting enzyme; Kuzbanian in *Drosophila*) (Mumm et al., 2000). This S2 cleavage generates the activated membrane anchored Notch form termed Notch extracellular truncation (NEXT) which in turn is

the substrate for S3 cleavage mediated by the intramembrane aspartyl protease complex γ -secretase, that contains presenillin, nicastrin, PEN2 and APH1 (Fortini, 2002). This leads to the release of the Notch intracellular signal transduction fragment, termed Notch intracellular domain (NICD), which due to its nuclear localization signal, enters the nucleus and activates the transcription of downstream genes. In the absence of NICD, most Notch target genes are maintained in an actively repressed state by large transcriptional complexes involving the CSL type transcription factors: CBF1 in vertebrates, Suppressor of Hairless ((Su(H)) in *Drosophila*, and LAG-1 in *Caenorhabditis elegans*. The CSL transcription factor acts together with various co repressors, including SMRT, SKIP, CIR, Hairless and Groucho. Upon nuclear translocation of the NICD fragment, co repressors are displaced by a transcriptionally active complex including CSL, its co activator Mastermind (Mam) and NICD (reviewed by Kopan and Ilagan, 2009). The direct translocation of the activated NICD fragment is the most striking feature of Notch signaling, and distinct from signaling pathways relying on phosphorylation cascades and second messengers. Given the high spatiotemporal sensitivity in Notch signaling, the signal down-regulation is as important as the activation process. Optimal signal duration is regulated by ensuring that the half-life of the NICD is short, sometimes only fractions of a cell cycle (Ambros, 1999). This is accomplished by phosphorylation within the PEST domain by the CDK8 kinase and subsequent degradation by E3 ubiquitin ligases. This process eliminates the NICD, disassembles the transcription activation complex, and resets the cell for new signaling (Fryer et al., 2004).

The regulation of Notch signaling

Given that the canonical Notch signaling pathway is used in such a wide array of developmental and physiological processes, using the same core components, regulation of the initiating of signaling, the duration of signal and the context dependent downstream effectors are critical aspects which result in distinct molecular and developmental outcomes. Recent studies have revealed much insight into the activation of the pathway, including posttranslational modification and trafficking of the Notch ligands and receptors.

It has been shown that solely ligand expression is not sufficient for their function; instead ligands have to be activated through ubiquitylation by E3 ubiquitin ligases i.e., Neuralized (Neur) and Mind bomb (Mib), in a context dependent manner. This is required for Epsin-mediated endocytosis and subsequent presentation of the activated ligand on the cell surface. The activity of E3 ubiquitin ligases is in turn regulated by members of the Bearded related family of small inhibitory polypeptides, which exemplifies the underlying complexity of the signal regulation (reviewed by Kopan and Ilagan, 2009).

Notch receptors are large glycoproteins where the EGF repeats is post-translationally modified in different ways by two forms of O-glycosylation; O-fucose and O-glucose, mediated by the GDP fucose protein O-fucosyltransferase (O-fut in *Drosophila*; profut1 in mammals). Fucosylation is essential for further modification mediated by Fringe glycosyltransferases. This modification can determine which ligands can bind to and activate the receptor. Fringe mediated addition of a single N-acetylglucosamine on EGF repeat 12 in *Drosophila* Notch is for instance sufficient for enhanced receptor binding to Delta and reduced affinity to Serrate (Xu et al., 2007).

Notch receptors are targeted by several ubiquitin ligases; for instance Nedd4 and suppressor of Deltex (Su(Dx)), which promotes Notch degradation. Recent studies show that a large fraction of Notch molecules are located inside endocytic vesicles in the cytoplasm. Unlike the ligands, where ubiquitylation and endosomal trafficking is essential for proper signaling, mutant studies show conflicting results with mostly subtle phenotypes regarding the role of endosomal trafficking upon the maturation of

the receptor (reviewed by Andersson et al., 2011; Bray, 2006). One well characterized inhibitor of Notch is Numb, which controls Notch endocytosis by directly recruiting Notch into endosomal vesicles (Berdnik et al., 2002). Furthermore, mammalian Numb promotes Notch ubiquitylation (McGill and McGlade, 2003). In asymmetrical divisions in *Drosophila*, Numb interacts with the four pass transmembrane protein Sanpodo and reduce its accumulation, which inhibits Notch signaling in an unknown way (Hutterer and Knoblich, 2005; O'Connor-Giles and Skeath, 2003).

A key regulatory event in productive Notch signaling has been shown to be the ligand-induced ADAM metalloprotease mediated S2-cleavage of the receptor. The S2 cleavage site resides in a negatively regulatory domain that prevents proteolysis of Notch in the absence of ligand. Mutations in this region have been shown to produce “leaky” Notch signaling, and for instance cause T cell acute lymphoblastic leukemia in humans (Weng et al., 2004). Studies of metalloproteases reveal that their activity is regulated by membrane environment and intracellular signaling pathways (Huovila et al., 2005). The cleavage of Notch by γ -secretase has been regarded as a constitutive proteolytic event, however recent studies show that intramembrane proteolysis is also regulated in a number of ways, where expression of different combinations of cofactors within the γ -secretase complex in different contexts are the most known (Jorissen and De Strooper, 2010).

NICD turnover is an essential part of the fidelity of the signal and its downstream effectors. Deletion of the PEST degradation domain can cause T cell acute lymphoblastic leukemia in humans (Weng et al., 2004), demonstrating the importance of a short half-life of the NICD fragment.

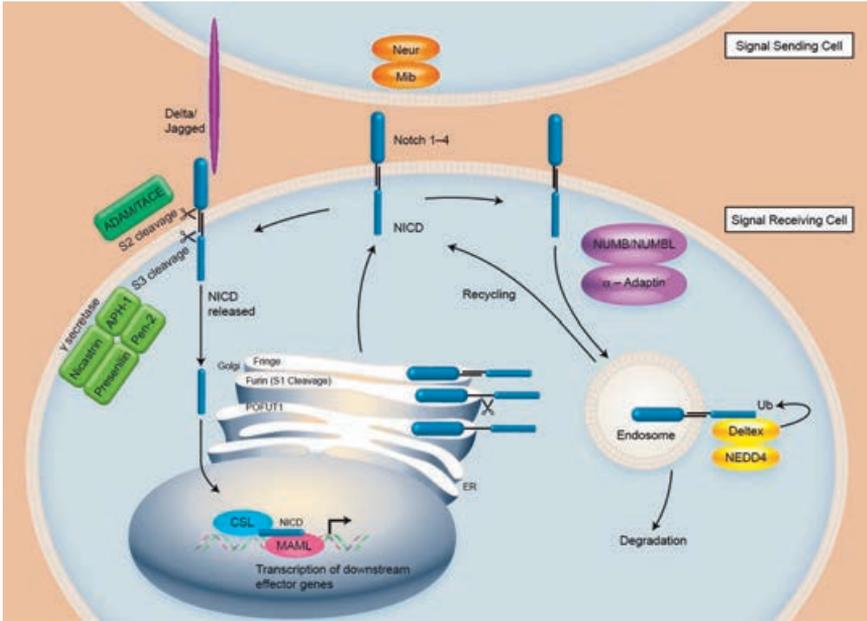


Figure 5. Model of the Notch signaling pathway. (Adapted from www.morphosys.com)

