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REGULATION OF NEUROGENESIS IN THE VERTEBRATE CNS

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ABSTRACT

During development, neurons are generated from self-renewing progenitor cells in the ventricular zone of the neural tube. Proneural bHLH proteins are involved in the regulation of neurogenesis and can direct the exit of neural progenitors from the cell cycle and promote the expression of downstream differentiation markers. The expression of proneural bHLH proteins is, in turn, regulated by the activation of the Notch signaling pathway. Thus, whether neural stem cells differentiate or remain as progenitors is dependent on the interaction between Notch receptors and their ligands and the expression of proneural proteins. However, proneural proteins are expressed in proliferating cells that are not yet committed to differentiate, indicating the presence of other factors that actively counteract neurogenesis and keep cells undifferentiated.

The HMG-box containing transcription factors, Sox1, Sox2 and Sox3 (Sox1-3) are expressed by most progenitor cells in the developing CNS. Sox1-3 are generally downregulated when neural cells start to differentiate, indicating a regulatory role of Sox1-3 during neuronal differentiation. In paper I, we found that Sox1-3 proteins have the ability to maintain progenitor cells in an undifferentiated state and suppress neuronal differentiation. Our analysis showed that the activity of Sox1-3 does not influence the expression of proneural bHLH genes, but instead blocks the ability of proneural proteins to induce downstream events of neuronal differentiation. Furthermore, the capacity of proneural proteins to induce differentiation seems to be dependent on their ability to repress the expression of Sox1-3 genes. We also showed that active repression of Sox1-3 target genes results in a premature differentiation of progenitor cells. Thus, in paper I we showed that Sox1-3 can maintain progenitors undifferentiated and that the proneural protein-mediated repression of Sox1-3 proteins represent an irreversible commitment step of neuronal differentiation.

Although proneural proteins are known to promote neuronal differentiation, it is not yet known how these factors that function as transcriptional activators can induce neural cells to suppress progenitor characteristics and commit to neuronal differentiation. The expression pattern of Sox21, a member of the Sox gene family, within the ventricular zone indicated that this protein might have a more general role in the regulation of neurogenesis and, therefore we investigated the role of Sox21 during the progression of neurogenesis in the chick spinal cord. In paper II, we showed that Sox21 has the ability to promote differentiation of neural cell. Interestingly, Sox21 has the opposite effect compared to Sox1-3, even though these proteins belong to the same group of the Sox gene family. We showed that the different activities of Sox21 and Sox1-3 appear to reside in the C-terminal domain of these proteins and that Sox21 mediates it function by counteracting the activity of Sox1-3. Thus, the balance between the repressive activity of Sox21 and the activity of Sox1-3 appears to determine if neural cells remain as progenitors or commit to differentiation. Finally, we demonstrated that the ability of Sox21 to promote neural cells to differentiation is independent of the cell intrinsic levels of proneural protein activity. However, the ability of proneural bHLH proteins to drive neurogenesis seems to be dependent on their ability to upregulate the levels of Sox21 expression. Together, these findings establish a key role for Sox21 in the progression of neurogenesis and further indicate that an important function of proneural proteins during neurogenesis is their capacity to upregulate the expression of Sox21.

Even though 50-70 million years has passed since the human and rodent genomes diverged, it is still possible to align 40% of the human and mouse genomes at the nucleotide level. A fraction of the aligned sequences are Highly Conserved Non-coding Regions (HCNRs) and Ultra Conserved Regions (UCRs) that exhibit an extremely high level of conservation. In paper III, we examined the functional role of these HCNRs by starting with a set of twelve genes encoding Homeodomain (HD) proteins that function as transcriptional repressors and are involved in dorsal-ventral patterning of the spinal cord. We could demonstrate that a majority of HCNRs associated with these HD genes are enriched for binding sites of Sox (S), POU (P) and HD transcription factors. By using a predictive computational model we could show that a significant portion of the HCNRs in vertebrate genomes contain binding sites of Sox, POU and HD transcription factors ($SPHD^{+}$). Furthermore, these $SPHD^{+}$ HCNRs can be linked to hundreds of genes that are expressed in the developing CNS. They are transcriptionally active in neural progenitor cells and the activity of these HCNRs are dependent on Sox and POU proteins. In summary, our data revealed an unifying feature for a large portion of vertebrate HCNRs and imply that $SPHD^{+}$ HCNRs are involved in a transcriptional core program, which is involved in the neural expression of a large set of genes during development. It also suggests a common transcriptional logic for these HCNR-linked genes in which Sox/POU proteins function as generic drivers of CNS expression, while the positional control of gene expression is regulated by HD-mediated transcriptional repression.

LIST OF PUBLICATIONS

This thesis is based on the following original papers, which are referred to in the text by their Roman numerals:

I. **Magdalena Bylund**, Elisabeth Andersson, Bennett G. Novitch, and Jonas Muhr (2003).
Vertebrate neurogenesis is counteracted by Sox1-3 activity.
Nature Neuroscience 11, 1162-1168.

II. Magnus Sandberg, **Magdalena Källström**, and Jonas Muhr (2005).
Sox21 promotes the progression of vertebrate neurogenesis.
Nature neuroscience 9, 995-1001.

III. Peter J. Bailey, Joanna M. Klos, Elisabeth Andersson, Mattias Karlén, **Magdalena Källström**, Jasmina Ponjavic, Jonas Muhr, Boris Lenhard, Albin Sandelin, Johan Ericsson (2006).
A global genomic transcriptional code associated with CNS-expressed genes.
Experimental Cell Research 13, 3108-3119.

CONTENTS

LIST OF ABBREVIATIONS

BMP	Bone morphogenetic protein
bHLH	Basic helix-loop-helix
CNS	Central nervous system
EC cells	Embryonic carcinoma cells
ES cells	Embryonic stem cells
FGF	Fibroblast growth factor
HMG	High-mobility group
PNS	Peripheral nervous system
POU	Pit-Oct-Unc
Shh	Sonic hedgehog
Sox	Sry related HMG box
SRR1	Sox-2 regulatory region 1
SRR2	Sox-2 regulatory region 2
Sry	Sex-determining region of Y chromosome
SVZ	Subventricular zone

1 INTRODUCTION

The human nervous system consists of the central nervous system (CNS) and the peripheral nervous system (PNS) and contains two cell types: neurons and glia. The CNS consists of approximately 100 billions of neurons and most of these neurons are generated during embryonic stages by neural stem cells, which are located in the ventricular zone of the developing CNS. During neurogenesis these cells receive both intrinsic as well as extrinsic signals that promote the development towards the neuronal lineage, while glial fates are blocked. Thus, neurogenic signals are important for the proper regulation of neurogenesis that results in the generation of the correct number of neurons at the right time and place.

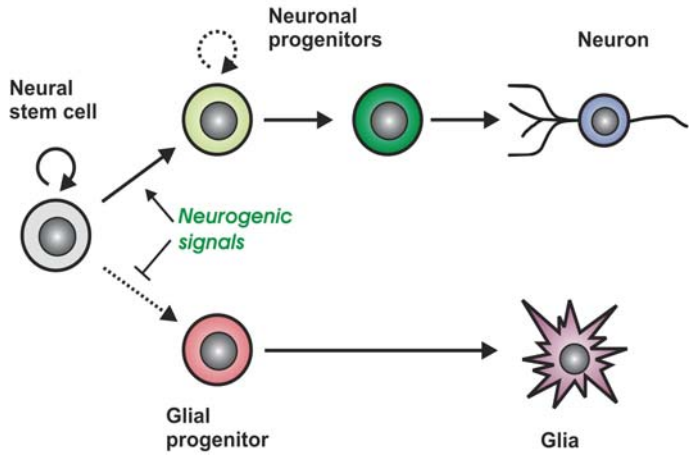

Figure 1 Generation of neurons from neural stem cells. During neurogenesis neural stem cells receive neurogenic signals that promote development towards the neuronal lineage, while blocking the glial fates.

1.1 EMBRYONIC STEM CELLS

Embryonic stem (ES) cells are derived from the inner cell mass (ICM) of the blastocyst and are capable of producing all cell types of an organism (Evans and Kaufman, 1981; Martin, 1981; Zhang, 2006). *In vitro*, these cells can both proliferate indefinitely and differentiate into all three germ layers (Liu et al., 2007; Pan and Thomson, 2007). However, maintaining ES cells as a pluripotent population requires a balance between survival, proliferation and self-renewing signals (Liu et al., 2007). In mice, there are several extrinsic signals that are important for the maintenance of self-renewal and pluripotency in ES cells (Liu et al., 2007; Pan and Thomson, 2007). Leukemia inhibitory factor (LIF) supports the undifferentiated state of mouse ES cells by activating the signal transducer STAT3 (Liu et al., 2007; Niwa et al., 1998). Interestingly, LIF cannot maintain human ES cells in an undifferentiated state, instead they need the presence of basic fibroblast growth factor (bFGF) (Amit et al., 2000; Xu et al., 2001) and mitogen-activated protein kinase (Kang et al., 2005) activity to be maintained as self-renewing cells. Another extrinsic factor is bone morphogenetic protein-4 (BMP4), which in the presence of LIF can enhance self-renewal and pluripotency of mouse ES cells by activating members of the Id (inhibitor of

differentiation) gene family (Liu et al., 2007; Pan and Thomson, 2007; Ying et al., 2003).

These external signals eventually lead to the regulation of genes that are involved in maintaining the pluripotent state of cells. The Oct4 (Niwa, 2001), Nanog (Pan and Thomson, 2007) and Sox2 (Avilion et al., 2003; Masui et al., 2007) genes are essential for keeping mouse ES cells in a pluripotent state. Loss of the POU protein Oct4 causes inappropriate differentiation of ES cells into trophectoderm, while the overexpression of Oct4 results in differentiation of ES cells into primitive endoderm and mesoderm (Liu et al., 2007; Niwa, 2001; Yeom et al., 1996). Nanog is a homeobox-containing transcription factor and disrupting the expression of this protein in ES cells results in the differentiation of these cells into endoderm lineages (Liu et al., 2007; Pan and Thomson, 2007). In addition, overexpression of Nanog causes mouse ES cells to self-renew independently of LIF, although the self-renewing capacity is reduced (Chambers et al., 2003; Liu et al., 2007; Mitsui et al., 2003; Pan and Thomson, 2007). These three factors have also been suggested to form an intrinsic regulatory core unit with the ability of maintaining ES cells in a pluripotent state at least *in vitro* (Boyer et al., 2005; Boyer et al., 2006; Ivanova et al., 2006; Loh et al., 2006; Rao and Orkin, 2006).

