TECHNISCHE UNIVERSITÄT MÜNCHEN

Fakultät Wissenschaftszentrum Weihenstephan für Ernährung, Landnutzung und Umwelt

Lehrstuhl für Entwicklungsgenetik

Examining the role of SoxC transcription factors in adult hippocampal neuronal differentiation

Dissertation von
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1 Zusammenfassung

Zahlreiche Forschungsergebnisse deuten auf eine Rolle der adulten Neurogenese beim Lernen, der Erinnerung und bei Depressionen hin. Während die Bildung neuer Nervenzellen im adulten Hippokampus als gesicherte Kenntniss angesehen wird, sind die zugrundeliegenden regulatorischen Mechanismen noch weitgehend unbekannt. Es gibt zwei fundamentale Fragen, die zum Verständnis des molekularen Codes der adulten Neurogenese beantwortet werden müssten: 1) Welche Transkriptionsfaktoren regulieren die adulte Neurogenese und 2) wie ist ihre Wirkungsweise? Mein Bestreben war es, diese zwei Fragen im Kontext der SoxC Transkriptionsfaktoren näher zu beleuchten. Die SoxC Faktoren Sox4 und Sox11 werden im sich entwickelnden Nervensystem der Maus und Rückenmark des Huhns exprimiert und regulieren während der embryonalen Neurogenese die neuronale Schicksalsbestimmung von Vorläuferzellen. In unserem wurde Labor die Hypothese aufgestellt, dass SoxC Proteine auch die adulte Neurogenese regulieren könnten. In dieser Studie konnte ich nachweisen, dass Sox4 und Sox11 essentielle Regulatoren der adulten Neurogenese im Hippokampus sind. Diese Schlussfolgerung wird durch folgende Beobachtungen unterstützt: Erstens fand ich heraus, dass die Expression der Sox4 und Sox11 Proteine im adulten Hippokampus mit dem Zeitpunkt der neuronalen Schicksalbestimmung zusammenfällt. Zweitens erlangte ich mittels in vitro Experimenten Hinweise darauf, dass Sox4 und Sox11 die neuronale Ausdifferenzierung adulter neuronaler Stammzellen induzieren. Des Weiteren aktivieren Sox4 und Sox11 direkt den Promotor des Nervenzell-spezifischen Gens Doublecortin (DCX). Drittens zeigten meine in vivo Experimente, dass der Verlust von Sox4 und Sox11 die Expression von DCX im adulten Hippokampus der Maus unterdrückt. Darüber hinaus verlängerte eine Überexpression von Sox11 in adulten neurogenen Nischen die Expression von DCX und verschob die Position der Zelle in Richtung der äußeren granulären Zellschicht. Gleichzeitig hatte die verlängerte Überexpression von Sox11 keinen Effekt auf die Länge und Verzweigung der Dendriten neugebildeter Nervenzellen. Abschießend konnte ich zeigen, dass Sox2 ein potentieller Aktivator der Sox11 Transkription in der adulten

hippokampalen Neurogenese sein könnte. Zusammenfassend kann ich demnach den SoxC Faktoren eine entscheidende Rolle in der adulten hippokampalen Neurogenese zuschreiben. Betrachtet man die zunehmende Bedeutung einer fehlgesteuerten hippokampalen Neurogenese bei Alzheimer Erkrankungen, Epilepsie und Depressionen, so werfen die Ergebnisse die Möglichkeit auf, dass mit Hippokampus assoziierten pathologischen Prozessen eine Störung der SoxC Aktivität einhergehen könnte.

2 Abstract

Adult neurogenesis has been suggested to play a role in learning, memory, and depression. While the observation and characterization of adding new neurons to adult hippocampus has been robust, the underlying regulatory mechanisms remain poorly understood. There are two fundamental questions for understanding the molecular code of adult neurogenesis: 1) Which transcription factors regulate it?; 2) What is their mode of action? I attempted to work out these two questions by focusing on SoxC group of transcription factors, Sox4 and Sox11. Their role in embryonic neurogenesis has been evidenced by findings that SoxC members are specifically expressed in the developing mouse central nervous system and chicken spinal cord and are functionally redundant. Meanwhile, our laboratory suggested that SoxC proteins could also regulate adult neurogenesis. In this study I give evidence that Sox4 and Sox11 are essential regulators of adult hippocampal neurogenesis. This conclusion is supported by the following observations. First, I found that Sox4 and Sox11 protein expression in adult hippocampus was initiated upon neuronal fate commitment. Second, my *in vitro* experiments showed that Sox4 and Sox11 potently induced neuronal differentiation of adult neural stem cells. In addition, Sox4 and Sox11 directly activated the promoter of the neuronal lineage-specific gene DCX. Third, my *in vivo* experiments showed that the loss of Sox4 and Sox11 abolished the expression of neuron-specific structural protein DCX in adult mouse hippocampus . Moreover, overexpressing Sox11 in adult neurogenic niche prolonged the DCX expression, shifted the cell position towards the outmost region of granule cell layer, but did not affect dendritic length and branching. Finally, I found that Sox2 might be one of the activators of Sox11 transcription in adult hippocampal neurogenic lineage. Based on these findings, I demonstrated a crucial role of SoxC factors in adult hippocampal neurogenesis. Given the emerging role of dysfunctional hippocampal neurogenesis in Alzheimer's disease, epilepsy and depression, the data raises the possibility that dysregulation of SoxC activity could be involved in hippocampus-associated pathological processes.