Notch downstream target genes

The primary targets of Notch signaling encode transcriptional factors of the basic helix-loop-helix class (bHLH), which mainly act as transcriptional repressors: the *hairy* and *Enhancer of split* (HES) genes. The most extensively studied targets are the *hairy* and *Enhancer of split* (*E(spl)*) genes in *Drosophila* and the related *Hes* and *Hey* genes in mammals (Fischer and Gessler, 2003). In *Drosophila*, *hairy* and seven clustered *E(spl)* genes (*m8*, *m7*, *m5*, *m3*, *mβ*, *mγ*, and *mδ*) encode proteins with the same overall protein structure: a DNA binding domain contiguous with the HLH dimerization domain. Following this is a protein binding domain called the Orange domain, which serves as a repressor. All HES genes also share the highly conserved C-terminal motif WRPW, which recruits the co-repressor Groucho (Fisher and Caudy, 1998). In addition to these HES genes, the *Drosophila* genome also contains a HES-related gene, *Hey*, encoding a protein with bHLH and Orange domains similar to those of *Hairy* and *E(spl)*, but instead of the C-terminal WRPW motif there is a YWRP motif. The function of the *Drosophila Hey* gene has until recently been unknown, but recent data show that *Drosophila Hey* is a target of Notch and seems to be expressed differently than the *Hairy* and *E(spl)* genes (Monastirioti et al., 2010 paperIII)

In the mouse and rat genomes, seven HES (*Hes 1-7*) and three *Hey* genes (*Hey-1*, *-2* and *-L*; or *Hrt-1*, *-2*, *-3*; or *Hesr-1*, *-2*) have been identified (reviewed by Fischer and Gessler, 2007). The mammalian HES proteins are highly similar to *Drosophila* *Hairy* and *E(spl)* proteins regarding the bHLH, Orange and WRPW domains. They bind the same DNA sequences and they recruit TLE-1-4 co-repressors, which are orthologs of Groucho (Iso et al., 2003). *Hes-1*, *Hes-5*, *Hes-7* and all three *Hey* genes can be induced by the Notch pathway, whereas *Hes-2*, *Hes-3* and *Hes-6* and possibly *Hes-4*, seems to act independently of Notch signaling.

HES-related proteins can interact with a large number of proteins, for instance HLH factors such as Mash1, other transcription factors such as c-myc, sox10, STAT3 and JAK 2, as well as transcriptional co-repressors such as histone deacetylases.

Drosophila E(spl) proteins form homo- and heterodimers with each other, and so do the other HES and *Hey* family members. Studies show that heterodimers often bind to

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DNA with higher affinity than the corresponding homodimers (Iso et al., 2001). Taken together, the HES proteins can form a multitude of different regulatory complexes depending on context, and compelling data show that with rare exceptions, they act as transcriptional repressors.

Roles of Notch signaling in non-neural stem cells

Knockout studies in mice have shown that Notch signaling plays a critical role in the generation of adult-type hematopoietic stem cells (Hadland et al., 2004). Notch also regulates blood vessel formation, by acting as a determinant in vascular formation (reviewed by Gridley, 2007). These studies demonstrate that Notch signaling is important for proliferation and migration of endothelial cells, smooth muscle differentiation, and seems to have an instructive role in arterial-venous fate choices. Interestingly, gain of function experiments in the vascular system often show the same results as loss-of-function experiments, which is opposite to experiments done in neural progenitors. It is known that Notch signals often function pro-apoptotically in endothelial cells, which can be an explanation for this phenomenon (Noseda et al., 2004). Notch signaling has also been shown to be critical for proper organogenesis of the mammalian kidney, heart pancreas and intestines (Apelqvist et al., 1999; Jensen et al., 2000; McCright et al., 2001; Xue et al., 1999). In the pancreas, mutants show hyperplastic phenotypes regarding one cell type, evident by for instance an excess of endocrine cells in the pancreas, at the cost of another cell type; the exocrine cells. The same mice exhibit excess differentiation to secretory cells in the intestinal mucosa at the cost of enterocytes (Jensen et al., 2000).

In adult stem cells, Notch signaling is involved in maintaining proper number of progenitors and differentiated cells throughout life. This has been most extensively studied in the hair follicles in the skin, where both gain and loss of function of Notch signaling leads to epidermal hyperplasia and cyst formation, as well as hyperkeratosis and hair loss (Uyttendaele et al., 2004). In the hematopoietic and immune systems, it has been shown that Notch signaling inhibits myeloid differentiation from progenitor cells. However, it is not clear if Notch signaling is critical for hematopoietic stem cell (HSC) maintenance in the bone marrow. On the other hand, a number of studies demonstrate that Notch signaling instructs further differentiation of HSC progeny; the hemato-lymphopoietic cells, where Notch signaling promotes T-lineage differentiation and blocks B-lineage differentiation (Koch et al., 2001). In the intestinal epithelial progenitor cells, Notch signaling is critical for maintaining the correct ratio of progenitor/differentiated cells (van Es et al., 2005).

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These studies demonstrate that Notch signaling has a number of roles in cell differentiation during development. In early development it affects the transition from primordial cells to tissue specific stem cells and later it regulates the maintenance of different progenitor pools. In mid- to late stage embryo development Notch signaling blocks the default pathway and promotes the alternative pathway (reviewed by Chiba, 2006).

Notch signaling in neurogenesis

During early *Drosophila* embryogenesis, Notch signaling acts via lateral inhibition among undifferentiated neuroectodermal cells with the same potential, and oscillations in Delta expression induces differences in Delta-Notch expression among otherwise seemingly equivalent cells. Cells that receive active Notch signaling will activate HES genes, down regulate the proneural *ac/sc* genes, and adopt an epidermal fate, while cells without Notch signal will delaminate and adopt a neuronal stem cell fate (Gaiano and Fishell, 2002). As a result thereof, perturbations of the Notch signaling pathway give hyperplastic nerve systems where super numerous cells turn into neuroblasts.

Conditional Notch-1, -3 and CSL knockout studies in mice also show that Notch signaling inhibits premature onset of neurogenesis, by negative regulation of the proneural genes *Neurogenin* (*Ngn*) and *MASH-1/-3*, and that the pathway is required for maintenance and expansion of the neural stem/progenitor pool. Inactivation of the different HES genes in mice variably induces precocious neural differentiation and decrease of neural progenitor numbers. Together, these studies indicate that Notch signaling and the downstream target HES genes are conserved regulators of the expansion and differentiation of neural stem cells (Kageyama et al., 2005).

During sensory organ development in *Drosophila*, Notch mediated lateral inhibition ensures that one cell in a proneural cluster adopts the neural fate; the sensory organ precursor (SOP). This precursor gives rise to the four different cells that form the external sensory organ, via a series of asymmetric cell divisions (Jan and Jan 2001). The Numb protein is asymmetrically segregated into one of the daughter cells in each division, where it inhibits Notch signaling. This will ensure that the siblings produced

in each division require a unique identity. In Numb mutants all four cells adopt the same socket fate (Rhyu et al., 1994; Uemura et al., 1989). This asymmetric division pattern, where Numb is inherited by one daughter cell to inhibit Notch signaling and by this separates the fate of the siblings, has been studied and confirmed in several lineages also in the *Drosophila* CNS (Skeath and Doe, 1998; Spana and Doe, 1996; Spana et al., 1995).