1.2 NEURAL STEM CELLS

During the development of the mammalian CNS, neural stem cells give rise to all neurons as well as being the source of glial cells (Gotz and Huttner, 2005). The self-renewal of neural stem cells can occur either by symmetric cell division, which generates two daughter cells with the same fate, or by asymmetric cell division that generates one daughter stem cell and a second cell, which differentiates into for example a neuron (Gotz and Huttner, 2005; Temple, 2001). At the onset of CNS development the neuroepithelial cells, which are localized in the ventricular zone, are regarded as neural stem cells, since they can self-renew and give rise to neurons, oligodendrocytes and astrocytes (Pevny and Rao, 2003). *In vitro*, the neurosphere assay has been used to demonstrate that neuroepithelial cells are stem cells. In the presence of bFGF, epidermal growth factor (EGF) or both, stem cells can be maintained as a source of undifferentiated progenitors, which can be expanded as neurospheres, or be induced to differentiate into neurons, astrocytes and oligodendrocytes (Gritti et al., 1995; Gritti et al., 1999; Gritti et al., 1996; Pevny and Rao, 2003; Qian et al., 1997; Reynolds and Weiss, 1992; Reynolds and Weiss, 1996; Weiss et al., 1996a; Zhang, 2006).

In general, glia cells are generated after neurons during development, an exception to this rule is radial glia cells (Gotz and Huttner, 2005; Malatesta et al., 2000; Noctor et al., 2001), which are generated already at the onset of neurogenesis. Radial glia cells contain long radial processes, express the glial marker glial fibrillary acidic protein (GFAP), contain glycogen granules and functions as guides for migrating neurons (Anthony et al., 2004; Gotz and

Barde, 2005; Gotz and Huttner, 2005; Malatesta et al., 2000; Noctor et al., 2001). Most of the radial glia cells are more fate-restricted compared to neuroepithelial stem cells and can only generate single type of cells, either astrocytes, oligodendrocytes or neurons (Gotz and Huttner, 2005; Malatesta et al., 2000; Noctor et al., 2001; Williams and Price, 1995). Furthermore, radial glia cells successively replaces the neuroepithelial stem cells (Gotz and Huttner, 2005). They are mitotically active throughout neurogenesis (Misson et al., 1988) and disappear or differentiate into astrocytes when neural migration is complete (Chanas-Sacre et al., 2000; Misson et al., 1991).

Adult stem cells within the mammalian CNS have been shown to reside within the subgranular zone of the dentate gyrus and the forebrain subventricular zone (SVZ) that lies next to the ependymal layer of the lateral ventricle (Alvarez-Buylla and Temple, 1998; Temple and Alvarez-Buylla, 1999). The dentate gyrus generates hippocampal interneurons (Kaplan and Bell, 1984), while the forebrain SVZ generates interneurons that migrate into the olfactory bulb (Lois and Alvarez-Buylla, 1994; Luskin, 1993), where neuronal incorporation continues into adulthood (Temple and Alvarez-Buylla, 1999). Self-renewing cells from the adult SVZ have been propagated in vitro in both adherent and non-adherent cultures by using high concentrations of EGF, bFGF or both (Gage et al., 1995; Morshead et al., 1994; Reynolds and Weiss, 1992; Weiss et al., 1996b). When these factors are removed cells can differentiate into neurons, astrocytes and oligodendrocytes (Alvarez-Buylla et al., 2001; McKay, 1997) and, therefore these cells are considered as neural stem cells (Alvarez-Buylla et al., 2001).

2 DEVELOPMENT OF THE CNS

Within the fertilized egg lies the information, which is needed for the generation of all the tissues and organs. Before these different structures can be produced the fertilized egg has to go through the cleavage stage, a process of rapid cell divisions that divide the egg into numerous cells and leads to the formation of a blastula. During gastrulation, the cells of the blastula are rearranged and the three germ layers, endoderm, mesoderm and ectoderm, are established. The cells that will give rise to the mesodermal and endodermal germ layers are brought into the interior of the embryo, while the cells that remain at the surface will form the ectoderm (Gilbert, 2003). These different cell movements are coordinated by the organizer, a structure that has the ability to organize embryonic development (Harland and Gerhart, 1997). The instructive properties of the organizer were discovered by Spemann and Mangold (1924). When they grafted a region containing the dorsal lip of the blastopore into the ventral side of a recipient gastrula newt a secondary axis with correct anterior-posterior and dorsal-ventral patterns developed in the host embryo (Chang and Hemmati-Brivanlou, 1998) and, therefore this structure is called the Spemann's organizer. The second nervous system that developed was not originating from the transplanted tissue, but originated from the ventral ectoderm of the host embryo, which if left undisturbed, would develop into epidermis (Hemmati-Brivanlou and Melton, 1997b; Stern, 2005). Soon after the discovery of the Spemann's organizer the equivalent region was identified in most vertebrate classes, including the embryonic shield in zebrafish and Hensen's node in birds and mammals (Stern, 2005). The three germ layers of the embryo respond differently to the signals from the organizer. Thus, the endoderm responds to the organizer's signals by becoming the anterior gut (by anteriorization of the endoderm), the mesoderm responds by becoming dorsal mesoderm (by dorsalization of the mesoderm) and the ectoderm responds by becoming the neural plate (by neural induction) (Harland and Gerhart, 1997).

2.1 NEURAL INDUCTION

Experiments conducted by Spemann and Mangold firmly established the concept of neural induction as an instructive interaction between the organizer and the neighboring ectoderm, which leads to the induction of the nervous system (Hemmati-Brivanlou and Melton, 1997b; Stern, 2005). Based on this it was assumed that the development of epidermis was a default fate for the gastrula ectoderm and that it did not require any cell-cell communication, whereas neural specification was induced with signals from the cells located in the dorsal mesoderm (Gilbert, 2003). However, experiments in which the ectodermal cells were dissociated revealed that neural induction in vertebrates could occur in the absence of instructive or positive signals from the dorsal mesoderm (Godsave and Slack, 1989; Grunz and Tacke, 1989; Sato and Sargent, 1989). Furthermore, misexpression of a dominant-negative activin receptor in *Xenopus* embryos both blocked mesoderm formation and generated ectopic

neural tissue (Hemmati-Brivanlou and Melton, 1992; Hemmati-Brivanlou and Melton, 1994). These findings suggested that there might be negative or inhibitory signals within the ectoderm that could prevent neuralization and promote the epidermal fate (Hemmati-Brivanlou and Melton, 1997b; Hemmati-Brivanlou and Melton, 1994; Stern, 2005). Thus, implying that it is the neural and not the epidermal fate, which is the default state of the ectodermal cells.

The organizer expresses a number of proteins, including Chordin (Sasai et al., 1995), Noggin (Lamb et al., 1993; Smith and Harland, 1992; Smith et al., 1993), Follistatin (Hemmati-Brivanlou et al., 1994) and Cerberus (Bouwmeester et al., 1996) and misexpression of these proteins leads to neural induction in animal caps (Bouwmeester et al., 1996; Hemmati-Brivanlou et al., 1994; Lamb et al., 1993; Sasai et al., 1995; Smith and Harland, 1992; Smith et al., 1993). All these factors have in common that they inhibit BMP signaling in *Xenopus* (Fainsod et al., 1997; Piccolo et al., 1996; Re'em-Kalma et al., 1995; Zimmerman et al., 1996). At the blastula stage, BMP-4 ventralizes mesoderm (Fainsod et al., 1997), while the BMP antagonists have dorsalising activity when injected into early embryos. At the late blastula/early gastrula stages BMPs act as epidermal inducers and neural inhibitors (Wilson and Hemmati-Brivanlou, 1995), whereas the BMP antagonists can suppress epidermal fates and promote neural induction. All these results have led to the widely accepted "default model", which proposes that ectodermal cells are fated to become neural by default, but are normally inhibited from neural fate by BMPs expressed throughout the ectoderm, from which they have to be released for neural induction to occur (Hemmati-Brivanlou and Melton, 1997a; Munoz-Sanjuan and Brivanlou, 2002; Weinstein and Hemmati-Brivanlou, 1999). In the chick, the expression patterns of BMPs or BMP antagonists do not fit with the default model and misexpression of neither BMPs nor the antagonists gives the effect expected from this model (Streit et al., 1998). Furthermore, mouse mutants that lack Noggin and/or Chordin still develop a nervous system (Bachiller et al., 2000; McMahon et al., 1998). These results indicate that inhibition of BMP signaling might not be all that is needed for neural induction to occur and that additional factors might be involved in this process (Lamb and Harland, 1995; Linker and Stern, 2004; Streit et al., 1998).

Another factor that has been implicated in neural induction is fibroblast growth factor (FGF) (Lamb and Harland, 1995; Mathis et al., 2001; Streit et al., 2000; Wilson et al., 2000). FGFs are expressed in the organizer of the gastrula stage embryo and in the cells of the early epiblast (Isaacs et al., 1992; Wilson and Edlund, 2001; Wilson et al., 2000). Interestingly, in the epiblast the expression of FGFs has been demonstrated to be an effective repressor of BMPs (Streit et al., 2000; Wilson and Edlund, 2001; Wilson et al., 2000) and, thus FGF signaling seems to be required for neural induction in chick (Linker and Stern, 2004; Streit et al., 2000; Wilson et al., 2000).

Wnts are a large class of secreted glycoproteins that have been implicated in the selection of neural or epidermal fate in both *Xenopus* and chick (Bainter et al., 2001; Baker et al., 1999; Heeg-Truesdell and LaBonne, 2006; Stern, 2005;

Wilson and Edlund, 2001; Wilson et al., 2001). Neural tissue is induced directly in *Xenopus* ectodermal explants by various components of the Wnt signaling pathway and the misexpression of Wnts during pregastrula, but not gastrula stages, is sufficient to initiate neural induction (Bainter et al., 2001; Baker et al., 1999). Furthermore, Wnt signaling seems to down-regulate the expression of BMP (Baker et al., 1999). Interestingly, a recent study has demonstrated that blocking Wnt signals in the ectoderm led to an increase in the size of the neural plate, while upregulation of Wnt signals could inhibit the formation of the neural plate (Heeg-Truesdell and LaBonne, 2006). These contradictory results might be explained by another study, in which it was shown that high levels of stabilized β-catenin could induce the expression of Noggin and Chordin in animal caps (Wessely et al., 2001). In chicken, Wnt signaling blocks the FGF signaling in ectoderm cells and thereby BMPs are upregulated that can induce the epidermal fate in the ectoderm (Wilson and Edlund, 2001; Wilson et al., 2000). Thus, it seems like neural induction in most vertebrates is a bit more complicated than what the "default model" predicts, and probably is dependent on a combination of antagonists and agonists of BMP, FGF and Wnt signaling.

2.2 NEURAL PATTERNING

After neural induction, the neural plate is pattern along both the anterior-posterior (A-P) and dorsal-ventral (D-V) axes (Chang and Hemmati-Brivanlou, 1998). Neural cells with similar developmental potential are induced to express specific molecular markers in broad domains along the A-P axis that corresponds with the future forebrain, midbrain, hindbrain and spinal cord (Chang and Hemmati-Brivanlou, 1998; Gould and Grainger, 1997; Ruiz i Altaba, 1993). Vertical signals from the underlying mesoderm are important for the patterning of the A-P axis (Chang and Hemmati-Brivanlou, 1998; Ruiz i Altaba, 1993). According to Nieuwkoop's "activation/transformation" model the A-P axis is generated by the action of two signaling mechanisms. An initial "activation" signal that induces the neural plate, but only results in anterior tissues (forebrain). This is followed by a "transformation" phase that induces more posterior neural character (midbrain, hindbrain and spinal cord) (Domingos et al., 2001; Gould and Grainger, 1997; Slack and Tannahill, 1992) in the newly formed neural plate. Retinoic acid (RA) (Glover et al., 2006; Maden, 2007), Wnts (Domingos et al., 2001; Wilson and Edlund, 2001) and FGFs (Lamb and Harland, 1995) are all candidates for mediating the transforming signal.