3 Introduction

3.1 Adult neurogenesis

The first observation of neurogenesis in adult mammalian brain was reported by Josef Altman and Gopal Das in 1965. They discovered newly generated adult-born neurons using [^{3}H]-thymidine labeling (Altman and Das, 1965). Adult neurogenesis, the generation of functionally integrated neurons from progenitor cells in adult brain, was later confirmed with techniques relying on the thymidine analogue 5-bromo-2'deoxyuridine (BrdU) (Kuhn et al., 1996) and on the retroviral labeling (Lie et al., 2004; Zhao et al., 2008).

Under physiological conditions, neurogenesis occurs throughout life in two anatomical regions of the mammalian brain: the subgranular zone (SGZ) of the dentate gyrus (DG) in the hippocampus, and the subventricular zone (SVZ) of the lateral ventricles (Figure 1A, Gage, 2000; Alvarez-Buylla and Garcia-Verdugo, 2002; Lie et al., 2004; Ming and Song, 2005; Hsieh, 2012). Adult neurogenesis consists of a stereotypic sequence of developmental steps and is regulated by physiological and pathological mechanisms at several levels: the proliferation of adult neural stem cells (NSCs) or progenitors, differentiation and fate specification of neural progenitors, and the migration, maturation and integration of newborn neurons (Figure 1, Alvarez-Buylla and Lim, 2004; Gage, 2000; Goh et al., 2003; Kempermann and Gage, 1999; Lie et al., 2004; Zhao et al., 2008). In the dentate gyrus, neural stem cells give rise to glutamatergic granule neurons that incorporate into the adult hippocampal circuitry (Laplagne et al., 2006). In the SVZ, neural stem cells generate a more diverse cohort of new neurons, including GABAergic, dopaminergic, and glutamatergic neurons. All of them migrate anteriorly through the rostral migratory stream (RMS), integrate in the olfactory bulb (OB) and become interneurons (Figure 1A, Merkle et al., 2004; Scheffler et al., 2005; Lledo et al., 2008; Brill et al., 2009; Ming and Song, 2005). Adult-generated olfactory interneurons contribute to odor discrimination and olfactory memory (Mouret et al., 2009; Sakamoto et al., 2011; Kageyama et al., 2012).

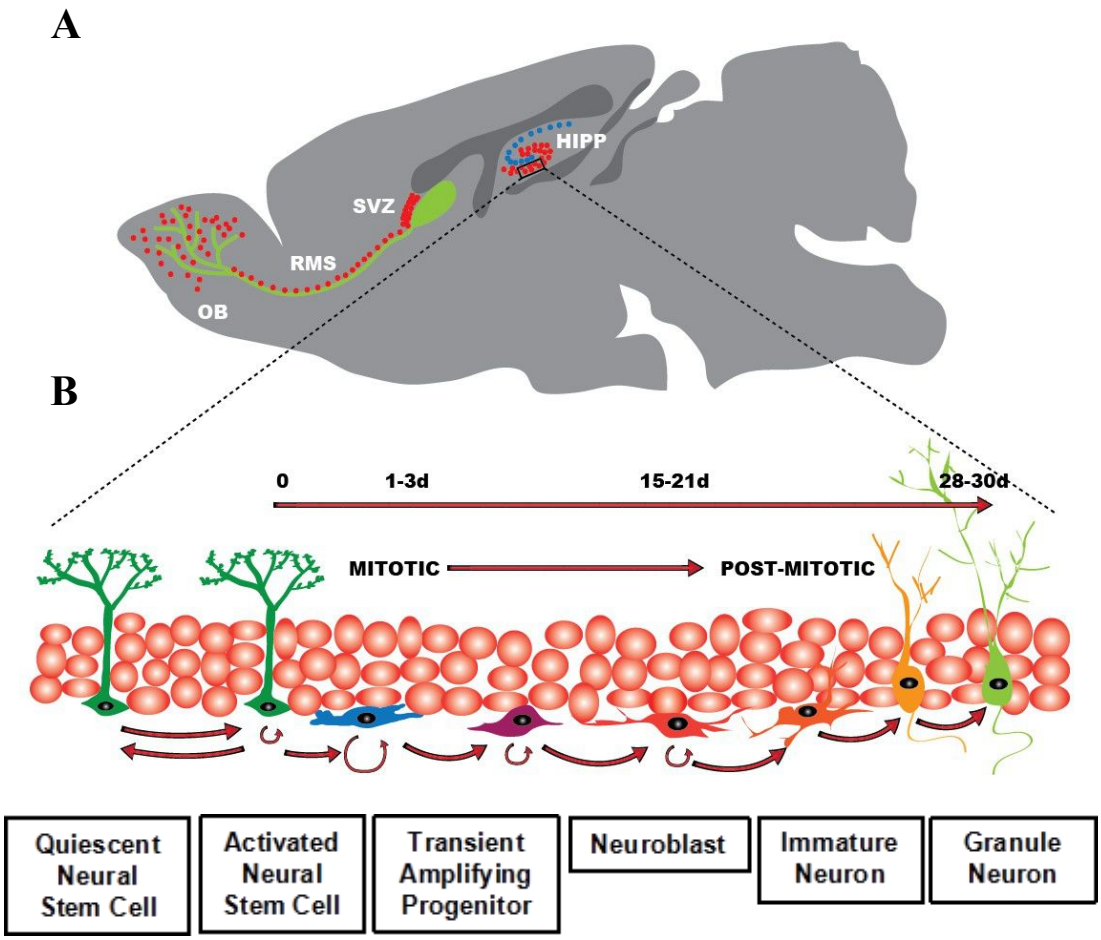

Figure 1. Neurogenesis in adult mouse brain

(A) **Sagittal overview of two adult neurogenic areas**
The subgranular zone of the dentate gyrus in the hippocampus (HIPP) and the subventricular zone (SVZ) of the lateral ventricles are the two neurogenic regions of the adult mammalian brain. In the SVZ, stem cells generate neuroblasts that migrate through the rostral migratory stream (RMS) and integrate in the olfactory bulb (OB) to become interneurons.