Notch signaling has also been shown to facilitate glial cell fate, by inhibiting neural cell fate, and also inducing astrocyte differentiation, by inhibiting differentiation to both neurons and oligodendrocytes (Grandbarbe et al., 2003; Tanigaki et al., 2001). This can be seen as examples of Notch signaling acting as a switch in binary cell fate decisions, and by that restrict sibling cell fates in a step wise manner.

COUP-TF orphan nuclear receptors

Nuclear receptors are transcription factors that are activated by ligand binding by steroid/thyroid hormones, as well as by other low-molecular weight molecules (reviewed by Sonoda et al., 2008; Tsai and O'Malley, 1994). The steroid/thyroid nuclear receptor superfamily represents the largest known family of transcription factors and consists of many transcriptional regulators acting in development, differentiation and homeostasis (reviewed by McKenna et al., 2009; Thummel, 1995; Tsai and O'Malley, 1994). A large number of these proteins are so called “orphan” receptors whose ligands have not yet been identified (Giguere, 1999).

One of the best characterized group of orphan nuclear receptors are the Chicken ovalbumin upstream promoter transcription factors (COUP-TFs) (Pereira et al., 2000). The first COUP-TF member discovered was the human COUP-TF I, and through homology screening, homologs and orthologs have been obtained from numerous species (Giguere, 1999). Based on alignment of their predicted ligand binding domains, vertebrate COUP-TFs can be subdivided into four groups (Tsai and Tsai, 1997). Humans have two COUP-TF family members (COUP-TF I/II), whereas zebrafish and *xenopus* have three members. *Drosophila*, *C. elegans* and the sea urchin contain only one member. The sequence conservation is strikingly high both in the DNA binding and ligand binding domains. The DNA binding domain of COUP-TF I/II is virtually identical among species, with predicted binding to identical DNA response elements. Even more striking is the conservation of the ligand binding domains, with 99.6% sequence identity among vertebrates and 90% between human and fly (Tsai and Tsai, 1997).

Biochemical studies indicate that COUP-TFs in general act as negative regulators, modifying the hormone responsiveness of a large number of nuclear receptors by competing for the same response elements (Tran et al., 1992). In addition to acting as repressors, COUP-TFs can also activate a large number of genes by interactions with co activators (Bailey et al., 1998). COUP-TFs have been shown to mainly regulate genes involved in fat metabolism. These include many apolipoproteins as well as enzymes involved in β -oxidation and in fatty acid synthesis. In addition to this, the

COUP-TFs can also regulate an ever growing list of genes involved in pattern formation, proliferation, differentiation and chromatin modification (Giguere, 1999).

Expression of COUP-TFs is regulated by retinoids and COUP-TFs have been shown to be an integrated part of retinoid signaling during development (reviewed by Pereira et al., 2000). In addition to this the COUP-TFII gene has been shown to be regulated by sonic hedgehog signaling (Krishnan et al., 1997). The expression patterns of COUP-TFs have been described in mouse, chick, zebrafish, *Xenopus*, *C.elegans* and *Drosophila*. The general expression patterns are approximately similar between species, and COUP-TFs are dynamically expressed in restricted regions of the CNS during embryonic development. COUP-TFs are also expressed in the mesenchymal tissues of many developing organs. The expression levels decrease after organogenesis, and are dramatically decreased in the adult animals (Qiu et al., 1994; Tsai and O'Malley, 1994; Tsai and Tsai, 1997)

Recent studies show that COUP-TFI and -II are expressed in a temporal dynamical way in the developing mammalian CNS (Qin et al., 2007; Yamaguchi et al., 2004), where it acts as a temporal switch to control the timing between early- and late born cells (Naka et al., 2008; Tomassy et al., 2010)

The *Drosophila* COUP-TF homolog *seven-up* (*svp*) is also dynamically expressed in the embryonic *Drosophila* CNS (Doe, 1992; Kohwi et al., 2011). *Svp* is expressed in 26 out of 30 neuroblasts in each hemi segment. The expression is temporally restricted with a first transient expression stereotyped within each neuroblast lineage (Broadus et al., 1995; Kanai et al., 2005). This first expression has been shown to be critical for the proper temporal progression of competence in neuroblasts. Mutant analyses show that *svp* has a critical role in limiting the number of neurons with early born fate, whereas misexpression of *svp* leads to a loss of early born neurons. This role of *svp* is due to its potency to repress the first temporal transcription factor *hunchback* (*hb*) in the temporal transcription cascade, and by this promote the temporal cascade to proceed (Kanai et al., 2005; Mettler et al., 2006). A second wave of *svp*-expression has also been observed within different lineages, but the function of this later expression was hitherto unknown (Kanai et al., 2005; Kohwi et al., 2011).

Svp has also been shown to specify photoreceptor subtype during compound eye development (Mlodzik et al., 1990). Ectopic expression of *svp* in cone cells in the

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developing *Drosophila* eye show that *svp* acts as a cell fate switch with the specific phenotype depending on the developmental stage at the time of *svp*-expression (Hiromi et al., 1993).

The NB 5-6T lineage and the Apterous neuron model

The studies presented in this thesis, have focused on one particular *Drosophila* embryonic neuroblast, the thoracic neuroblast 5-6 (NB 5-6T), and its progeny. In the three thoracic segments NB 5-6 generates a lineage of 20 cells. The last four cells born in the lineage, are thoracic-specific and form a cluster of cells, all expressing the LIM-HD protein Apterous (Ap) and the transcription cofactor Eyes absent (Eya) (Baumgardt et al., 2009; Baumgardt et al., 2007; Miguel-Aliaga et al., 2004). NB 5-6 and its progeny can be identified by using reporter genes under the control of an enhancer fragment from the *ladybird early* gene (*lbe(K)*) (Baumgardt et al., 2007; De Graeve et al., 2004). Extensive studies performed on this neuroblast show that NB 5-6T delaminates at stage 8 and produces a stereotyped early lineage of GMC's dividing into neurons or glia. At stage 12, the NB switches into a direct-neuron division mode, and generates directly differentiating neurons in a sequential manner, without an intermediate GMC. By this alternate division mode, the NB generates the four Ap neurons in a sequential manner. The four Ap neurons can be further sub-divided by their selective neuropeptides expression: Ap1 expresses the neuropeptide Nplp1, the two Ap2/Ap3 interneurons and the FMRFa expressing Ap4 neuron. After this, the NB exits the cell cycle and dies by apoptosis at stage 16 (Baumgardt et al., 2007) (Fig. 6).