The generation of different neuronal subtypes along the D-V axis of the neural tube is dependent on extracellular signals. Both BMPs secreted from the ectoderm overlaying the neural tube (Lee and Jessell, 1999) and Sonic hedgehog (Shh) released by the notochord underneath the neural tube (Briscoe and Ericson, 1999) are important for the generation of specific neuronal subclasses (Lee and Pfaff, 2001; Novitch et al., 2001). BMPs and Shh from these non-neural sources lead to the formation of the roof plate and floor plate, which also express BMPs and Shh and serve as secondary signaling centers for the

establishment of cell identity along the D-V axis (Jessell, 2000; Lee and Pfaff, 2001). The position at which the different neuronal subclasses are generated is dependent on the concentration of both BMPs and Shh (Briscoe and Ericson, 1999; Briscoe et al., 2000). The graded expression Shh induces the expression of homeodomain proteins, which are important both for the specification of progenitor cell identity (Briscoe et al., 2000) and for the generated neurons. The expression of homeodomain proteins is maintained in progenitor cells through a cell-autonomous mechanism (Muhr et al., 2001).

2.3 NEUROGENESIS

The ventricular zone of the neural tube, contains dividing progenitor cells that have the potential to generate both neurons and glia (Bertrand et al., 2002; Chang and Hemmati-Brivanlou, 1998; Gilbert, 2003; Lee and Pfaff, 2001). Generally, neurons are generated before glia, and the switch from neurogenesis to gliogenesis is controlled by both extrinsic and intrinsic signals (Qian et al., 2000; Temple, 2001). Proneural genes, which encode transcription factors of the basic helix-loop-helix (bHLH) proteins, are intrinsic determinants that have been suggested to take part in the switch between neurogenesis to gliogenesis (Bertrand et al., 2002). These factors are both necessary and sufficient to initiate the development of neuronal lineages and to promote the generation of progenitors that are committed to differentiation (Bertrand et al., 2002; Kageyama and Nakanishi, 1997; Lee, 1997).

The bHLH genes were first identified in *Drosophila*, including achaete, scute, lethal of scute, asense (the achaete-scute complex) and atonal (Bertrand et al., 2002). In vertebrates, many genes that are related to both the achaete-scute complex and atonal genes have been found. These bHLH genes are expressed at different stages of neuronal development, some of them are expressed early in dividing neural precursor cells, while others are expressed later in postmitotic cells (Kageyama and Nakanishi, 1997; Kageyama et al., 2005; Lee, 1997). Depending on whether they act early or late during the differentiation they can either be classified as determination factors or differentiation factors (Lee, 1997; Lee et al., 1995). In mammals, bHLH proteins such as Mash1 (mammalian achaete-scute homolog) (Gradwohl et al., 1996; Guillemot and Joyner, 1993; Guillemot et al., 1993), Ngns (neurogenins) (Ma et al., 1996) and Math1 (mammalian atonal homolog) (Akazawa et al., 1995) are expressed in neural precursor cells, while NeuroD (neurogenic differentiation) (Lee et al., 1995), NeuroD2 (McCormick et al., 1996) and Math2 (Shimizu et al., 1995) are expressed in postmitotic cells.

The neural bHLH proteins in mammals bind DNA as heterodimeric complexes that are formed with other bHLH proteins or E proteins, such as E2A (E12 and E47), HEB or E2-2 (Johnson et al., 1992; Massari and Murre, 2000). This heterodimerization has been proposed to be a prerequisite for proneural proteins to bind DNA. Interestingly, Id proteins have a higher affinity for E-proteins compared with proneural proteins and can compete with proneural

proteins and, thereby they function as passive repressors of proneural gene activity (Bertrand et al., 2002; Massari and Murre, 2000; Yokota, 2001).

Proneural proteins inhibit their own expression in adjacent cells, which prevent these cells from differentiating (Bertrand et al., 2002; Lee and Pfaff, 2001). This is achieved through a process termed lateral inhibition, in which the Notch signaling pathway is activated (Bertrand et al., 2002; Kageyama and Nakanishi, 1997; Kageyama et al., 2005; Lee, 1997). This signaling pathway is highly conserved across species and it is widely used in the regulation of cell fate during both invertebrate and vertebrate development (Artavanis-Tsakonas et al., 1999; Gaiano and Fishell, 2002; Lewis, 1998; Louvi and Artavanis-Tsakonas, 2006). The Notch family of proteins are cell-surface receptors that are activated by the ligands Delta and Jagged/Serrate (Artavanis-Tsakonas et al., 1999; Lewis, 1998). Both receptors and ligands are single-pass transmembrane proteins, which suggests that signaling through the Notch pathway requires cell-cell contact (Gaiano and Fishell, 2002). Expression of ligands in the future progenitor cells activates the Notch signaling cascade in neighboring cells, and this results in the expression of the bHLH Hes/Her/Esr genes in vertebrates (Artavanis-Tsakonas et al., 1999; Bertrand et al., 2002; Gaiano and Fishell, 2002; Lewis, 1998; Louvi and Artavanis-Tsakonas, 2006; Ohtsuka et al., 1999). The Hes/Her/Esr transcription factors can prevent cells from differentiating by repressing the transcription of proneural genes, through direct binding to the promoters of these genes and by the recruitment of the corepressor TLE/Groucho. In addition, Hes/Her/Esr proteins can also inhibit the activity of proneural proteins by sequestering E proteins (Hatakeyama et al., 2004; Kageyama and Nakanishi, 1997; Kageyama et al., 2005). Thus, the regulation of neurogenesis, which only allows a subset of cell to undergo differentiation, while keeping others as neural stem cells is important for generating a CNS with appropriate size, shape and cell arrangement.

3 THE SOX GENE FAMILY

3.1 THE DISCOVERY OF SOX PROTEINS

The mammalian testis-determining factor Sry was discovered 1990 in both human and mouse (Gubbay et al., 1990; Sinclair et al., 1990). This discovery led to the identification of the Sox proteins. All members of the Sox family contain a DNA binding motif of approximately 80 amino acids called the HMG domain (Jantzen et al., 1990), which is present in a large number of proteins belonging to the HMG box superfamily.

The HMG domain consists of three α-helices that are arranged in a twisted L-shape and the overall structure of this domain is maintained by a hydrophobic core (Wegner, 1999). Both the amino acids that constitute the hydrophobic core and the amino acids which provide base-specific DNA contact are highly conserved among Sox proteins (Werner et al., 1995). To be classified as a Sox protein, the HMG domain has to be at least 50% identical with the HMG domain of Sry (Bowles et al., 2000). Sox proteins have the ability to recognize specific DNA structures *in vitro* (Ferrari et al., 1992; Peters et al., 1995; Trimmer et al., 1998; Vriz et al., 1995) and they also have the capacity to bind to specific DNA sequences. The consensus DNA binding site for Sox proteins has been identified as 5'-(A/T)(A/T)CAA(A/T)G-3' (Harley et al., 1994). Among the HMG box superfamily, the ability to bind DNA in a sequence specific manner is unique for Sox proteins and the distant related TCF/LEF family (Wegner, 1999). Sox proteins bind DNA in the minor groove (van de Wetering and Clevers, 1992) and when doing so they introduce large conformational changes in the DNA, such as helix unwinding, minor groove expansion and a 70-85º bending of the DNA molecule (Connor et al., 1994; Ferrari et al., 1992; Ma et al., 1998; Pontiggia et al., 1994; Werner et al., 1995). Several Sox proteins from both mouse, chicken and human have been shown to either activate or repress the transcription of reporter constructs (Hosking et al., 1995; Kamachi et al., 1995; Uchikawa et al., 1999). Thus, the Sox proteins have the ability to function both as classical transcription factors and as architectural proteins of chromatin.

3.2 CLASSIFICATION OF SOX PROTEINS

Sox transcription factors are present throughout the animal kingdom and they appear to be important both in the regulation of embryonic development and during cell type specification (Pevny and Lovell-Badge, 1997; Wegner, 1999). Around 20 different Sox proteins in mammals, 8 in *Caenorhabditis elegans* and 5 in *Drosophila* have been identified (Bowles et al., 2000; Cremazy et al., 1998; Kamachi et al., 2000; Schepers et al., 2002). These proteins have been grouped into eight groups (A-H), group A being assigned to Sry (Kamachi et al., 2000). The similarity in the amino acid sequence of the HMG domain within an individual group is ≥90%, although it decreases to roughly 60%

between different groups. Once the complete coding sequences were known for many Sox proteins, it became evident that members of the same group also display significant homology even outside the HMG domain (Kamachi et al., 2000). Some groups, such as group B, C and E, have regions flanking the HMG domain that are highly conserved. In group B this region is located C-terminal of the HMG domain and consist of a short amino acid motif, while the conserved region of group C and E is located N-terminal of the HMG domain. The transactivation domains comprise yet another conserved region within the eight groups. Finally, members of group D have, in addition to the HMG domain (Wegner, 1999), a leucine zipper motif, which is a highly conserved coiled-coil domain that confers on them the ability to dimerize (Lefebvre, 2002; Lefebvre et al., 1998).

The Sox genes are distributed randomly throughout the genome and there is no evidence of gene clustering within the Sox family. The mammalian Sox genes of group A, B and C are intronless, at least in the coding sequence. However, the mammalian group D-G exhibit an exon-intron structure. Sox genes are usually transcribed into proteins of small to moderate sizes and even Sox proteins that are generated from genes containing introns are of fairly small sizes (Pevny and Lovell-Badge, 1997; Wegner, 1999).

3.3 EXPRESSION AND FUNCTION OF SOX PROTEINS IN THE CNS

The expression pattern of Sox genes during embryogenesis and in adult tissues is both diverse and dynamic. So far, all tissues and cell types that have been analyzed have turned out to express at least one Sox protein at some point during their development. In the CNS a subset of Sox proteins are expressed both during the development and throughout adulthood. Some of these Sox proteins are essential for maintaining progenitor cell characteristics of uncommitted progenitor cells, while others specify progenitors or are responsible for the terminal differentiation of progenitor cells (Wegner, 2005).