(B) **Schematic illustration of morphologically and physiologically distinct development stages of hippocampal neurogenesis**
The cell bodies of stem cells are located within the subgranular zone of the dentate gyrus. Their radial processes extend towards the granular cell layer and their short processes extend along the border of the granule cell layer and hilus. Stem cells give rise to transient amplifying cells that differentiate into immature neurons, which then mature into glutamatergic neurons and integrate into the granular cell layer.

Newborn neurons in the dentate gyrus of the hippocampus go through several stages of morphological and physiological development, express distinct sets of marker proteins and display striking morphological and electrophysiological differences in different developmental stages (Figure 1B, Zhao et al., 2008; Hsieh, 2012). Stem cells with their cell bodies located within the subgranular zone in the dentate gyrus extend their radial processes towards the granular cell layer and their short processes along the border of the granule cell layer and hilus. These neural stem cells are known as radial glia like stem cells or Type 1 cells, and express glia fibrilary acidic protein (GFAP) (Seri et al., 2001). Radial glia like stem cells or Type 1 cells are thought to be quiescent or to divide very rarely (Bonaguidi et al., 2011). When activated by certain extrinsic signals, these Type 1 cells divide and give rise to transient amplifying cells (Type 2 cells), which express transcription factors Mash1 and Tbr2. The type 2 precursor cells commit to a neuronal fate to differentiate into neuroblasts. These undergo a limited number of cell divisions (Steiner et al., 2006) prior to differentiating into immature neurons, which express Doublecortin (DCX) and Calretinin (after cell cycle exit) and start to extend axonal projections toward the CA3 pyramidal cell layer and to send their dendrites in the opposite direction toward the molecular layer to receive inputs from the entorhinal cortex. At a certain stage, these immature neurons cease to proliferate and become postmitotic. The immature neurons migrate into the granule layer of the dentate gyrus in hippocampus and mature into new glutamatergic granule neurons (Cameron et al., 1993; Kempermann et al., 2004; Ming and Song, 2005; Steiner et al., 2006). The newly generated granule neurons are excitatory and the principal neurons of dentate gyrus. The DG neurons provide excitatory input to the pyramidal cells of CA3 area and express markers of mature dentate granule neurons like NeuN and Calbindin. The structural and functional maturation of adult-born hippocampal neurons lasts between 6 and 8 weeks (van Praag et al., 2002; Zhao et al., 2006; Ge et al., 2008).

The discovery of adult neurogenesis has radically changed our idea about the ways of brain adaption to physiological and environmental challenges. By continuously generating cohorts of new neurons, adult hippocampus retains

an enhanced form of plasticity through the adulthood, so that the adult brain maintains the ability to adapt to new experiences. Contrary to mature DG neurons, adult-born DG neurons are more responsive and show increased plasticity during the first 1-2 months after their birth (Snyder et al., 2001; Schmidt-Hieber et al., 2004). This difference is due to a lower threshold for induction and a larger amplitude of long-term potentiation (LTP) in immature DG neurons. Their temporarily enhanced plasticity is mediated through transient expression of NR2B-containing NMDA receptors (Snyder et al., 2001; Ge et al., 2007), and the protracted development of GABAergic inhibition (Li et al., 2012; Marin-Burgin et al., 2012). Such distinct neurophysiological properties of immature DG neurons contribute to hippocampal plasticity, and have been linked to spatial memory, in particular to pattern separation (Clelland et al., 2009; Aimone et al., 2011; Sahay et al., 2011; Aimone et al., 2014). Moreover, as immature adult-born DG neurons age, their function seems to switch from pattern separation to pattern completion (Nakashiba et al., 2012). In other words, the maturation process of adult-born DG neurons contributes to the ability to distinguish among memories and to recall memory from partial sensory cues.

Hippocampal neurogenesis in adult brain may also be involved or dysregulated in neuropsychiatric disorders (Noonan et al., 2010; Aimone et al., 2011; Sahay et al., 2011; Snyder et al., 2011; Petrik et al., 2012). Initial studies reported that the rate of hippocampal neurogenesis would correlate with emotional states. However, subsequent studies provided evidence suggesting that there is a rather complicated link between hippocampal neurogenesis and affective states in animal models. For example, antidepressant-related increase in NPC proliferation was observed only in some mice strains (David et al., 2009; Holick et al., 2008; Miller et al., 2008; Santarelli et al., 2003), and stress does not always reduce in neurogenesis (Kirby et al., 2013). However, in certain tests for anxiety- and depression-like behaviors, the efficacy of some antidepressants such as fluoxetine (an antidepressant of the SSRI class) depended on the hippocampal neurogenesis (Santarelli et al., 2003). Moreover, the neurodegenerative disorders correlate with a gradual loss of relatively well-defined neuronal

populations in adult brain. Yet, it has been unknown whether the symptoms of some neurodegenerative diseases are the consequence of reduced adult neurogenesis or its cause (Armstrong and Barker, 2001; Jordan et al., 2006). Therefore, understanding the basic mechanisms regulating adult neurogenesis and their contribution to brain functions may be crucial for prevention and/or therapy of neurological and psychiatric pathologies such as Alzheimer's and Parkinson's disease, anxiety and depression.