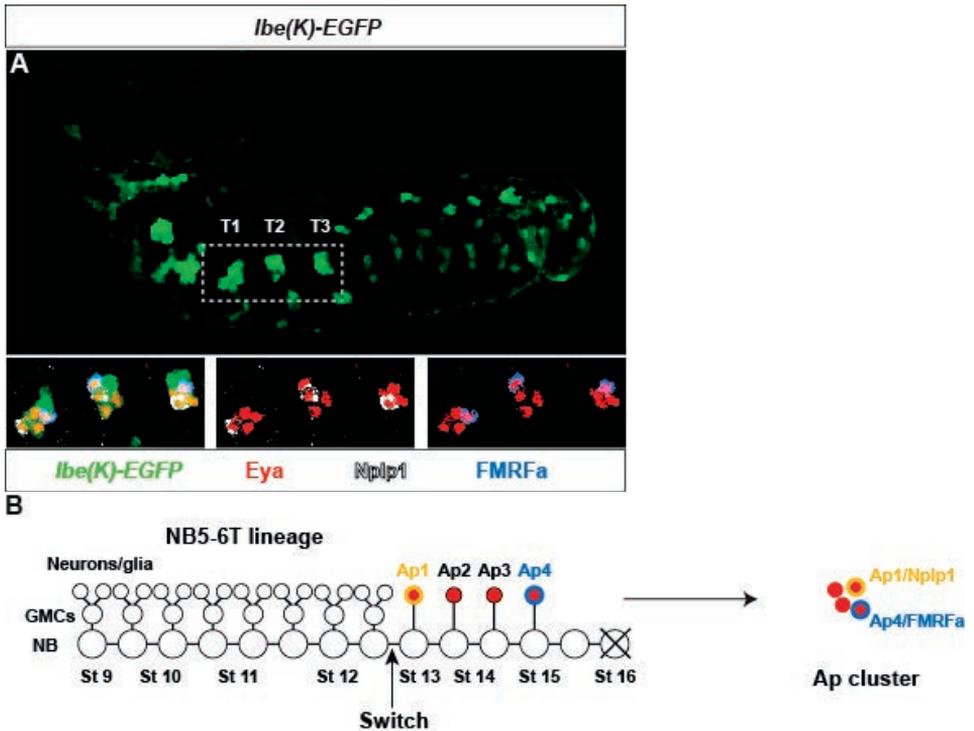


Figure 6. The lineage of thoracic neuroblast 5-6. Expression of *ibe-EGFP* reveals the NB 5-6 lineage in the embryonic *Drosophila* VNC. In the three thoracic segments, the Ap cluster is generated at the end of the lineage and can be identified by *Eya* expression. The Ap4 neuron is identified by *FMRFa* expression. Lateral view of a *Drosophila* CNS. The cartoon shows the NB 5-6 lineage progression. (From Paper III).

The Ap neurons have been extensively studied, and a number of genes have been shown to be critical for their proper formation and specification. The Ap neurons are produced in a competence window in the temporal cascade dictated by the temporal genes *castor* (*cas*) and *grainyhead* (*grh*), and both these genes are critical for Ap neuron specification. *cas* acts as a central activator of a number of downstream genes, which act in combinatorial codes to individually specify the four consecutively born Ap neurons. This is accomplished by simultaneous triggering of two different feed forward loops. One loop plays out in the post mitotic Ap1 neuron, where *cas* activates

the *collier* → *ap/eya* → *dimmed* (*dimm*) genes. This combination of genes establishes the code that activates the expression of Nplp1. In the second loop, which progresses within the NB during sequential divisions, *cas* activates *squeeze* (*sqz*) → *nab*. During these NB divisions the Ap2-Ap4 neurons are generated. Here, *sqz* and *nab* act together in the post mitotic neurons to suppress *col*. This will oppose the action of the first loop and lead to the expression of an alternate code which includes *dachshund* (*dac*) and *grh* (Baumgardt et al., 2009; Baumgardt et al., 2007). This detailed analysis has revealed that *col* plays dual roles during Ap cluster specification; first by dictating a “generic” Ap neuron fate in all four Ap neurons and then specifically by acting only in the Ap1 neuron. By this *col* acts to divide the Cas window into one early and one late part (Fig.7).

The Ap cluster is produced only in the thoracic NB 5-6 lineage, and recent studies on the Hox homeotic genes reveal that the Hox gene *Antennapedia* (*Antp*), together with the Hox cofactors *homothorax* (*hth*) and *extradenticle* (*exd*), are critical for Ap neuron specification, by integrating with the temporal gene *cas* (Karlsson et al., 2010).

In addition to the intrinsic input provided by the temporal and Hox genes, FMRFa expression is critically dependent upon a retrograde TGFβ/BMP signal received from the axonal target (Allan et al 2003). The Ap4/FMRFa neuron has a unique axonal trajectory, perpendicular to the axonal trajectories of the other Ap-neurons. The axon of the Ap4 neuron projects out of the VNC at the dorsal midline, and innervates the neurohemal organ (DNH) (Gorczyca et al., 1994; Nassel, 1996). In the DNH the BMP ligand Glass bottom boat (*Gbb*) activates the type II BMP receptor Wishful thinking (*Wit*). This activates the downstream effector phosphorylated receptor-Smad protein Mad (pMad), which enters the nucleus of the Ap4 neuron and acts together with the intrinsic transcription factor code to activate FMRFa expression (Allan et al., 2003) (Fig. 7).

Introduction

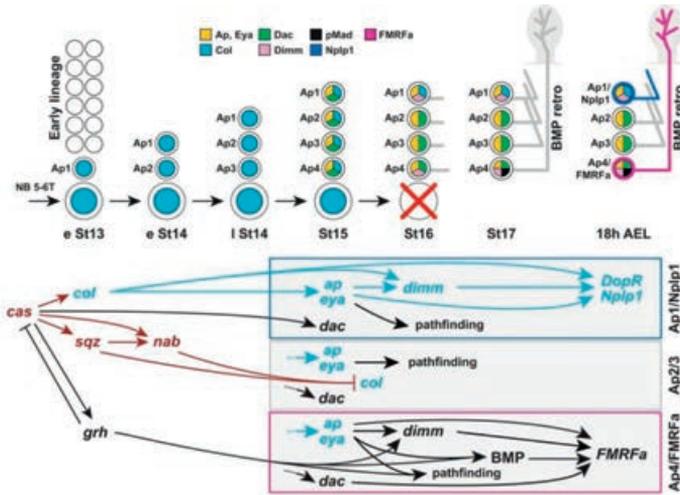


Figure 7. Model summarizing the genetic mechanisms underlying terminal cell fate specification of the Ap neurons (Baumgardt et al., 2009).

Taken together, the biological features of the NB 5-6 lineage makes it a favorable model for understanding biological mechanisms underlying neural development. The lineage is comparable large and relatively complex, which makes it possible to identify the temporal appearance of different progeny. The segmental difference between the abdominal 5-6 lineage and the thoracic 5-6 lineage makes it possible to address spatial cues acting on cell cycle decisions as well as cell specification. The different axon pathway choices within the lineage, together with the need of a retrograde TGF β /BMP signal in the Ap4 neuron allows for detailed genetic analyses of axon path finding, axon transport and retrograde signaling. The cell cycle progression switch, where the neuroblast produces progeny with a more restricted mitotic potential give opportunities to address questions about cell proliferation decisions. In addition to these advantages, previous work using this model has provided a multitude of markers and genetic tools, which enable us to address these questions in single cell resolution within an identifiable lineage.