Sox8, Sox9 and Sox10 are all members of the SoxE group in vertebrates and they are important for proper development of oligodendrocytes (Stolt et al., 2004; Stolt et al., 2003; Stolt et al., 2002), the myelin-forming glia in the CNS. There are some differences in the expression pattern between the SoxE proteins during gliogenesis, and in the spinal cord the expression of Sox9 precedes the expression of the other two SoxE proteins. The expression of Sox9 is first detected in neural precursor cells before the onset of gliogenesis, which might suggest that Sox9 is a component in the mechanism that causes neural stem cells to switch from neurogenesis to gliogenesis (Stolt et al., 2003). The specification defects in oligodendrocytes and astrocytes that occur in the Sox9-deficient mice demonstrate that Sox9 has an important role in the specification of the two main types of glial cells in the CNS (Stolt et al., 2003). Sox8 is also expressed in the neuroepithelial cells, however, the expression of Sox8 only appears after the onset of Sox9 (Stolt et al., 2004). The expression of Sox10 coincides with the specification of oligodendrocyte progenitors (Stolt et al., 2002). After the

specification event, all three SoxE proteins are co-expressed in the developing oligodendrocytes until the onset of terminal differentiation when Sox9 expression is downregulated (Stolt et al., 2003). The expression of Sox8 and Sox10 is maintained both throughout the terminal differentiation and thereafter (Stolt et al., 2004; Stolt et al., 2005). Loss-of-function data show that oligodendrocyte progenitors in Sox10-deficient spinal cords develop normally, but that terminal differentiation is disrupted (Stolt et al., 2002). The phenotype of Sox8-deficient mice is similar, but not as severe as in Sox10-deficient mice. This imply that Sox8 and Sox10 have partially redundant functions and are important during terminal differentiation of oligodendrocytes (Stolt et al., 2004). Additionally, Sox10 controls the expression of several myelin genes, such as myelin basic protein and proteolipid protein, by directly binding to the regulatory regions of these genes (Stolt et al., 2002). Sox8 also seems to have a role in regulating some of these myelin genes, even though the effect of Sox8 on these genes is not as prominent as the effect of Sox10 (Stolt et al., 2004). Thus, Sox9 and, to a lesser extent, Sox8 specify the gliogenic fate of neuronal precursors, while Sox10 and, to a lesser extent, Sox8 are expressed during terminal differentiation and are required for myelination (Lefebvre et al., 2007; Stolt et al., 2002).

In addition to their function during the oligodendrocyte development, the SoxE proteins are also involved in the development of neural crest cells and their derivatives (Aoki et al., 2003; Cheung and Briscoe, 2003; Hong and Saint-Jeannet, 2005; Honore et al., 2003; Kim et al., 2003; O'Donnell et al., 2006; Spokony et al., 2002). All of the SoxE proteins are expressed in neural crest progenitor cells (Cheung and Briscoe, 2003; Kim et al., 2003; O'Donnell et al., 2006; Spokony et al., 2002), although there are some differences in the onset of expression between these proteins in different species (Hong and Saint-Jeannet, 2005). Both Sox9 and Sox10 have been shown to regulate the neural crest precursor formation and later on they influence the differentiation pathway that migrating neural crest cells adopt (Aoki et al., 2003; Cheung and Briscoe, 2003; Honore et al., 2003; Spokony et al., 2002). Importantly, Sox10 also seems to contribute to the maintenance of stem cell properties of the neural crest cells (Kim et al., 2003).

The SoxD proteins, Sox5 and Sox6, have also been implicated in the regulation of oligodendrocyte development. They repress specification and terminal differentiation of oligodendrocytes and they also influence the migratory pattern of these cells. In the absence of SoxD proteins, specification of oligodendrocyte precursors and terminal differentiation of oligodendrocytes in the spinal cord occur prematurely (Stolt et al., 2006). Interestingly, this effect appears to be dependent on the ability of Sox5 and Sox6 to interfere with the function of SoxE proteins (Stolt et al., 2006).

Finally, neural cells that have committed to neuronal differentiation express both Sox4 and Sox11, two members of the SoxC group (Cheung et al., 2000; Uwanogho et al., 1995). Recent results suggest that these two proteins have an essential role during neuronal maturation and that the induction of Sox4 and

Sox11 in neural cells could be important for the acquisition of a neuronal phenotype (Bergsland et al., 2006). When either Sox4 or Sox11 are deleted in mice no obvious role has been revealed for these proteins during neurogenesis (Cheung et al., 2000; Sock et al., 2004). However, they exhibit structural similarities and they also have a conserved expression pattern, implying that functional redundancy can compensate for the loss of either one of the genes (Bergsland et al., 2006; Cheung et al., 2000; Sock et al., 2004). In the oligodendrocyte lineage both Sox4 and Sox11 are expressed in the precursor cells, but they are down-regulated during the early stages of differentiation (Kuhlbrodt et al., 1998). Prolonged expression of Sox4 in the oligodendrocyte lineage results in a severe hypomyelination. Interestingly, hypomyelination does not result in an increased rate of apoptosis and the proliferation rate in the oligodendrocyte lineage is only mildly increased. Instead, postmitotic oligodendrocytes become arrested in a premyelinating or early myelinating state, suggesting that Sox4 normally prevents precocious terminal differentiation of oligodendrocyte precursors, and it is likely that Sox11 has the same role due to functional redundancy (Potzner et al., 2007). Thus, it seems like SoxC proteins have opposite function during neurogenesis and gliogenesis.

3.4 EXPRESSION OF SOXB PROTEINS

Members of the SoxB group are among the earliest proteins to be specifically expressed in the developing CNS. They are expressed throughout the proliferating ventricular zone of the spinal cord and brain, but as cells exit mitosis and migrate to more lateral sites where they start to differentiate, the expression of these proteins is downregulated (Uwanogho et al., 1995). Sox1, Sox2, Sox3, Sox14 and Sox21 are all members of the SoxB group. Since the resemblance between these proteins only exist in the HMG domain and the immediate C-proximal group B homology domain this group has been divided into two subgroups, namely subgroup B1 consisting of Sox1, Sox2 and Sox3 (Sox1-3) and subgroup B2, which consists of Sox14 and Sox21 (Uchikawa et al., 1999). The C-terminal domain of Sox1-3 has been proposed to harbor an activation domain, since this group can bind and activate the DC5 minimal enhancer of the chicken δ1-crystallin gene (Kamachi et al., 1995; Kamachi et al., 1998). In contrast, members of the subgroup B2 are considered to have a repressor domain in their C-terminus, since these proteins can repress the DC5 minimal enhancer (Uchikawa et al., 1999). Thus, the similarities within each subgroup also extend to the remaining parts of these proteins.

3.4.1 Subgroup B1

The SoxB1 proteins are expressed from early embryonic stages. Within the developing CNS, the expression domain of these proteins are highly overlapping, although there are sites where they are uniquely expressed (Collignon et al., 1996; Uwanogho et al., 1995; Wood and Episkopou, 1999; Zhao et al., 2004). Due to the expression pattern SoxB1 proteins are classified as

pan-neural factors (Rex et al., 1997a). This broad expression pattern within the neural primordium is similar for the SoxB1 orthologs in *Drosophila*, *Xenopus*, zebrafish, avian and rodent (Bowles et al., 2000; Penzel et al., 1997; Pevny et al., 1998; Rex et al., 1997a; Uchikawa et al., 2003; Uchikawa et al., 1999; Uwanogho et al., 1995; Wood and Episkopou, 1999). Functional studies of SoxB1 proteins in *Xenopus* (Kishi et al., 2000; Wood and Episkopou, 1999) and *Drosophila* (Cremazy et al., 2000; Overton et al., 2002) embryos suggest that these proteins have a role in neural induction. Furthermore, disrupting the expression of SoxB1 in *Xenopus* embryos inhibits the formation of CNS and neural crest cells (Kishi et al., 2000), and deleting both Sox orthologs in *Drosophila* (SoxN and Dichaete) results in neural hypoplasia (Buescher et al., 2002; Overton et al., 2002). Together these results suggest a role of SoxB1 proteins in the specification of the neural fate. Furthermore, SoxB1 proteins are widely expressed in proliferating neural stem and progenitor cells both in the developing and adult CNS suggesting that they might also have a role in the maintenance of neural progenitor fate (Graham et al., 2003; Pevny and Rao, 2003; Uchikawa et al., 2003; Zappone et al., 2000).

The SoxB1 proteins recognize the same DNA binding sites (Harley et al., 1994; Wegner, 1999) and have similar expression patterns (Kamachi et al., 2000; Uchikawa et al., 1999; Uwanogho et al., 1995; Wood and Episkopou, 1999). These findings together with both loss-of-function (Ferri et al., 2004; Nishiguchi et al., 1998; Rizzoti et al., 2004) and gain-of-function (Bylund et al., 2003) studies suggest that these proteins exhibit functional redundancy. Thus, it is difficult to elucidate the specific role for each one of the SoxB1 proteins during both development and adulthood, although some knowledge has been obtained by analyzing areas in the CNS where they are uniquely expressed.

The Sox1 knock-out mice are viable, but suffer from spontaneous seizures (Nishiguchi et al., 1998). It has been shown that the epilepsy in the homozygous mice is likely due to the loss of the telencephalic neurons in the ventral striatum. Sox1 is expressed in this region and seems to be important for the appropriate migration and positioning of these neurons within the ventral striatum (Ekonomou et al., 2005; Malas et al., 2003). Furthermore, Sox1-deficient mice also have small eyes with opaque lenses, which is a result of an impairment in the elongation of lens fiber cells and because the expression of the γ-crystallin gene, which is one of several classes of crystallin genes that are expressed in lens fiber cells, is severely downregulated. The ability of Sox1 to bind to a promoter element in the γ-crystallin gene, which is essential for its expression both *in vivo* and *in vitro*, demonstrates that Sox1 has a direct role in the development of the lens, at least partly though its action on this specific gene (Kamachi et al., 1998; Nishiguchi et al., 1998). Additionally, the induced expression of Sox1 in P19 embryonal carcinoma (EC) cells is sufficient to promote these cells to develop towards the neural lineage, thus providing evidence that Sox1 has an important role in biasing ectodermal cells towards the neural fate (Pevny et al., 1998).

The expression of Sox2 is first detected at the blastocyst stage, in the multipotent ICM, but it is also expressed in the epiblast, the trophoblast stem cells and in germ cells (Avilion et al., 2003; Wood and Episkopou, 1999). In many species, including *Xenopus*, chick and mouse, Sox2 is expressed throughout the development of the CNS, where the expression is first detected in the neural plate and later becomes restricted to neural stem and progenitor cells (Avilion et al., 2003; Ekonomou et al., 2005; Graham et al., 2003; Kamachi et al., 2000; Kishi et al., 2000; Rex et al., 1997a; Uwanogho et al., 1995; Wood and Episkopou, 1999; Zappone et al., 2000). Sox2 is also expressed both in the gut endoderm (Collignon et al., 1996) and during the development of the sensory systems. Sox2 has an essential role in maintaining stem cells of the mouse epiblast in an undifferentiated state and Sox2-deficient mice die at implantation (Avilion et al., 2003). However, it has been possible to study the function of this protein in various tissues by generating mutants, including gene-dosage allelic variations of the Sox2 mutations, hypomorphic and conditional mutants, a regulatory mutant allele, in which a neural cell-specific enhancer is deleted, and a null allele of Sox2 (Ferri et al., 2004; Kiernan et al., 2005; Taranova et al., 2006). The use of such mutants has demonstrated that the levels of Sox2 are important for maintaining neural progenitor cells in the retina, inner ear and in the tongue (Kiernan et al., 2005; Neves et al., 2007; Okubo et al., 2006; Taranova et al., 2006). In addition, Sox2 is also involved in the development of the lenses and disruption of Sox2 leads to malformation of the eyes (Fantes et al., 2003). Finally, Sox2 appears to be important both in the developing neocortex and for maintaining neurons in selected adult brain areas, such as thalamus and striatum (Bani-Yaghoub et al., 2006; Ferri et al., 2004), as well as keeping the immature Schwann cell state in the PNS (Le et al., 2005; Wakamatsu et al., 2004).