A crucial step in adult neurogenesis is the commitment of the multipotent precursor cell towards a neuronal fate. Several transcription factors have been shown to direct fate specification and lineage commitment in both adult neurogenic niches (see review Hsieh, 2012). Neuronal fate commitment is accompanied by downregulation of the multipotency factor Sox2 (Ellis et al., 2004; Steiner et al., 2006) and onset of expression of transcription factors NeuroD1 (Steiner et al., 2006; Gao et al., 2009), Prox1 (Steiner et al., 2008; Lavado et al., 2010) and Mash1 (Raposo et al., 2015; Andersen et al., 2014). These transcription factors are regulated by the Wnt-signaling pathway (Kuwabara et al., 2009; Karalay et al., 2011) whose activity is required for the generation of new hippocampal neurons during adulthood (Lie et al., 2005). Loss of NeuroD1 (Gao et al., 2009) and Prox1 (Lavado et al., 2010; Karalay et al., 2011) in the hippocampal neurogenic lineage results in impaired maturation and survival of new neurons. Nevertheless, NeuroD1-deficient (Gao et al., 2009) and Prox1-deficient (Lavado et al., 2010) NSCs still give rise to immature neurons, indicating that additional transcriptional factors contribute to neuronal fate commitment of NSCs in the adult hippocampus.

3.2 The SoxC family of transcription factors

Sox (Sex-determining region Y (Sry)-box protein) family members are characterized by a conserved high mobility group (HMG) DNA binding domain (Gubbay et al., 1990; Sinclair et al., 1990). So far, 20 different Sox genes have been identified in mice and humans (Schepers et al., 2002). According to the similarities of their HMG box region, Sox proteins are divided into eight

different groups termed A to H (Sarkar et al., 2013). Members with the same group preserve higher than 80% identity in their HMG-domain and share other well-conserved regions (Bowles J et al., 2000). In addition, individual members with a group share biochemical properties, have overlapping expression patterns and perform synergistic or redundant functions. In contrast, members from different groups usually perform distinct functions (Wegner, 2010).

The SoxC group is composed of three members in mice, humans and most other vertebrates: Sox4, Sox11 and Sox12. The three proteins feature two conserved and functional domains: an Sry-related HMG box (Sox) DNA-binding domain, located in the N-terminal half of the protein, and a transactivation domain (TAD), located at the C-terminus (Dy et al., 2008; Alfredo et al., 2010; Kamachi et al., 2013).

Sox family members have homologs in chicken, gull, frog, turtle, zabrafish, marsupials and Drosophila (Laudet et al., 1993; Coriat et al., 1993; Chardard et al., 1993; Denny et al., 1992; Foster et al., 1994; Griffiths et al., 1991; Uwarogho et al., 1995; Vriz et al., 1995). In addition, two Sox-like genes have been identified in the unicellular choanoflagellate *Monosiga brevicollis*, an animal that is most closely related to multicellular metazoans, suggesting that the origin of Sox proteins predates multicellularity or possibly marks the transition of unicellular to multicellular organnisms (Guth and Wegner, 2008; King et al., 2008).

Sox genes are critical developmental regulators. They regulate sex determination, chondrogenesis, hematopoiesis, neural crest development and neurogenesis. Moreover, Sox proteins control the maintenance of stem cell characteristics and pluripotency, they influence cell death, survival, proliferation, cell fate decisions, consecutive lineage progression, and they participate in terminal differentiation processes (for review see Wegner and Stolt., 2005; Sarkar et al., 2013).

These highly versatile functions of Sox proteins are largely dependent on the presence of their interaction partners that allows their spatial- and timely-

restricted activity. The specificity of Sox trancription factors for their target genes seems to be regulated through several ways: i) Differential affinity for particular flanking sequences next to consensus Sox sites; ii) Homo- or heterodimerization among Sox proteins; iii) Post-translational modifications; iv) Interaction with other co-factors (Wegner, 2010; Sarkar et al., 2013).

3.3 The role of SoxC factors in neurogenesis

In 2006, Jonas Muhr's group reported that the induction of two SoxC family members --Sox4 and Sox11-- is a critical step in the acquisition of a neuronal phenotype in the developing chicken spinal cord. The authors suggested that Sox4 and Sox11 are critical initiators of both generic and subtype specific neuronal properties. They identified *β*-tubulin III and Map2 as potential SoxC targets (Bergsland et al., 2006). In 2011, the same group reported the genome-wide binding analysis of SoxB1 and SoxC proteins, which have vital functions in embryonic cells, neural precursor cells, and maturing neurons, respectively. They demonstrated that Sox factor binding depended on developmental stage-specific constraints and become activated during neural lineage development through remarkable sequentially acting Sox2-->Sox3-->Sox11 complexes. In embryonic cells, Sox2 preselected for neural lineage specific genes destined to be bound and activated by Sox3 in neural precursor cells, where Sox3 bound genes that were later bound and activated by Sox11 in differentiating neurons. This observation indicated that a single family of transcription factors may coordinate the whole neural lineage-specific gene expression program, starting from the early pluripotent stem cell stage to the final onset of neuronal and glial gene expression in differentiated cells (Bergsland et al., 2011).

In 2008, Veronique Lefebvre's group found that all three SoxC proteins -- Sox4, Sox11 and Sox12 -- functionally interact with each other in many lineages and play distinct roles in developmental, physiological and possibly pathological processes. They found all three genes co-expressed at high levels in neuronal and mesenchymal tissues in the developing mouse. Moreover, they found that Sox11 activates transcription several times more

efficiently than Sox4 and up to one order of magnitude more efficiently than Sox12, owing to a more stable a-helical structure of its transactivation domain (Dy et al., 2008).