Genetic screening in *Drosophila*

Drosophila melanogaster has been in use as a genetic model organism for longer than any other. The foundation of genetics were built on studies in *Drosophila*, and in the last 90 years, subsequent genetic studies have provided the major part of our knowledge about the mechanisms of genetic cause and phenotypic effect. *Drosophila* mutants with detectable phenotypes have provided the key to identification and characterization of genetic mechanisms, and these mutants have most often been obtained in large scale genetic screens. Forward genetic mutagenesis screens, in which genetic variation is artificially introduced and mutants are screened for phenotypes of interest, are powerful tools for identifying genes involved in developmental processes. In contrary to reverse genetics where a gene of interest is knocked down and the resulting phenotype is studied, forward genetics identifies unpredicted genes (Greenspan, 2004).

In *Drosophila*, four main approaches are commonly used to induce loss of protein function. First; chemical mutagenesis with EMS, which is the most commonly, used chemical mutagen for de novo induction of DNA lesions. It gives a wide and unbiased coverage of the genome with high mutation frequency. As a consequence of this, the genomic position of the point mutation that causes the phenotype of interest can be very difficult to map. Second; disruption of coding sequences by insertion of a transposable element. This approach most often gives just one disruption per chromosome, which increases the number of stocks that need to be screened. On the other hand, dominant markers on the transposon make it amenable to mapping with recombination mapping. Genes can also be disrupted by imprecise excisions of such elements. If the dominant markers were lost in the process, these mutations can be almost as difficult to map as the EMS induced ones. Third; chromosomes with chemical- or radiation induced deleted regions that are mapped with high precision. The phenotype in this case will be caused by a coding region within the breakpoints of the deletion. Fourth; RNA-interference, where the transcript of a gene is prevented from being translated by complementary binding of siRNA, is also increasingly utilized. An advantage with this approach is that the identity of the interfered gene is already known. However the effectiveness of RNAi is variable in *Drosophila* in

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general, for unknown reasons. Moreover, siRNA-based RNAi is not used endogenously during the development of the *Drosophila* CNS, although miRNA-based RNAi is, which makes siRNA even less effective for studies in the CNS (Whyard, 2009).

Mapping the genomic position of a mutation of interest can, as mentioned above, vary in practice, depending on the screen setup. Standard practice of genetic mapping typically occur in three phases were linkage and recombination analysis is followed by deletion mapping, where the mutant is tested for failure to complement genomic deletions. Finally, alleles of candidate genes within the identified interval can be tested by complementation analysis, or the identified region can be sequenced. New whole genome sequence techniques show promising opportunities for reducing the time in identifying causative mutations by comparing mutated chromosomes to the un-mutagenized sequence and by this identify single base pair mutations (Blumenstiel et al., 2009).

AIMS

The goal of my thesis project was to take a comprehensive approach of finding new, undiscovered genes and study their actions in the development of one specific *Drosophila* CNS stem cell, the neuroblast 5-6 (NB 5-6), which generates the Ap neurons. We took advantage of the Ap neuron model system and the restricted embryonic FMRFa expression pattern revealed by the FMRFa enhancer to identify novel genes that can lead to a more complete understanding of the generation of neural diversity.

The aims of this thesis were:

1. To design and conduct a forward genetic screen in *Drosophila* utilizing a GFP reporter construct coupled to an end marker of our cells of interest, and thereby hopefully find novel genes involved in neural development.
2. To perform detailed studies on mutants identified in the screen, in order to address unsolved issues in developmental neurobiology.

MATERIALS AND METHODS

The results presented in this thesis have mainly been obtained by analyzing protein expression in developing *Drosophila* embryonic ventral nerve cords. This was accomplished by immunolabeling of dissected CNS's in combination with confocal microscopy. With the help of the lineage specific *ladybird* marker we could identify NB 5-6 and all progeny derived from it. In addition to this, we could identify each progeny in the latter part of the lineage thanks to their unique expression of specific markers. This made it possible to compare embryos with manipulated genotypes to wild type embryos, both regarding temporal specification and cell numbers in single cell resolution.

In the first study (Paper I) we used a genetic construct, where *GFP* is expressed in the same pattern as the endogenous *FMRFa* neuropeptide. The advantage with this approach is that GFP expression can be analyzed directly in intact, living embryos, without the need of dissecting and staining with antibodies. These fly stocks were treated with a mutagenizing agent; ethyl methanesulfonate (EMS), which induces point mutations. The mutated fly stocks were thereafter screened for aberrant *FMRFa-GFP* expression as a sign of perturbations in NB 5-6 development.

The mutants identified were analyzed regarding their expression of a number of known proteins, used as distinguishing markers. These markers included transcription factors, transcriptional cofactors, effector proteins and neuropeptides. The extensive knowledge about this lineage enabled us to use these expression patterns to categorize the mutants into different phenotypic groups.

In this type of random mutagenesis, the affected gene is unknown. In order to identify the loci of the different mutations, we used complementation analysis. This approach relies on the assumption that the induced mutation is recessive and will reveal the expected phenotype when crossed to a deficient allele of the same locus. In this work we used both alleles of previously known genes, and a set of overlapping genetic deletions that have been mapped with high precision; the Bloomington Deficiency Kit.

Material and Methods

In Paper II and Paper III two principle types of genetic experiments were used: loss-of-function and gain-of-function experiments. A number of these experiments were conducted by the use of the GAL4/UAS-system which is used to express genetic constructs in a spatial- or temporal manner. The GAL4 protein is a yeast derived transcription factor that binds selectively to the UAS sequence. By fusing a gene of interest to the UAS sequence, the expression can be controlled by a promoter controlling the expression of the GAL4 protein.

Performing Loss-of-function experiments is a classical way of analyzing the function of genes by observing the phenotype obtained when one, or several genes are disrupted. In these studies we used a number of different ways to disrupt the function of the genes of interest; for instance EMS-induced mutant alleles, as well as alleles produced by insertions of transposable elements in the coding region of the gene.

In paper III we studied globally important developmental genes i.e., the Notch signaling pathway, and here homozygous embryos are often severely malformed and die earlier than in the stage of interest. To overcome this problem we used transgenic flies where the GAL4/UAS-system is used to drive expression of hairpin RNA. These double stranded RNA's are processed into siRNAs which direct sequence specific degradation of the target mRNA. By using a late, neural specific GAL4-driver and UAS-dsRNA targeting genes in the Notch pathway we were able to knock down genes in a restricted spatial and temporal manner. We could also use the GAL4/UAS-system to drive repressors of Notch effectors as well as dominant-negative forms of molecules in the Notch pathway, and by this approach interfere with the Notch signal.

In gain of function experiments, a gene is expressed in a context where it normally not is expressed. The purpose of these experiments is to serve as complement to a loss-of-function-experiment and as a proof-of-principle. These types of experiments can be valuable, but it should be noted that both the context and the expression levels of a gene may influence the phenotype in unpredicted ways, and the results can be difficult to interpret.