Prior to primitive streak formation both in mouse and chick the expression of Sox3 is initiated throughout the epiblast. However, as the formation of CNS proceeds the expression of Sox3 becomes progressively more restricted to the immature neural epithelium of the developing CNS (Penzel et al., 1997; Rex et al., 1997a; Uwanogho et al., 1995; Wood and Episkopou, 1999). In addition, Sox3 has also been shown to be expressed in developing gonads as well as in non-neural tissues, including the gut endoderm and the placodal epithelium (Collignon et al., 1996; Gubbay et al., 1990; Rizzoti et al., 2004; Weiss et al., 2003) and during the development of the sensory system of the inner ear, Sox3 is expressed in the neurogenic domain of the otic cup (Neves et al., 2007). In humans, mutations in Sox3, which is located on the X chromosome, are associated with hypopituitarism and mental retardation (Laumonnier et al., 2002). In mice the deletion of Sox3 leads to a range of phenotypes, including craniofacial abnormalities, defects in functions of specific CNS midline structures and a reduction in both fertility and size (Rizzoti et al., 2004; Weiss et al., 2003). The latter one is due to defects in the pituitary gland and, thus these knockout mice confirm that Sox3 is involved in the formation of the hypothalamic-pituitary axis (Rizzoti et al., 2004).

3.4.2 Subgroup B2

The Sox14 and Sox21 proteins exhibit a more restricted expression pattern in the CNS than the broadly expressed SoxB1 proteins (Hargrave et al., 2000; Rex et al., 1997b; Uchikawa et al., 1999). Also, the co-expression of the SoxB2 proteins only occurs at highly restricted sites in the CNS, including a subgroup of interneurons in the spinal cord where Sox21 expression precedes the expression of Sox14 (Uchikawa et al., 1999).

In both mouse and chicken the expression of Sox14 is restricted to a limited population of interneurons in the developing brain and spinal cord and Sox14 has been implicated in their cell-type specification (Hargrave et al., 2000; Uchikawa et al., 1999). Additionally, Sox14 has also been detected in the apical ectodermal ridge of the developing chick limb (Wilmore et al., 2000). In humans, Sox14 is expressed in the fetal CNS and in various adult tissues, including heart, liver, gut, kidney and testis (Arsic et al., 1998; Malas et al., 1999).

In chicken, the expression of Sox21 is first detected in the ectoderm although it rapidly becomes restricted to the rostral half of the embryo, where neural induction occurs. The predominant area of Sox21 expression is in the CNS, where it appears as longitudinal stripes within the spinal cord, while the expression in the brain is more complex (Rex et al., 1997b). Sox21 is also detected in gut endoderm and the sensory systems, where it is observed in the nasal placode, in neural retina and lenses, and sensory epithelia of the inner ear (Uchikawa et al., 1999). In some tissues, such as the spinal cord, early lenses and the rostral part of the alimentary tract, Sox21 expression overlaps extensively with the expression of Sox1-3 (Sandberg et al., 2005; Uchikawa et al., 1999). In zebrafish, Sox21a is detectable as a maternal transcript in the oocytes and its expression is limited to the embryonic stage of development (Argenton et al., 2004). At the onset of neurulation, Sox21a becomes specifically localized to the presumptive midbrain-hindbrain boundary, which remains as the major site for the expression of Sox21a in zebrafish (De Martino et al., 1999; Rimini et al., 1999).

3.5 REGULATION OF SOXB PROTEINS IN THE CNS

Although, SoxB proteins are key players during the development of CNS and are also widely expressed within the developing CNS (Collignon et al., 1996; Pevny et al., 1998; Rex et al., 1997a; Uchikawa et al., 1999; Uwanogho et al., 1995; Wood and Episkopou, 1999), not much is known about the transcriptional regulation of these genes. Sox2 has been used as an early marker for neural induction and its expression in the developing neural plate is induced by signals from the organizer (Fernandez-Garre et al., 2002; Streit et al., 1997; Uchikawa et al., 2003). These data suggest that Sox2 might have a role in neural induction.

The murine Sox2 promoter harbors two regulatory elements that are capable of activating gene transcription. Examination of these promoter elements has demonstrated that they might be involved in the regulation of the endogenous Sox2 expression during differentiation, since these constructs are expressed at significantly lower levels in differentiated EC cells compared with undifferentiated cells (Wiebe et al., 2000). Also, two transcriptional enhancer regions, Sox-2 regulatory region 1 (SRR1) and Sox-2 regulatory region 2 (SSR2), have been identified in the Sox2 gene (Miyagi et al., 2004; Tomioka et al., 2002). SSR1 and SSR2 are active in neural progenitor cells *in vitro* and activate the expression of a reporter gene *in vivo*, specifically in the developing telencephalon (Miyagi et al., 2004; Tomioka et al., 2002; Zappone et al., 2000). Thus, this suggests that SSR1 and SSR2 might at least take part in maintaining the high levels of Sox2 expression in the developing brain (Miyagi et al., 2004). Both SSR1 and SSR2 contain binding sites for Sox and POU proteins (Catena et al., 2004; Tomioka et al., 2002) and in ES cells Oct4 and Sox2 are necessary for the ability of SSR1 and SSR2 to transactivate a reporter gene (Tomioka et al., 2002). Furthermore, the same POU binding sites that are occupied by Oct4 in the ES cells are also utilized by the neural specific POU proteins, Brn1 and Brn2 in neural stem cells, suggesting that these binding sites have a role in regulating Sox2 both in ES and neural stem cells (Catena et al., 2004).

Functional analysis of the chicken Sox2 gene has revealed that the pan-neural expression of Sox2 in the CNS is actually pieced together by five different enhancers, N-1 to N-5 (Uchikawa et al., 2003). These enhancers are located in extragenic sequences and are highly conserved between chicken, human and mouse. These different enhancers exhibit distinct spatial and temporal activities (Uchikawa et al., 2003). For instance, during the development of the visual system, the Sox2 gene is partly under the control of the N-3 enhancer. This enhancer contains a core element, which is activated through the cooperative interaction between Sox2 and Pax6 in transfected cells. Thus, suggesting that the N-3 enhancer is dependent on Sox2 for its activity and once activate the expression of Sox2 is amplified though an auto-regulatory loop involving the same enhancer (Inoue et al., 2007). The enhancers N-1 and N-2 are important for the early expression of the Sox2 gene, where N-2 is responsible for the initial expression and N-1 for the subsequent expression of Sox2 in neural plate precursors of the future caudal hindbrain and spinal cord (Uchikawa et al., 2003). The core element of the N-1 enhancer is synergistically activated by Wnt and FGF signaling, but is not affected by BMP signaling. This core element also harbors a region that inhibits the activity of the N-1 enhancer in the mesendodermal precursor cells (Takemoto et al., 2006). In the neural plate the activity of the N-2 enhancer is regulated by three coiled-coil domain proteins, ERNI (early response to neural induction), BERT and Geminin, that interacts with each other and with chromatin-remodeling factors, such as Brg1 and Bramha (Nielsen et al., 2002; Seo et al., 2005a), and heterochromatin proteins, such as HP1α and HP1γ (Papanayotou et al., 2008). Bramha can activate Sox2 through direct binding to the N-2 enhancer (Kondo and Raff, 2004), but even though Brahma is ubiquitously expressed in the embryo (Elfring et al., 1998) Sox2 is not prematurely expressed. This is because of the repressor HP1α that

has the ability to interact directly with Brahma and thereby inhibit the expression of Sox2 (Nielsen et al., 2002; Papanayotou et al., 2008). Another protein that can interact with Bramha at the same site as HP1α is Geminin (Seo et al., 2005a), which has the opposite effect of HP1α and can active the expression of Sox2 (Papanayotou et al., 2008). Although, Geminin is present during gastrulation there is no activation of Sox2, which is due to the protein ERNI that has the ability to both interact with Geminin, through the coiled-coil domain, and with HP1γ. When BERT, a small coiled-coil domain protein, is up-regulated within the neural plate, where it binds both ERNI and Geminin and displaces the ERNI-HP1γ complex away from Brahma, the repression of Sox2 via the N-2 enhancer is relieved (Papanayotou et al., 2008). Thus, this would be a way of regulating the activation of Sox2 in the early neural plate.

In mice, sequences both 5' and 3' of the Sox3 open reading frame are necessary for driving the expression of Sox3 along the anterior-posterior axis from the onset of neurogenesis until at least midgestation stages. These regions are also involved in the regulation of Sox3 along the dorsal-ventral axis of the neural tube (Brunelli et al., 2003). In *Xenopus*, the maintenance, but not the induction of Sox2 and Sox3 requires FGF. However, both genes are induced by the inhibition of BMP (Rogers et al., 2008). The human and the mouse Sox3 promoters share extensive homology, especially in regions both upstream and downstream of the TATA box, but there are no similarities with the *Xenopus* Sox3 promoter (Kovacevic Grujicic et al., 2005; Rogers et al., 2008). The expression of human Sox3, mouse Sox2 and mouse Sox3 is influenced by the transcription factor nuclear factor Y, which is an ubiquitously expressed transcription factor that is involved in the expression of many tissue-specific genes (Mantovani, 1998). Also, both the human and mouse Sox3 promoters contain putative binding sites for two additional transcription factors, namely Sp1 and USF. Thus, this implies that these three factors are involved in regulating the basal activation of both the human and mouse Sox3 gene (Kovacevic Grujicic et al., 2005).

3.6 THE SPECIFICITY OF SOX PROTEINS

Even though Sox proteins recognize the same consensus DNA binding site, have similar DNA bending activity and their affinity for binding DNA is very low, they can activate gene transcription in a highly cell-specific manner (Kamachi et al., 2000). A possible explanation for this specific target selection and activation is that Sox proteins are cooperating with partner factors. Indeed when the regulatory regions in several of the target genes, including Fgf4 (fibroblast growth factor 4) (Ambrosetti et al., 1997; Dailey et al., 1994; Johnson et al., 1998; Yuan et al., 1995), UTF1 (undifferentiated embryonic cell transcription factor 1) (Nishimoto et al., 1999), Nanog (Kuroda et al., 2005; Rodda et al., 2005), Nestin (Tanaka et al., 2004), δ-crystallin (Kamachi et al., 1995; Kamachi et al., 1998) and Col2a1 (collagen type II) (Lefebvre et al., 1997), were analyzed it became evident that a second site that can bind a partner

factor is located nearby the Sox-binding site (Kamachi et al., 2000). With the assistance of partner factors the Sox proteins might achieve a more stable DNA binding, which would allow the activation of target genes *in vivo*. It appears that the distribution of the partner factors is cell specific and therefore, the specific gene activation attributed to Sox proteins is due to the interaction with these partner factors (Kamachi et al., 2000).