SoxC factors regulate embryonic neurogenesis and the survival of neural precursors and their differentiated progeny in a highly redundant manner (Hoser et al., 2008; Bhattaram et al., 2010; Potzner et al., 2010; Thein et al., 2010). Thein and colleagues reported that Sox4 and Sox11 regulate cell survival during spinal cord development in a largely redundant manner (Thein et al., 2010). Bhattaram and colleagues found that the three SoxC genes work redundantly during mouse organogenesis, in part by directly targeting the Tead2 gene (Bhattaram et al., 2010). Finally, Hoser and colleagues reported that Sox4 and Sox11 function redundantly with Sox12 and can compensate its loss during mouse development (Hoser et al., 2008).

The very first evidence that Sox11 is expressed in adult neurogenic niche was presented by Haslinger and colleagues (Haslinger et al., 2009). This study based on immunohistochemical analysis of adult mouse brain revealed that Sox11 expression is strictly confined to doublecortin-expressing neuronally committed precursors and immature neurons in both neurogenic areas. Haslinger and colleagues reported that Sox11 is not expressed in non-committed Sox2-expressing precursor cells and in mature neurons of both adult neurogenic lineages. Moreover, Sox11 overexpression enhanced the neuronal fate commitment of adult NSCs *in vitro* (Haslinger et al., 2009). This suggested that the transcription factor Sox11 could be one of the regulators of adult mouse brain neurogenesis and became the main motivation for my PhD study.

3.4 The objectives of this study

Impaired hippocampal neurogenesis typically seen in aged or stressed mice is associated with cognitive and memory impairments in clinical human populations. This suggested that neurogenesis may be potentially lower in psychiatric and neurological conditions. Elucidation of the transcriptional code

regulating adult neurogenesis represents an important step towards strategies aiming to enhance hippocampal function via stimulation of neurogenesis in these diseases. There is strong evidence that SoxC transcription factors -- Sox4 and Sox11-- are critical initiators of both generic and subtype specific neuronal properties in the developing chicken spinal cord (Bergsland et al., 2006). Moreover, loss-of-function mouse studies revealed that SoxC proteins play an important role in proliferation and survival of developing sympathetic neurons (Potzner et al., 2010). Yet, whether SoxC factors regulate adult neurogenesis remains poorly understood. Previous evidence from our laboratory showed that Sox11 protein is almost exclusively expressed in immature neurons of adult mouse brain (Haslinger et al., 2009). Moreover, preliminary experiments suggested that Sox11 may activate the expression of immature neuronal specific protein Dcx.

The topic of my PhD was to investigate whether adult neurogenesis is regulated by two members of the SoxC family of transcription factors, Sox4 and Sox11. I assessed the following aims:

1) Analyze the expression pattern of Sox4 and Sox11 proteins in neurogenic regions of the adult mouse brain
2) Examine whether SoxC factors are essential for *in vitro* neurogenesis of adult neural precursors using gain- and loss-of-function experiments
3) Investigate whether the ablation of Sox4 and Sox11 in the adult hippocampal neurogenic lineage inhibits neurogenesis
4) Elucidate whether prolonged expression of Sox11 in maturing adult-born hippocampal neurons sustains the expression of immature neuron-specific protein DCX and affects neuronal morphology.
5) Investigate candidate upstream regulators and downstream targets of SoxC transcription factors.

4 Results

The aim of my PhD project was to investigate whether adult neurogenesis is regulated by two SoxC transcription factors, Sox4 and Sox11. Their role in embryonic neurogenesis has been evidenced: SoxC members are specifically expressed in the developing mouse CNS and chicken spinal cord and are functionally redundant during neuronal development (Bergsland et al., 2006; Dy et al., 2008). This may be explained by the high conservation of their Sry-related high-mobility group (HMG) box DNA-binding domain and in the C-terminal region (Wetering et al.,1993; Kuhlbrodt et al., 1998; Jay et al., 1995; Maschhoff et al., 2003). That SoxC proteins regulate also adult neurogenesis has been suggested by our laboratory. Haslinger et al found that SOX11 protein is almost exclusively expressed in immature neurons of adult mouse brain (Haslinger et al., 2009). My aim was to analyze the expression pattern of Sox4 protein in the adult hippocampal neurogenic lineage and compare it to the Sox11 expression pattern. Secondly, I performed gain- and loss-of-function experiments *in vitro* to examine whether SOXC factors are essential for neuronal differentiation in adult neural precursor cells (NPCs). Thirdly, I performed loss of function experiment in adult mouse hippocampus to see if *in vivo* ablation of Sox4 and Sox11 could affect neuronal differentiation. Fourthly, I prolonged expression of Sox11 in hippocampal mature neurons to see if it would change their morphology and reactivate DCX expression. Finally, I performed luciferase assays on Sox11 and DCX promoters to get first clues about upstream regulation and downstream targets of Sox11.

4.1 Overlapping expression of Sox4 and Sox11 in adult neurogenic niches of the adult mouse brain

In order to examine the expression patterns of SoxC protein in the adult neurogenesis niche, I performed immunohistochemistry using Sox4 and Sox11-specific antibodies (Potzner et al., 2007). Similar to the Sox11 expression pattern in the adult mouse brain (Haslinger et al., 2009), Sox4 expression was primarily confined to areas of adult neurogenesis (Figure 2),

i.e. the hippocampal dentate gyrus (Figure 2B), the subventricular zone of the lateral ventricles (Figure 2C), the rostral migratory stream (Figure 2D) and the olfactory bulb (Figure 2E).

The expression of Sox4 overlapped to a high degree with the expression of Sox11 in the subgranular zone of the dentate gyrus (88.4±10.6% of the Sox 4+ cells were also Sox11 positive) in the hippocampus (Figure 3A, 3A', 3A'' and 3G). Similarly, Sox4 and Sox11 showed overlapping expression in areas of the second adult neurogenic niche, i.e., the subventricular zone of the lateral ventricles (Figure 4C) and the rostral migratory stream (Figure 4G).