RESULTS AND DISCUSSION

Paper I

Introduction

Earlier work had shown how the NB 5-6 lineage develops and how temporal and positional cues in an increasingly restrictive manner controls the specification of the progeny produced by the NB5-6T. In addition, studies had identified combinatorial transcription factor codes playing out in the post mitotic neurons, which acted to dictate final sub-type identity. It has also been shown that the last cell produced by the NB, Ap4, is critically dependent upon a retrograde BMP signal, provided by the axonal target, for its proper specification and subsequent expression of the *FMRFa* neuropeptide. However, these studies also indicated that there are unknown genes acting in these different mechanisms including pattern formation, stem cell competence, cell cycle control, cell specification, axon path finding, axon transport and retrograde signaling. The Ap neuron model system thus presented an advantageous opportunity to identify novel genes acting on any level of neuronal development.

In the developing *Drosophila* ventral nerve cord, *FMRFa* is only expressed in the six Ap4 neurons. We took advantage of the restricted expression pattern of this terminal differentiation gene, generated *FMRFa-GFP* transgenic flies and conducted a forward genetic screen to identify genes involved in NB 5-6 development. The screen identified many known Ap neuron determinants, confirming the utility of the screen, however the majority of the mutants represent novel genes with previously unknown functions in NB 5-6 development.

Results and Discussion

Previous studies had identified an enhancer fragment upstream of the *FMRFa* gene that drives reporter expression in the Ap4/*FMRFa* neurons (Schneider et al., 1993).

Results and discussion

Using this enhancer, transgenic fly lines were generated where GFP is co-expressed with *FMRFa*.

We treated the *FMRFa-GFP* reporter flies with the mutagenizing agent ethyl methanesulfonate (EMS), and established 9,781 balanced stocks of single mutated chromosomes, on the 2nd or 3rd chromosome. Embryos from these mutated stocks were screened for *FMRFa-GFP* expression as intact preparations under a fluorescent microscope, and 611 mutants with aberrant GFP expression were identified.

The mutants were categorized into different phenotypic classes based on their GFP expression. These groups could be further subdivided by staining with antibodies against different identity markers. The mutants among each of these phenotypic categories were then tested for failure to complement each other, as well as alleles of previously known genes that give the same phenotype. This work resulted in the identification of several new alleles of previously known genes. However, because the initial complementation analysis did not identify the affected gene in the vast majority of the mutants, we initiated a mapping procedure based on failure to complement a series of overlapping deletions. This work is not finished at the writing of this thesis, but to this date genes with several different molecular functions have been identified in the screen.

One group of mutants that did not express the Ap neuron determinant *Eya* in the Ap cluster was identified to belong to the Polycomb group. Polycomb group proteins are known to repress Hox homeotic genes through histone modifications (Francis et al. 2001). Earlier work have demonstrated that the Hox gene *Ultrabithorax (Ubx)* is critical for the control of the lineage-size difference between abdominal NB 5-6 and thoracic NB 5-6, where misexpression of *Ubx* in the thoracic segments truncates the thoracic NB 5-6 lineage and the Ap cells are never generated (Karlsson et al. 2010). Moreover, the Hox gene *Antp* acts together with the temporal gene *cas* to specify the Ap neurons (Karlsson et al. 2010). A number of different members of the Polycomb group were identified in the screen, and they could be further subdivided regarding two main differences in their *Eya* expression, where one group fail to express *Eya* globally in the VNC and the other group displayed a specific loss of *Eya* expression in the Ap neurons. The identified mutants belong to the same protein complex; the *Drosophila* Polycomb repressive complex 1 (PRC1). Strikingly, recent work have

demonstrated that the target genes of the PRC1 can be divided in two groups depending on the different subunits present in the complex (Gutiérrez et al., 2012). Further characterization of Polycomb group proteins using the Ap neuron model can give more insight in their role in Hox gene silencing in the CNS.

One group of mutants was identified where each hemi segment contained not one, as in wild type, but instead two or three *FMRFa-GFP* expressing cells. Of these mutants, a number were mapped to two critical positive components of the Notch signaling pathway; the metalloprotease Kuzbanian and the ubiquitin ligase Neuralized. The Notch pathway has several known roles in neural development, but the possible role during Ap neuron specification was unknown. The identification of the Notch pathway genes in this screen inspired us to address the role of Notch signaling in Ap neuron specification (Paper III).

Another group of mutants displayed none or low *FMRFa* expression in the Ap neurons, but in the subsequent categorization these mutants showed more *Eya* expressing cells in the Ap cluster than normal. One mutant in this group was mapped to the *seven up (svp)* gene. *Svp* is the *Drosophila* ortholog of COUP-TF1/2, an orphan receptor of the steroid/thyroid receptor family. Previous studies have identified *svp* as critical for proper progression of the temporal transcription factor cascade that control competence changes in neuroblasts. However, this function did not explain the phenotype identified in this screen. To explore the function of *svp* in Ap neuron specification we conducted detailed studies of the expression and function of *svp* in the NB 5-6 lineage (Paper II). This revealed that *svp* plays dual roles in this lineage: first, controlling a switch in the early canonical temporal cascade, by controlling the down-regulation of *Hb*, and second, to sub-divide the late temporal Cas window, thereby ensuring proper Ap neuron specification.

In this screen we identified both known and novel genes acting during neural development, and the high resolution of the NB5-6 lineage gave us opportunities to discover new actions of previously identified genes.

Paper II

Introduction

One group of mutants identified in the screen described above, was mapped to the *seven-up (svp)* gene, which is the *Drosophila* ortholog to the mammalian *COUP-TF1/2* gene; an orphan receptor of the steroid /thyroid receptor family. The Ap cluster consists of four neurons that are born sequentially directly from the neuroblast. All four Ap neurons express the transcriptional co-factor Eya, which can be used as an Ap neuron marker. The first born neuron is the Ap1 neuron, which expresses the neuropeptide Nplp1. Thereafter two non-peptidergic neurons, Ap2 and Ap3, are born, followed by the last born neuron, Ap4, which expresses the neuropeptide FMRFa (Baumgardt et al 2007). In the identified *svp* mutant, the number of Eya expressing neurons in the Ap cluster were elevated from 4 to ~6 cells. In addition to this, Nplp1 was often ectopically expressed while FMRFa expression was absent.

Neural stem cells in both insects and vertebrates undergo temporal changes in their competence. In *Drosophila*, temporal competence is under the control of an intrinsic cascade of transcription factors. This cascade consist of progressive expression of the Hunchback (Hb), Kruppel (Kr), Pdm, Castor (Cas) and Grainyhead (Grh) transcription factors. It has previously been shown that a first wave of Svp expression is required, together with other factors, to down regulate Hb at the proper time (Kanai et al. 2005; Mettler et al 2005; Kohwi et al 2011).

We found it unlikely that this known function of *svp* would cause the phenotype identified in our screen. Since Eya expression is critically dependent upon Cas (Baumgardt et al., 2007), a premature stop in the temporal cascade would rather abolish Eya expression in the Ap neurons, than activate Eya expression ectopically as in our mutant. We analyzed the expression of Svp in the NB 5-6T lineage, and as expected from previous studies in other lineages, we found that Svp is expressed in two distinct temporal pulses. Mutant analyses further revealed that Svp has two distinct functions in the specification of the Ap-neurons.