Synergistic interaction between Sox2 and Oct4 has been detected on the enhancers of Fgf4, Nanog, Nestin, UTF1, Oct4 (Okumura-Nakanishi et al., 2005) and Sox2 (Tomioka et al., 2002). These genes are involved in maintaining ES cells and EC cell lines in a pluripotent state and are good examples of how Sox proteins together with partner factors can support specific target gene activation. Although, Sox2 can form a complex with both Oct1, yet another POU protein, and Oct4 on the Fgf4 enhancer, it is only the Sox2:Oct4 combination that promotes transcriptional activation (Yuan et al., 1995). Furthermore, the DNA binding domains of these two proteins, namely the HMG domain of Sox2 and the POU domain of Oct4, are responsible for both the direct protein-protein interaction and the cooperative binding to the Fgf4 enhancer. Changing the spacing between the Oct- and Sox-binding elements reduces both the ability of the Sox2:Oct4 complex to assemble on the enhancer and decreases the transcriptional activation of the Fgf4 gene (Ambrosetti et al., 1997). In addition, regions outside the DNA binding domains of Sox2 and Oct4 also contribute to the activation of Fgf4 (Ambrosetti et al., 2000).

The Pax family of transcription factors is another group of proteins that can interact with Sox proteins and the regulation of the δ–crystallin gene by Sox2 and Pax6 is an example of this (Kamachi et al., 1998; Kamachi et al., 2001). Pax6 binds cooperatively together with Sox2 to the DC5 enhancer in the δ–crystallin gene resulting in the formation of a complex that can activate the enhancer (Kamachi et al., 2001).

The gene (Col2a1) for collagen II, an early and abundant marker of chondrocyte differentiation, contains an enhancer that is a direct target for Sox9 (Bell et al., 1997; Lefebvre et al., 1997). Sox9 is coexpressed with Sox5 and Sox6 at all chondrogenic sites of the mouse embryo (Lefebvre et al., 2001; Lefebvre et al., 1998). The Col2a1 gene and other genes important for chondrogenesis, including Col11a2 and aggrecan, are activated by the cooperatively action between Sox9, Sox5 and Sox6 (Ikeda et al., 2005; Lefebvre et al., 2001; Lefebvre et al., 1998). In addition, it has been demonstrated that this Sox trio is not only necessary for cartilage development, but is also sufficient for the formation of permanent cartilage (Ikeda et al., 2004). Thus, the partner factors, which are both interacting and mediating the specificity of Sox proteins, constitute a diverse set of transcription factors. Interestingly Sox proteins are also included in this group.

4 AIMS

The aim of the thesis was to unveil fundamental principles regulating the growth and differentiation behaviour of neural stem cells in the developing vertebrate CNS. The main goals were to:

1. Understand the molecular pathways that maintain neural stem cells in an undifferentiated proliferative state.

2. Identify factors that are involved in directing neural progenitor cells towards neuronal differentiation.

3. Identify CNS expressed genes regulated by Sox proteins.

5 RESULTS AND DISCUSSION

5.1 VERTEBRATE NEUROGENESIS IS COUNTERACTED BY SOX1-3 ACTIVITY (PAPER I)

It has been well established that proneural bHLH proteins can drive neurogenesis by directing the exit of neural progenitors from the cell cycle and by promoting the expression of proteins characteristic of post-mitotic neurons (Farah et al., 2000; Guillemot, 1999; Ma et al., 1996; Morrow et al., 1999; Scardigli et al., 2001). The expression of proneural bHLH proteins is, in turn, regulated by the activation of the Notch signaling pathway (Kageyama and Nakanishi, 1997; Kageyama et al., 2005). Thus, the ability of neural progenitor cells to commit to neuronal differentiation is dependent on the interaction between Notch receptors and their ligands and the expression of proneural proteins. However, proneural bHLH proteins are expressed in mitotically active cells that are not yet committed to neuronal differentiation (Gradwohl et al., 1996; Lo et al., 2002; Ma et al., 1996), which indicates the presence of other factors that can actively counteract neurogenesis and keep cells in an undifferentiated state.

5.1.1 Sox1-3 are expressed in progenitor cells and inhibit neuronal differentiation

The Sox1-3 proteins are expressed by most progenitor cells in the developing CNS and are generally downregulated when these cells start to differentiate (Graham et al., 2003; Pevny et al., 1998; Uwanogho et al., 1995), indicating that they might have a regulatory role during neuronal differentiation. This prompted us to examine what role Sox1-3 have in the regulation of neurogenesis using the chick spinal cord as a model system. By comparing the expression of Sox1-3 proteins with molecular markers for mitotic, differentiating and post-mitotic cells, it became evident that Sox1-3 are coexpressed in self-renewing progenitor cells, but are downregulated as progenitor cells leave the cell cycle and start to express post-mitotic neuronal markers. Overexpression of Sox1-3 in the chick spinal cord demonstrated that these proteins act in a redundant fashion to maintain the expression of progenitor cell characters and prevent cells from upregulating post-mitotic neuronal markers. The ability of Sox1-3 to maintain cells in a proliferative state, did not seem to depend on their ability to interfere with the cell cycle regulatory machinery, because forced expression of Sox1-3 had no effect on the expression of key components of the cell-cycle machinery.

5.1.2 Sox1-3 are transcriptional activators and repression of Sox3 target genes promotes differentiation

To determine whether Sox1-3 proteins suppress neuronal differentiation by either activating or repressing gene transcription, obligate activator (HMG-Vp16) and repressor (HMG-EnR) versions of the Sox3 protein were generated. Forced expression of the HMG-Vp16 mimicked the activity of full-length Sox1-3 proteins and could suppress neuronal differentiation, while the HMG-EnR had the opposite affect and promoted differentiation. These results implied that Sox1-3 inhibit neurogenesis by acting as transcriptional activators.

The results obtained with the HMG-EnR construct indicated that active repression of Sox1-3 target genes promotes neural progenitor cells to initiate differentiation prematurely. Indeed all transfected cells had exit the cell cycle and initiated the expression of the post-mitotic markers Lim2 and NeuN, at time points where normally only a few neurons, at most, have been generated. However, no expression of Neurofilament or Tuj1 could be detected,. Thus, when Sox3 target genes are repressed cells differentiate prematurely and upregulate a partial repertoire of neuronal markers.

5.1.3 Ngn2 promotes neuronal differentiation by suppressing Sox1-3

Proneural bHLH proteins can promote neurogenesis (Mizuguchi et al., 2001; Novitch et al., 2001). For instance overexpression of Ngn2 caused cells to exit the cell cycle and commit to differentiation. Furthermore, neurons generated by forced expression of Ngn2 were not detected until 12 hours after transfection. The different activities measured with HMG-EnR and Ngn2 indicated that HMG-EnR drives progenitor cells towards differentiation independently of endogenous proneural bHLH protein activity. Indeed, when HMG-EnR is coexpressed with Ngn2 a full array of neuronal markers is induced already with in 3-10 hours and, thus the active repression of Sox1-3 target genes in the presence of high levels of Ngn2 activity is sufficient to induce a complete neuronal phenotype prematurely. Finally, the capacity of Ngn2 to promote neuronal differentiation is dependent on its ability to suppress the expression of Sox1-3, because high levels of Ngn2 activity could suppress the expression of Sox1-3. In addition, overexpression of Sox3 completely block the ability of co-electroporated Ngn2 to induce premature differentiation.

5.1.4 Discussion

In summary, we showed that neurogenesis is regulated by the interplay between proneural proteins and Sox1-3. The Sox1-3 proteins counteracted neurogenesis by repressing differentiation events downstream of proneural bHLH activity, whereas the capacity of proneural proteins to drive cells toward

differentiation is based on their ability to suppress the expression of Sox1-3. The fraction of progenitor cells expressing proneural bHLH proteins is selected by the interaction by Notch receptors and their ligands (Bertrand et al., 2002). However, Sox1-3 proteins seem to act in parallel with this process, to prevent cells that express proneural bHLH proteins from differentiating prematurely.

Sox1-3 act as transcriptional activators and therefore it is likely that Sox1-3 normally induce the expression of a factor(s) that, in turn, represses the expression of proteins necessary for the differentiation of progenitor cells. Furthermore, our results showed that under normal conditions, Ngn2 is extensively coexpressed with Sox1-3 proteins in neural progenitors, whereas overexpression of Ngn2 efficiently represses the expression of Sox1-3. These findings indicate that the level of proneural protein expression or activity controls whether Sox1-3 are downregulated or not. Thus, together these data reveals a central role for the Sox1-3 proteins in maintaining cell in an undifferentiated state, and further suggest that suppression of Sox1-3 proteins, mediated by proneural proteins, reflect a critical commitment step in the acquisition of a definitive neuronal fate.

5.2 SOX21 PROMOTES THE PROGRESSION OF VERTEBRATE NEUROGENESIS (PAPER II)

Although proneural bHLH proteins are know to promote neuronal differentiation, it is not yet know how these factors that function as transcriptional activators can induce neural cells to suppress progenitor characteristics and commit to the irreversible set of events, which eventually results in terminal neuronal differentiation (Bertrand et al., 2002; Kintner, 2002). The SoxB group consists of Sox1-3, Sox14 and Sox21 and transcriptional analyses have shown that Sox14 and Sox21 contain a transcriptional repressor motif in their C-terminal portions (Kamachi et al., 2000; Uchikawa et al., 1999). Whereas the expression of Sox14 is limited to a population of post-mitotic interneurons, Sox21 is expressed within the ventricular zone and at all axial levels of the CNS (Rex et al., 1997b; Uchikawa et al., 1999).

5.2.1 Sox21 is expressed in progenitor cells and promotes neuronal differentiation by acting as a transcriptional repressor

To examine if Sox21 could have a regulatory role in the progression of neurogenesis, we first examined the expression pattern of Sox21 in the chick neural tube. At spinal cord levels, Sox21 mRNA was detected at most positions along the dorsoventral axis, but was predominately expressed in three stripes that encompassed a dorsal, medial and ventral domain of the ventricular zone. Sox21 was generally coexpressed with Sox1-3 and was also detected in all proliferating cells. In addition, the majority of cells expressing Ngn2 also expressed Sox21, while the expression of NeuroM (Roztocil et al., 1997), another bHLH protein, was only detected in the most lateral $Sox21^+$ cells. In general, cells expressing post-mitotic markers, were Sox21-negative. Thus, the expression of Sox21 is mainly limited to $Sox1\text{-}3^+$ progenitor cells and the expression of Sox21 is downregulated before differentiating cells upregulate the expression of neuronal markers.