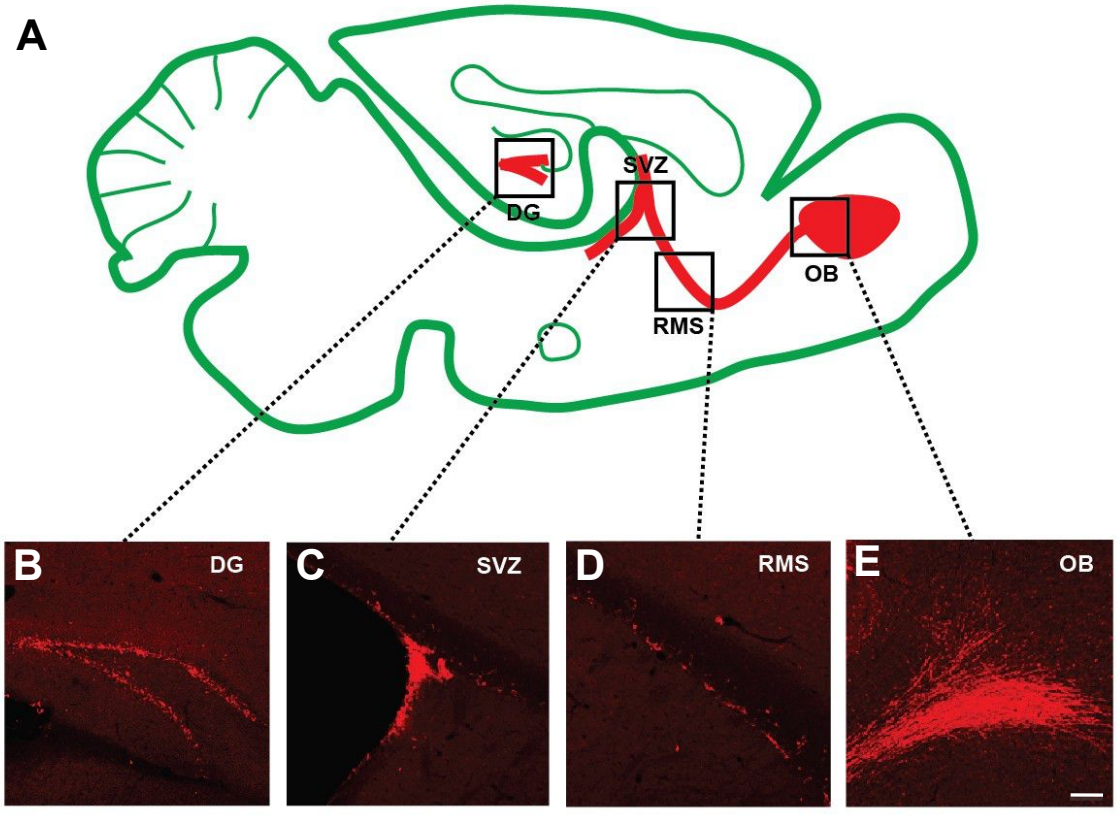

Figure 2. Sox4 transcription factor expression in the adult neurogenic niches
(A) Depictions of sagittal views of mouse brain in areas where neurogenesis occurs. Red areas indicate the germinal zones in the adult mouse brain: the subgranular zone of the dentate gyrus (DG) in the hippocampus and the subventribular zone (SVZ) of the lateral ventricles. Neurons generated in the SVZ migrate through the rostral migratory stream (RMS) and are incorporated into the olfactory bulb (OB). Sox4 (red) is highly expressed in the subgranular zone of the DG (B), and SVZ (C), RMS (D), OB (E). Scale bars: 100μm.

The prominent expression of SoxC proteins in the neurogenic regions of the adult brain prompted me to perform a detailed analysis of SoxC expression patterns in the hippocampal neurogenic lineage (Figure 3; Figure 4). To this end, I compared the expression of Sox4 and Sox11 with the expression of markers that are expressed at different stages of adult neurogenesis (summarized in Figure 5). Kempermann and colleagues reported three types of dividing precursor cells in the adult hippocampal neurogenic lineage (Kempermann et al., 2004). Type 1 precursor cells are slowly dividing and display a radial glia like morphology. They express Sox2 and glial fibrillary acidic protein (GFAP) and have the unique capability to give rise to highly proliferative transient amplifying Type 2 cells. Type 2 precursor cells are rapidly dividing and express Sox2 but not GFAP (Ellis et al., 2004; Steiner et al., 2006; Suh et al., 2007). Both type 1 and type 2 cells are considered to be multipotent whereas type 3 precursor cells are committed to the neuronal lineage. They show no Sox2 and express characteristic proteins of immature neurons like microtubule-associated protein Doublecortin (DCX).

In a detailed analysis of the dentate gyrus of hippocampus, I found that Sox4 expression was more common in committed, type 3 cell than multipotent, type 1 and 2 cells. GFAP expression overlapped with 1.2±1.1% of Sox4-positive cells whereas Sox2 overlapped with 8.7±4.1% of Sox4-positive cells (Figure 3B, 3G). To the contrary, 99.3±1.2%, of the Sox4-positive cells co-expressed immature neuronal marker DCX (Figure 3D, 3G) and 62.5±6.1% of Sox4-positive cells co-expressed calcium-binding protein Calretinin (Figure 3A, 3G). As the triple immunostaining of Sox4, DCX and proliferation marker Ki67 showed that 8.0±5.8% of Sox4-positive cells co-expressed Ki67 and DCX (Figure 3D, 3G), I concluded that Sox4 expression was most common in neuronally-committed precursor cells.