Results and discussion

To address the expression and function of Svp in the NB 5-6 lineage we used the lineage-specific *lbe-GFP* transgenic marker. We observed a short pulse of Svp expression at stage 10 in NB 5-6, which correlates with our finding that in *svp* mutants Hb is not always properly down-regulated. The late pulse of Svp expression has previously been observed in other lineages, but no function of this late expression has been reported. In the NB 5-6 lineage we found a dynamic expression pattern that commences in the neuroblast at stage 14, and later in all four Ap neurons. The expression of Svp is then down-regulated in the two peptidergic neurons Ap1 and Ap4, but maintained in the Ap2 and Ap3. In *svp* mutants there are two types of Ap clusters. One type where Ap neurons are missing entirely, which correlates in prevalence with the number of cases where Hb is not down regulated, and another type where there is an increase in the number of Ap neurons. Furthermore, *svp* mutants display ectopic expression of the peptidergic regulator Dimmed (Dimm). In wild type, Dimm is only expressed in the Ap1 and Ap4 neurons, whereas the Ap2 and Ap3 neurons expressing Svp, do not express Dimm. Earlier work showed that an intriguing genetic feed forward mechanism is responsible for the specification of the four different identities of the Ap neurons (Baumgardt et al 2009). In this mechanism the Ap neuron determinant Collier (Col) has an important role. In *svp* mutants Col is expressed in all Ap neurons, but the critical down regulation of Col that is needed for proper sub-specification among the Ap-neurons does not occur. In a gain-of-function experiment where Svp was misexpressed from a post-mitotic driver, the results were in line with the loss-of-function phenotypes: Loss of Dimm expression and a weakened expression of Col, as well as a total loss of Nplp1 and FMRFa.

The Ap-neurons are born in the Cas/Grh window, and previous work has shown that Cas itself is initiating the feed forward loop that sub-divides the Cas-window and specifies the Ap neurons. In line with this we find that the late pulse of Svp expression is regulated by Cas. However we find no evidence for Svp regulating Cas nor down regulating the last temporal gene in the cascade, Grh.

Based upon these results, we can conclude that Svp in the late expression pulse acts as a sub-temporal factor, fine-tuning the Cas window into three different temporal parts.

To our knowledge it is the first report of a gene playing dual temporal roles in the same neural lineage.

Paper III

Introduction

It is well established that neural progenitors, in both vertebrates and invertebrates, generates daughter cells with different proliferation potential i.e., daughter cells can divide multiple times, once or directly differentiate into neurons/glia. Previous studies had demonstrated that the four Ap neurons are born at the very end of the NB 5-6 lineage, and that there is a switch in division mode so that the NB instead of dividing into a new NB and a GMC, the NB now divides into a NB and a differentiating neuron (Baumgardt et al 2007, Baumgardt et. al 2009).

In our genetic screen we identified mutants in the Notch pathway that had an excess number of Ap neurons born from NB 5-6. To confirm that we were indeed dealing with the canonical Notch signaling pathway, we analyzed core components from all different parts of the Notch pathway with regard on the number of Ap cells and their final identity. In all cases we found additional cells compared to wild type, but interestingly the specification of the neurons seemed unaltered.

Notch signaling is known to act early in neurogenesis to restrict the number of neuroectodermal cells that acquire a neuroblast fate. Mutations in genes in the Notch pathway show hyper plastic CNS's due to this overproduction of neural stem cells. Thus we hypothesized that these extra Ap neurons were the result of additional NB 5-6T producing ectopic lineages. However, the fact that *kuzbanian* (*kuz*), one of the Notch pathway genes identified in the screen, is maternally provided, and thereby allowing for proper early Notch signaling, together with the fact that we never saw more than two FMRFa positive cells in each hemisegment in *kuz* mutants, made us speculate that the extra Ap neurons in our mutants had another origin than ectopic NB 5-6T lineages. Initial analysis of our *neur* and *kuz* alleles, using the lineage specific *lbe* marker, revealed that there are indeed two distinct mechanisms that produce extra Ap neurons in Notch pathway mutants. In strong Notch pathway mutants, such as *neur*, the extra Ap neurons were accompanied by a total increase in NB 5-6 cells, as well as extra NB 5-6 neuroblasts, revealed by the expression of the neuroblast marker Deadpan (Dpn). However, in *kuz* mutants we saw no sign of increased early NB

lineage size, nor could we see any extra Dpn positive neuroblasts. Thus, these extra Ap neurons must be generated by another Notch mediated mechanism, acting in a single NB 5-6 lineage. To address this we initiated a detailed analysis of the mitotic events, and the temporal progression of Ap neuron determinants in the latter part of the NB 5-6T lineage.

Results and discussion

In order to address the role of Notch signaling late in the lineage progression we used a multitude of different late perturbations of the Notch pathway, using the Gal4/UAS system, as well mutants of the maternally provided *kuz* gene. In all cases we could see extra Ap neurons produced, within one single NB 5-6T lineage. These extra neurons can be the result of three different scenarios:

- I: a failure of the neuroblast to exit the cell cycle,
- II: a premature initiation of the production of neurons with the Ap-neuron fate,
- III: a failure of the neuroblast to switch from producing direct neurons and instead proceed with the production of GMC's, which in turn divides into two neurons.

To distinguish between these possibilities we analyzed the temporal progression of different Ap determinants in control and in *kuz* mutants. There was no evidence for a shift in the NB 5-6T temporal progression in the mutant background. We also stained with the cell death marker cleaved caspase-3 in both control and *kuz* mutants to address if the neuroblast exits the cell cycle and dies by apoptosis in a normal way, revealing no difference between mutants and control. In addition to this, we could not find any cell divisions in the NB in *kuz* mutants after stage 15, which showed that the terminal cell cycle exit is unaltered in the mutant background. To analyze the division pattern in the late part of the lineage, we used pH3-labelling, which labels cells from prophase to cytokinesis. In control, we find only one dividing cell; the neuroblast. However, in *kuz* mutants we find two simultaneously dividing cells in 37% of the cases, which indicates that in addition to the neuroblast, there is a daughter cell dividing.

Finally, to determine sibling cell relationship i.e., to determine if the cells are born sequentially from the neuroblast or are results of a GMC dividing into two neurons, we performed DNA-labeling with EdU-injections. In several different genetic perturbations of Notch signaling, the two FMRFa neurons and the two Nplp1 neurons could never be separated from each other, and must thus be generated as sibling pairs.

This detailed analysis of the role of the canonical Notch signaling pathway reveals that Notch signaling is critical for controlling the intriguing “direct-neuron switch” in the latter part of the NB 5-6T lineage. Notch is not involved in controlling the GMC divisions earlier in the lineage, where instead *prospero* (*pros*), as anticipated, (Li and Vaessin, 2000), restricts their mitotic potential. These data implies that Notch is activated in the neuroblast prior to the switch and our results show expression of the Notch target gene *E(spl)m8* in the neuroblast, in this temporal window. *Pros* is expressed in the late part of the lineage but is not involved in the topology-switch, as in *pros* mutants the number of Ap neurons resembles wild type. To address if there is cross-regulation between *prospero* and Notch we analyzed the activation of Notch target genes in a *pros* mutant background and analyzed *Pros* expression and trafficking in Notch mutants. In no case we could find evidence for cross regulation between the two pathways.