Forced expression of Sox21 caused cells to exit the cell cycle, downregulate the expression of the progenitor markers Sox3 and Pax6, and initiate premature expression of the post-mitotic markers Lim2 and NeuN, but not Tuj1 or Neurofilament. Electroporation of Sox21 did not result in an upregulation of Ngn2 expression, nor did the co-electroporation with Id2, a repressor of proneural protein activity, affect Sox21's ability to promote the formation of differentiated cells expressing neuronal markers. Thus, the ability of Sox21 to direct neural cells towards a differentiated state appear to be independent of the expression of proneural genes. Furthermore, by fusing the HMG domain of Sox21 to the repressor domain EnR (HMG-EnR) it was shown that Sox21 promotes neuronal differentiation by acting as a transcriptional repressor, since the HMG-EnR construct mimicked the activity of full-length Sox21.

5.2.2 Sox21 and Sox1-3 have similar activities and counterbalance each others activity

An activator version of Sox21 could maintain neural cells as progenitors in similar manner as Sox1-3. These findings might suggest that these proteins regulate a similar set of target genes. A construct in which the C-terminal domain of Sox21 was maintained, but the HMG domain of Sox21 was changed with the HMG domain of Sox3, induced cells to exit the cell cycle, suppress Pax6 expression and upregulate neuronal markers, all within 15 hours after electroporation, while the construct containing the C-terminal part of Sox3 fused to the Sox21 HMG domain had the opposite effect. Hence, the distinct activities of Sox21 and Sox3 appear to be conferred to the different regulatory capacities of their C-terminal regions, but seem to be independent of their DNA-binding HMG domains.

The varying expression levels of Sox21 within Sox1-3$^+$ progenitor cells suggest that the intrinsic balance between Sox21 and Sox1-3 expression might determine whether neural cells will remain as progenitor cells or commit to neuronal differentiation. Cells that were co-electroporated with a fixed amount of vector expressing Sox3 and with different amounts of vector expressing Sox21 expressed either progenitor, post-mitotic or neural traits dependent on the amount of misexpressed Sox21 and Sox3 expression vectors. These findings indicate that the intrinsic balance of Sox21 and Sox1-3 activities determines the status of differentiation in neural cells.

5.2.3 Neurogenesis is inhibit by low levels of Sox21

Transfection of RNAi against Sox21 mRNA decreased the level of Sox21 protein expression and demonstrated that the activity of Sox21 is required for the generation of neurons. Furthermore, Sox21 seems to counteract the activity of Sox1-3 and promotes the step at which differentiating progenitor cells leave the cell cycle and upregulate the expression of NeuroM. Misexpression of RNAi against Sox21 reduced the number of NeuroM$^+$ cells and increased the number of Ngn2$^+$ cells that remained in a self-renewing state. The inability of Ngn2$^+$ cells to exit the cell cycle when Sox21 expression was reduced prompted us to determined if Ngn2 could regulate the levels of Sox21 expression. Indeed, 10 hours after electroporation, Sox21 was substantially upregulated in Ngn2-transfected cells and this occurred before Ngn2 had induced the expression of post-mitotic neuronal markers. Thus, the generation of neurons by overexpressing Ngn2 seems to be preceded by an increased expression of Sox21. Furthermore, transfection of Sox21 together with Ngn2 promoted the expression of a full array of neuronal markers, including Neurofilament and Tuj1, implying that Sox21 and Ngn2 seem to function synergistically in the formation of neurons from progenitor cells.

5.2.4 Discussion

In summary, these data suggest that the generation of neurons from precursor cells is dependent on the repressor activity of Sox21, which promotes neurogenesis by counteracting the function of Sox1-3. These findings also position Sox21 in a central role during neurogenesis and suggest that an important function of proneural bHLH proteins is their capacity to upregulate the expression of Sox21. In addition, the distinct regulatory functions of Sox21 and Sox1-3 during neurogenesis seem to be conferred to regions outside their DNA-binding HMG domains and whether neural cells will remain as progenitors or commit to neuronal differentiation seems to be depending on the intrinsic balance of Sox21 and Sox1-3 activity. Thus, one possible developmental mechanism is that the amount of Sox21 expression progressively increases in progenitor cells until a critical level is reached at which Sox1-3 activated genes are repressed, inducing these cells to commit to differentiation. However, an alternative explanation is that Sox21 interacts with a heterodimerizing protein that modulate the activity of Sox21 and its ability to promote neural cells to commit to differentiation.

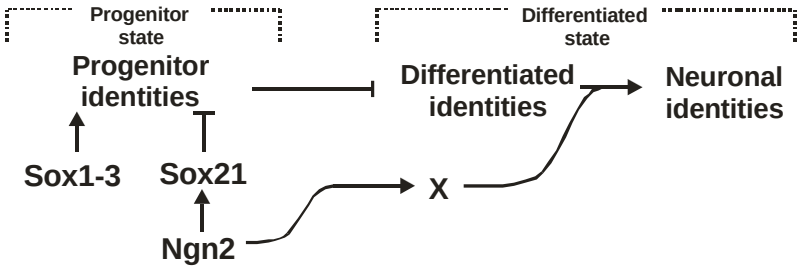

Figure 2 Proposed model of a transcriptional pathway regulating neurogenesis. The progenitor state is maintained by Sox1-3 and suppressed by Sox21. Proneural proteins upregulate the expression of Sox21 and thereby promote neural cells to commit to differentiation.

5.3 A GLOBAL GENOMIC TRANSCRIPTIONAL CODE ASSOCIATED WITH CNS-EXPRESSED GENES (PAPER III)

Even though 50-70 million years has passed since the human and rodent genomes diverged, it is still possible to align 40% of the human and mouse genomes at the nucleotide level (Waterston et al., 2002). A fraction of the aligned sequences are Highly Conserved Non-coding Regions (HCNRs) and Ultra Conserved Regions (UCRs) that exhibit an extremely high level of conservation (Bejerano et al., 2004; Sandelin et al., 2004; Woolfe et al., 2005), but apart from having a high degree of conservation, there is no obvious shared sequence identity between the distinct HCNRs. However, it has previously been shown that clusters of HCNRs often span the loci of developmentally expressed genes, implying and important role(s) during embryonic development (Bejerano et al., 2004; Sandelin et al., 2004; Woolfe et al., 2005).

5.3.1 HCNRs associated with Homeodomain (HD) proteins contain binding sites for Sox, POU and HD proteins

In order to determine the developmental function of HCNRs, we analyzed a set of twelve genes encoding Homeodomain (HD) proteins that function as transcriptional repressors and are involved in the dorsal-ventral (DV) patterning of the spinal cord (Jessell, 2000; Muhr et al., 2001). All twelve HD genes were associated with HCNRs and more importantly the majority of these regions contained a significant over-representation of transcription factor binding sites (TFBS) for Sox (S), POU (P) and Homeodomain (HD) proteins. Both Sox (Sox1-3) and POU (Brn1, -2 and -4) proteins are broadly expressed in the developing CNS and the enrichment of SPHD TFBS in the HCNRs might therefore indicate that these sequences are involved in controlling transcription in the CNS. Furthermore, these findings indicated that Sox and POU proteins might act as common activators of HD genes involved in DV patterning. Indeed forced expression of a repressor variant of Sox3 ($Sox3^{EnR}$) led to a rapid suppression of HD proteins in the chick spinal cord.

5.3.2 $SPHD^{+}$ HCNRs can be found in many genes expressed in the developing CNS

The association of $SPHD^{+}$ HCNRs with several HD genes raised the possibility that other CNS-expressed genes might also be linked to such sequences. Scanning the HCNRs associated with the DACH locus, a gene that is expressed in the CNS, for SPHD TFBS revealed that each of the previously defined DACH enhancers that can recapitulate the DACH expression within the CNS (Nobrega et al., 2003), were highly enriched in SPHD TFBS. These results indicated that the $SPHD^{+}$ HCNRs could be part of a more general transcriptional program for genes expressed within the developing CNS and that scanning HCNRs with the SPHD prediction method could be highly predictive for CNS-specific enhancers. This possibility was address by extending the screen

SPHD$^+$ HCNRs to the vertebrate genome and indeed a significant portion of the HCNRs identified in the vertebrate genome contain binding sites for Sox, POU and HD transcription factors.

5.3.3 The binding sites of Sox and POU in HCNRs are necessary for neural expression

Analyzing the developmental expression pattern of a number of randomly selected genes within the CNS using in situ hybridization together with a search in the available literature for gene expression, demonstrated that at lest 80% of the genes linked to SPHD$^+$ HCNRs where expressed in the developing CNS. Thus, revealing that the association of genes with SPHD$^+$ HCNRs is a strong predictor of expression within the developing CNS. Furthermore, these results also suggest that a large number of CNS-expressed genes are regulated by a general transcriptional program involving Sox, POU and HD proteins. One of the identified HCNRs, which was located in an intron of TCF12, a bHLH E-protein that is broadly expressed in neural progenitor cells (Uittenbogaard and Chiaramello, 2002) and that exhibit a conserved expression pattern between mouse and chick (Neuman et al., 1993), contained four consensus Sox and POU TFBS. This element was referred to as TCF12-HCNR and when cloned into a LacZ vector it had an enhancer activity in neural progenitors *in vivo*. Furthermore mutating any of the Sox or POU binding sites, except for one of the Sox sites, resulted in a reduction or complete loss of the LacZ expression. These results demonstrate that the TCF12-HCNR enhancer activity depends on Sox and POU proteins *in vivo*. In addition, the reduction in activity when individual Sox or POU TFBS were mutated implies that these proteins act cooperatively. This was also shown *in vitro* through the synergistic activation of the TCF12-HCNR enhancer by Sox3 and Brn4.

5.3.4 Discussion

In summary, we showed that up to 30% of HCNRs in vertebrate genomes are enriched for TFBS for Sox, POU and HD proteins and that this type of HCNRs is tightly associated with genes expressed in the developing CNS. The isolated SPHD$^+$ HCNRs function as Sox/POU-dependent enhancers in neural progenitors *in vivo* and HCNRs known to be active in the CNS (Nobrega et al., 2003) are highly enriched for conserved Sox, POU and HD TFBS. In addition, our data also indicate that at least 80% of genes connected to SPHD$^+$ HCNRs in the genome are expressed in the developing CNS. This suggests that SPHD$^+$ HCNRs have a general role in controlling the transcription of CNS-expressed genes and that Sox1-3 and POU proteins act as a generic activator complex in this process. Also, the majority of genes linked to SPHD$^+$ HCNRs have specific gene expression patterns in the developing CNS, including HD genes, suggesting that additional mechanisms must control the spatial expression pattern of genes in the neural tube. Studies of DV pattering strongly suggest that such positional control of expression involves repression by HD proteins. Thus,

we propose a common transcriptional logic for SPHD^{+} HCNR-linked genes in which Sox/POU proteins serve as generic drivers of CNS expression, while the positional control of gene expression is regulated by HD protein-mediated transcriptional repression.