Sox4 expression also overlapped with some transcription factors: 94.0±1.0% of Sox4-positive cells co-expressed NeuroD1(Figure 3E, 3E’ and 3G) and 91.0±4.0% of the Sox4-positive cells overlapped with Prox1 (Figure 3E, 3G). Moreover, 19.0±2.0% of Sox4- and Sox11- positive cells co-expressed Tbr2

(Figure 3C, 3G), a transcription factors that is common in Type 2a/b and Type 3 cells (Hodge et al., 2008).

In contrast to immature neurons, Sox4 and Sox11 expression in the mature dentate granule cells was very rare. Only 3.4±2.9% of the Sox11-positive cells overlapped with calcium-binding protein Calbindin (Figure 3F, 3G).

Taken together, these data demonstrated that the expression of Sox4 was confined to areas of adult neurogenesis, and almost completely overlapped with the expression of Sox11. Their expression appeared to be triggered at the time of neuronal fate commitment, i.e. in late Type 2a and Type 2b cells, was maintained in immature neurons, and was extinguished during neuronal maturation (Figure 5).

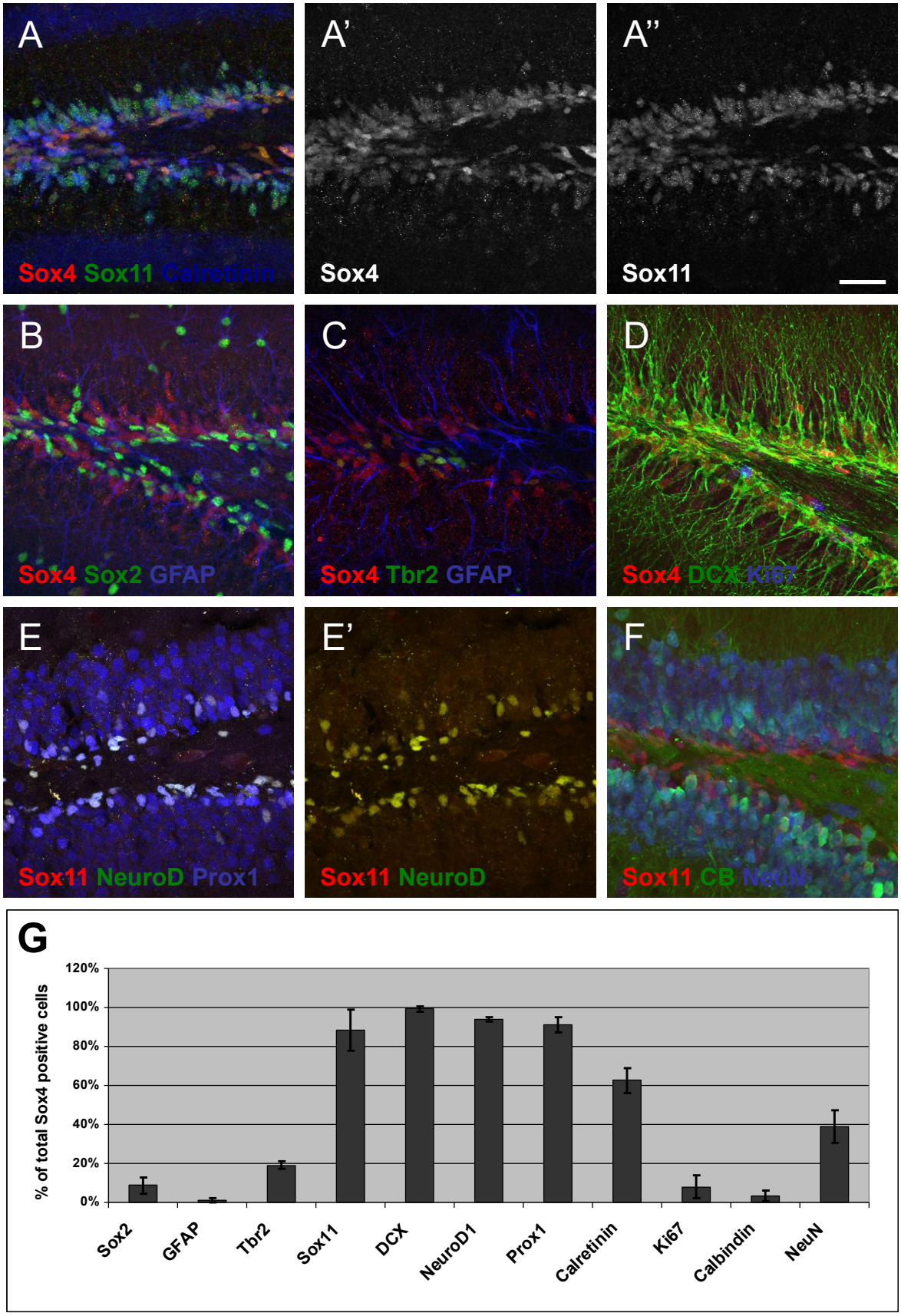

Figure 3. SoxC transcription factors expression in the adult hippocampal neurogenic lineage