Previous studies in other lineages e.g., in the larval brain, have identified switches in lineage topology, where for instance neuroblasts go from producing GMC's to intermediate precursors with higher mitotic potential. There are also examples of lineages with a similar direct-neuron switch as in the NB 5-6T lineage. However to our knowledge, our data for the first time demonstrate that active Notch signaling in the neuroblast is a critical input for the proper switching in stem cell division mode.

When we mutated each pathway alone the result was over-proliferation of daughter cells, but in different parts of the lineage. Mutating both pathways simultaneously, and thereby removing both proliferation control mechanisms, resulted in massive over-proliferation of all daughter cells. Finally, since we found no evidence of regulation between the Notch pathway and the temporal cascade, it allowed us to test the concept of topology-temporal interplay, by independently and simultaneously manipulating daughter cell proliferation and temporal progression. In this way we were able to predictable control cell fate and cell numbers born from a single

Results and discussion

progenitor. This demonstrates that the switch in proliferation potential, is integrated with temporal progression and thereby controls the numbers of different Ap neuron subtypes produced.

CONCLUDING REMARKS

The results presented in this thesis, provides new genetic insights into developmental neurobiology and stem cell biology. The initial screen was designed to give a high phenotypic resolution, and help identify genes that are involved in mechanisms underlying both the control of cell identity and of cell numbers. The terminal cell fate marker *FMRFa-GFP*, in combination with high throughput methods, allowed us to screen some 10,000 mutated chromosomes, and thereby to identify several hundreds of mutants affecting NB 5-6 development.

In the screen we identified the previously described *svp* gene, but the phenotype we found did not resemble the expected. We initiated a detailed expression analysis of Svp in the NB 5-6 lineage and we could find that Svp has a dynamic expression pattern with two distinct pulses. Mutant analysis further revealed that *svp* plays two different roles in the NB 5-6 lineage. First it acts as a switching factor in the temporal cascade to ensure proper temporal progression. In the late expression pulse *svp* acts as a sub temporal gene, now controlled by the temporal gene *cas*. In this phase Svp expression is again dynamic and is critical for sub specification of the Ap neurons. These data show that a temporal gene can play dual roles in the same lineage. Vertebrate neural progenitors generate stereotypic lineage trees, with different types of neurons and glial cells born in determined temporal sequences. In mammals, the *svp* orthologues *COUP-TF1/2* are also expressed dynamically in the developing CNS (Qin et al., 2007). Interestingly, recent data show that COUP-TF1/2 acts in a temporal manner to control the timing generation of subclasses of neurons and glia in the mouse brain (Naka et al., 2008).

The restricted expression pattern of our reporter revealed mutants with apparently subtle phenotypes: two cells instead of one in each hemi segment. However the following detailed analysis of these mutants using mitotic and identity markers revealed that the origin of these extra cells was not random. Instead they were the

Concluding remarks

result of a genetically programmed switch in the mitotic potential of the daughter cells produced in the end of the lineage.

We could identify two different proliferation controls, within the same lineage; Prospero and Notch, which act in concert to control daughter proliferation. Prospero acts in the early lineage to control the mitotic potential of the intermediate progenitor, the GMC. In the latter part of the lineage we identified Notch signaling as the daughter proliferation switch, which restricts the mitotic potential of the daughter cell even more. This demonstrates that a neural lineage can depend on two complementary regulatory mechanisms controlling the proliferation behavior of the daughter cells, and thereby achieve distinct branching of the lineage tree.

We could also conclude that these pathways do not regulate each other. Moreover, they do not regulate or are regulated by the temporal genes. By mutant analysis we could study the integration between temporal progression and lineage branching, and by this identify a novel mechanism for a stem cell to control the number of progeny with distinct identities. Alternate daughter proliferation patterns in both vertebrates and invertebrates give rise to the proper branching of lineage trees, ensuring the right proportions of stem cells, intermediate precursors and postmitotic cells. In addition to this, stereotyped temporal transitions have been shown to occur in vertebrates in the same manner as in invertebrates. Given the high conservation in the regulators controlling these events, this novel perspective of proliferation and temporal integration, can be utilized in a variety of stem cell systems.

FUTURE CHALLENGES

In the genetic screen we identified several both known and novel genes, and we chose to further study two already known genes; *svp* and *Notch*. The high resolution of the screen and the extensive knowledge regarding the Ap neuron model revealed unknown functions of these genes, which led to the discovery of new genetic mechanisms. Given that, it is tempting to speculate that among the remaining mutants identified in the screen, a myriad of unexplored biological mechanisms are hiding. Further mutant analysis, and identification of the causative genes involved in the phenotypes would contribute to our understanding of neural development.

A number of mutants identified in the screen belong to the Polycomb group. These genes have been shown to repress the transcription of developmental regulator genes through chromatin modification. The genes identified in the screen belong to the same complex: the *Drosophila* polycomb repressive complex 1 (PRC1). Despite belonging to the same complex they show distinct phenotypes regarding expression of Ap neuron determinants. Recent work show that the target genes of the PRC1 complex differs depending on distinct subunits present in the complex. Further analysis of the Polycomb genes, using the Ap neuron model could give new insights in chromatin modification as a mechanism for cell specification in neural development.

To this date there are a number of unanswered questions regarding the NB 5-6 lineage progression. For example, what is the mechanism behind the activation of Notch signaling in the NB prior to the proliferation switch? When we analyzed ligand expression using different GAL4 drivers, we could see that we need to abolish ligand expression pan-neurally to interfere with the Notch signal in the NB 5-6T. This indicates that ligand expression is critical for the proliferation switch, but the ligand does not specifically come from within the 5-6 lineage. Is the increasing sensitivity to ligand expression intrinsically controlled in the neuroblast? The most obvious intrinsic regulator of the Notch mediated proliferation switch would be Cas, however in Cas mutants we do not see any negative impact on the Notch signal in NB 5-6T. Addressing the intersection of Notch signaling with other temporal transitions in the NB could give new insight in both regulation of Notch signaling, as well as the use of signaling pathways in transcriptional control.

Future challenges

Another intriguing question is the identity of the target genes that are activated downstream this Notch mediated switch. Obvious target genes would be cell cycle regulators. Little is known about how cell signaling pathways interact with the cell cycle machinery, even though Notch signaling is implicated in a number of mammalian cancers. The *Drosophila* system can give new insights with high resolution in the causative relationships between Notch signaling and the regulation of cell cycle control.

Another interesting topic is the underlying control of unique lineage sizes in the CNS. The lineage size produced by the different NBs within a hemi segment varies from 2 to 40 cells. Given the stereotyped progression of the temporal transcription factor cascade, what is the underlying mechanism for this variation? An interesting phenotype we could see in *svp* mutants is the finding of two extra Ap neurons, indicating imprecise cell cycle exit. Studies in other neuroblasts, both embryonic and postembryonic have also shown roles for *svp* as well as the temporal genes *grh* and *cas* in these decisions. These findings indicate that lineage size is dependent upon at least two independent cell cycle decisions where branching of the lineage tree and terminal NB cell cycle exit depend upon distinct mechanisms.

The mechanisms identified in this thesis are hitherto studied in one unique *Drosophila* neural lineage. It would be interesting to explore how global they are utilized both in other *Drosophila* neural lineages as well as in vertebrate model systems.

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