5.4 PRELIMINARY RESULTS

According to the model presented in paper II, whether neural cells differentiate or remain as progenitor cells is dependent on the relative balance between Sox1-3 and Sox21 activities. However, Sox21 is broadly expressed within the ventricular zone and is also expressed in self-renewing progenitors. Thus, although the expression level of Sox21 vary within progenitor cells, it is likely that the activity of Sox21 and its ability to promote progenitor differentiation is also regulated at the post-transcriptional level. The specificity and stability of the interactions between Sox proteins and DNA have previously been shown to be influenced by the presence of specific partner factors (Kamachi et al., 2000). For instance, in the developing CNS the POU proteins Brn1, -2 and -4 can function as specific partner factors for Sox21. However, these proteins are broadly expressed within the developing neural tube, which argues against the possibility that they regulate the activity level of Sox21. In an attempt to search for possible proteins that could influence the capacity of Sox21 to promote neurogenesis, we initiated an yeast two-hybrid screen, using the entire Sox21 protein as a bait. With this technique we screened an e11 cDNA library and identified for example the homeodomain transcription factor Cux2 and DNA-dependent ATPase Brg1, which is a component of the SWI/SNF complex.

Cux2 is one of the two vertebrate homologous (Cux1 and Cux2) to the *Drosophila* Cut homeodomain transcription factors (Neufeld et al., 1992; Quaggin et al., 1996; Tavares et al., 2000; Valarche et al., 1993). In *Drosophila*, the Cut proteins have been demonstrated to have an important role in the development of external sensory organs (Blochlinger et al., 1990; Blochlinger et al., 1991; Bodmer et al., 1987). To begin to examine the role of Cux2 we analyzed its pattern of expression in the developing chick spinal cord, using in situ hybridization. Interestingly, Cux2 expression was mostly detected in the intermediate zone, in post-mitotic progenitors that are under the process of downregulating progenitor proteins and differentiate into neurons. To elucidate the role of Cux2, we next misexpressed Cux2 in the chick neural tube. Misexpression of full-length Cux2 did not have any detectable effect on neurogenesis in the chick spinal cord. If Cux2 would have an important role as a partner factor of Sox21, misexpression of Cux2 would be expected to enhance the rate of neuronal differentiation. A resent study by Iulianella et al (2008) demonstrates in the developing mice that Cux2 has a vital role during neurogenesis by promoting the expression of the cell-cycle inhibitor and CDK-inhibitor $p27^{kip}$ (Iulianella et al., 2008). Thus, although we could not detect any functional synergism between Cux2 and Sox21, it is interestingly to note that Sox21 and Cux2 appear to function at similar steps during neurogenesis, when differentiating cells are exiting the cell cycle.

The mammalian SWI/SNF chromatin remodeling complexes consist of 7-13 subunits and use energy provided by ATP hydrolysis to locally disrupt histone-DNA associations and relocate nucleosomes to alternate positions (Kingston and Narlikar, 1999; Seo et al., 2005b; Whitehouse et al., 1999).

SWI/SNF complexes in mammalian cells have either one of the two catalytic subunits, Brahma or Brg1 (Martens and Winston, 2003; Seo et al., 2005b). Brg1 has been suggested to have a role during neuronal development (Seo et al., 2005b) and in mice embryos, expression of Brg1 is enriched in neural tissue, especially within the brain, retina and spinal cord (Randazzo et al., 1994). Also, Brg1-null mice die at pre-implantation stages and heterozygotes are predisposed to tumors of neural origin (Bultman et al., 2000). In addition, Brg1 has been shown to be required both for neuronal differentiation, by mediating the transcriptional activities of proneural bHLH proteins (Seo et al., 2005b), and for repressing neuronal differentiation and allowing glial differentiation (Matsumoto et al., 2006).

To begin to examine whether Brg1 might have a role in regulating the activity of Sox21 we first confirmed the interaction between Sox21 and Brg1 using co-immunoprecipitation (co-IP) (**fig. 3**). We also determined the expression pattern of Brg1 in the chick spinal cord.

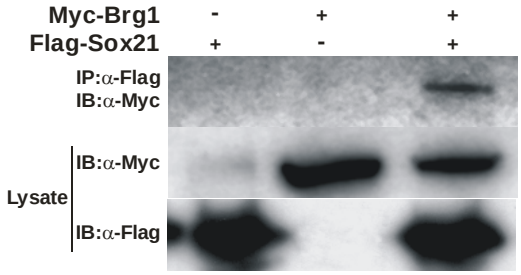

Figure 3 Brg1 physically interacts with Sox21. HEK293 cells were transfected as indicated and applied to co-IP assay. Lysates were immunoprecipitated (IP) with anti-Flag antibodies and immunoblotted (IB) with anti-Myc antibodies. Protein expression levels were monitored by western blotting of lysates.

Interestingly, although Brg1 has been regarded as ubiquitously expressed our analysis showed hat the expression of Brg1 was mostly localized to cells within the intermediate zone (**fig. 4**). Thus, Brg1 appears to interact with Sox21 and is highly expressed in cells that are in the process of differentiating into neurons.

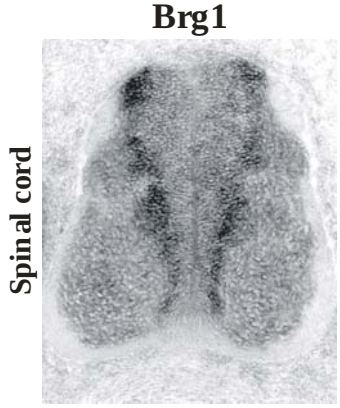

Figure 4 Expression pattern of Brg1 in E4.5 chick spinal cord. High levels of Brg1 expression could be detected in the intermediate zone.

Overexpression of Brg1 in the chick neural tube did not have any detectable effect on neurogenesis. However, the SWI/SNF complex consists of 7-13 subunits and overexpression of the subunit Brg1 alone might not be sufficient to elicit an effect on neurogenesis. To address this issue we next used loss-of-function approaches. Misexpression of a dominant negative version of Brg1, which can be incorporated into the SWI/SNF complex but is non functional (Khavari et al., 1993), or suppression of Brg1 expression using siRNA, resulted in a reduction (40%) in the number of post-mitotic neurons. Although, these results indicate a role for Brg1 in neuronal differentiation, the loss of Brg1 only had a minor effect on the formation of differentiated neurons. Thus, it is not likely that Brg1 has a major role in regulating the activity of Sox21. Furthermore, we could also demonstrate that Brg1 had a similar capacity to interact with Sox3 as it had with Sox21. Nevertheless, although these findings argue against the idea that Brg1 is a specific partner factor of Sox21, Brg1 is likely to have a regulatory role during neurogenesis even though it may be of a more general character.

6 POPULÄRVETENSKAPLIG SAMMANFATTNING

Nervsystemet hos ryggradsdjuren, dvs vertebraterna, har flera olika uppgifter. Det centrala nervsystemet, som består av hjärnan, förlängda märgen och ryggmärgen har till uppgift att ta emot signaler från alla sinnesorgan, behandla dem och ge order till både muskler och andra organ. Det periferar nervsystemet, som består av nerverna har till uppgift att förmedla signalerna från sinnesorganen till hjärnan, men förmedlar dessutom också hjärnans kommandon.

Det centrala nervsystemet består av cirka 100 miljader nervceller och många av dessa celler bildas under fostertiden från speciella celler, som kallas för stamceller. En stamcell är ganska märklig, eftersom den är ospecialiserad och dess slutgiltiga funktion ännu inte bestämts. Stamceller kan utvecklas (mogna) till många olika celltyper genom en process som kallas för differentiering. De stamceller som finns i nervsystemet brukar kallas för neurala stamceller, eftersom de endast kan ge upphov till nerver. Det är viktigt att de neurala stamcellerna bibehålls tillräckligt länge så att de hinner dela sig och ge upphov till alla nervceller i det centrala nervsystemet, om de inte gör det finns risken att alldeles för få nervceller bildas.

Från början trodde forskarna att nevceller inte kunde nybildas efter födseln. Det har dock visat sig att de faktiskt kan göra det, vilket innebär att de neurala stamcellerna finns kvar efter det att vi har fötts. Denna upptäckt har inneburit att nervceller som förstörts, t ex vid en olycka eller på grund av en sjukdom (Parkinson sjukdom och Alzheimers sjukdom), skulle kunna ersättas med nya celler. För att det här överhuvudtaget ska kunna bli verklighet är det viktig att ta reda på vad det är som reglerar stamcellernas tillväxt och differentiering.

Huvudsyftet men den här avhandlingen har varit att försöka ta reda på vad det är som reglerar tillväxten och differentieringen av neurala stamceller under utvecklingen av det centrala nervsystemet. För att bättra på den kunskap som redan finns har vi tittat närmare på en grupp faktorer som kallas för Sox proteiner. Det finns ett tjugotal olika Sox proteiner, som kan delas in i åtta olika grupp (A-H). Medlemmarna som tillhör samma grupp är väldigt lika varandra. Dessutom brukar de ha ungefär samma funktion i cellerna och kan därför oftast ersätta varandra vid behov. Vi har tittat närmare på Sox proteinerna som tillhör grupp B och vilken roll de har i utvecklingen av det centrala nervsystemet. Grupp B har delats in i två undergrupper, grupp B1 och grupp B2, eftersom några av medlemmarna har motsatta funktioner jämfört med den andra i denna grupp.

Avhandling är uppdelad i tre delar och i den första delen tittar vi närmare på medlemmarna i grupp B1, Sox1, Sox2 och Sox3 (Sox1-3), och vilken roll de har i regleringen av tillväxten och differentieringen av neurala stamceller. Vi kan visa att när mängden Sox1-3 ökas till nivåer som ligger långt över de normala,

så kan inga nya nervceller bildas. Eftersom en stamcell antingen kan dela på sig, för att bilda fler stamceller, eller ge upphov till nya nervceller, tyder det här på att Sox1-3 är viktiga för att stamcellerna ska bibehållas.

Syftet med det andra delarbete var att ta reda på om medlemmarna i grupp B2 också är viktiga för tillväxten och differentieringen av neurala stamceller. Denna grupp består av två medlemmar, men vi har bara studerat en medlem, nämligen Sox21. När mängden Sox21 översteg den normala mängden, så bildades det flera ny nervceller än vad som normalt bildas. Vilket tyder på att Sox21 har en motsatt funktion jämfört med Sox1-3, dvs Sox21 verkar vara viktig för att stamceller ska kunna bilda nya nervceller.

I den tredje delen tittade vi närmare på många av de gener som är viktiga för att det centrala nervsystemet ska bildas. Vi var intresserad av att ta reda på hur dessa gener styrs, så att de aktiveras vid rätt tillfälle och i rätt mängd. Det visade sig att många av dessa gener styrs med hjälp av flera olika grupper av faktorer och att en av dessa grupper är Sox proteinerna som tillhör grupp B1.

Sammanfattningsvis, så har vi visat att medlemmarna i grupp B1 är viktiga för att stamceller ska finnas kvar, medan en medlem i grupp B2 är viktig för att nya nervceller ska kunna bildas. Dessutom så har vi visat att många av de gener, som är viktiga för att det centrala nervsystemet ska bildas, styrs av bland annat Sox proteinerna som tillhör grupp B1.

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