(A) Sox4 (red) and Sox11 (green) are co-expressed in the dentate gyrus. Sox4/Sox11 double-positive cells appear in yellow. A large fraction of Sox4 and Sox11 positive cells are also positive for calretinin (blue), which is transiently expressed by postmitotic immature dentate granule neurons (Brandt et al., 2003). Single channels for Sox4 and Sox11 are presented in (A', A''). (B) Sox4 (red) is rarely expressed in Sox2 (green) expressing precursor cells. GFAP is in blue. (C) A fraction of Tbr2-positive precursor cells (green) expresses Sox4 (red). GFAP is in blue. (D) Sox4 (red) and DCX (green) show almost completely overlapping expression patterns. (E, E') Sox11 (red) is co-expressed with NeuroD1 (green) and Prox1 (blue). Triple-labelled cells in E appear in white and double-labelled cells in E' in yellow. (F) Mature calbindin-positive (CB, green) dentate granule cells are primarily negative for Sox11 (red). NeuN is in blue. (G) Quantification for expression of stage-specific molecular markers in total Sox4 positive cells in adult hippocampal neurogenesis. Scale bars: 30μm

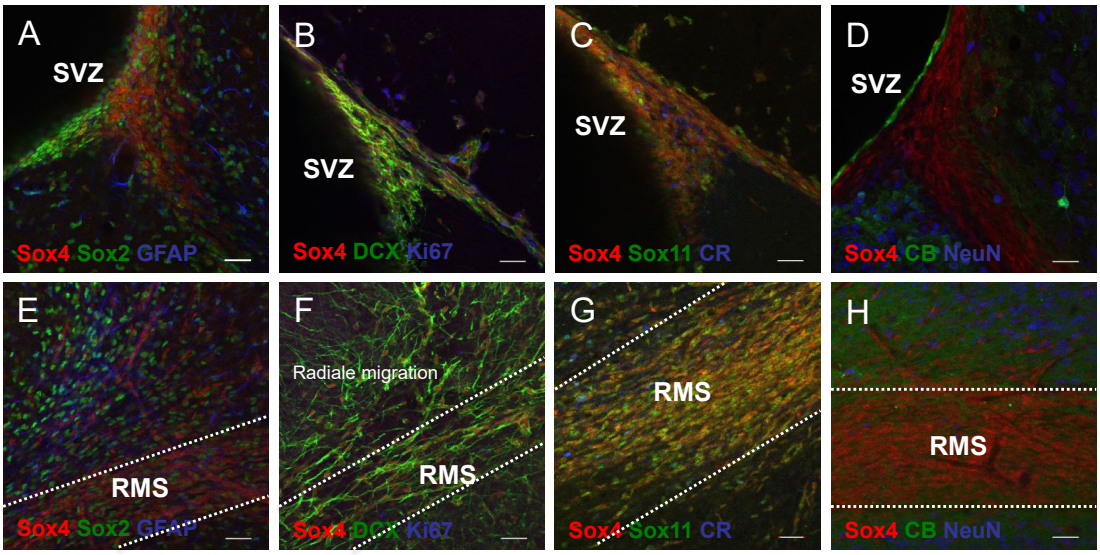

Figure 4. SoxC transcription factor expression in subventricular zone of the lateral ventricles and the rostral migratory stream in adult neurogenic niche.
Immunohistochemistry analysis of Sox4 expression pattern in the adult neurogenic niche of subventricular zone (SVZ; Figure 3 A-D) and the rostral migratory stream (RMS; Figure 3 E-H) with stage-specific markers. Sox4 is coexpressed with Sox11 (B, F) and DCX at high levels (C,G), and only occasionally expressed together with Sox2 and GFAP (A, E). No overlap is observed for Calbindin (CB) and NeuN (D, H). Scale bars: 100μm

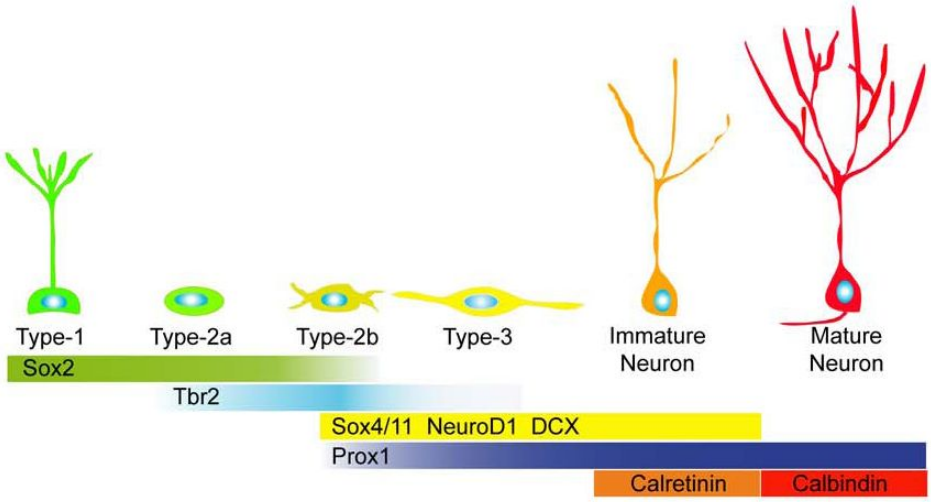

Figure 5. Schematic illustration of a model of adult hippocampal neurogenesis and Sox4/Sox11 expression in relation to expression of other markers along the cell lineage of the dentate gyrus

4.2 SoxC transcription factors are essential for *in vitro* neurogenesis from adult neural precursor cells

The finding that the onset of SoxC expression coincides with the onset of expression of the early neuronal lineage specific marker DCX suggests that SoxC transcription factors could participate in the regulation of neuronal fate determination in adult hippocampal neurogenesis.

To test this hypothesis, I firstly investigated the consequences of Sox4/Sox11 ablation on neural stem cell differentiation *in vitro*. Neural stem cells derived from hippocampus of 8-weeks-old adult Sox4$^{flox/flox}$; Sox11$^{flox/flox}$ double conditional knockout mice (Sox4/Sox11 dcKO) were maintained as monolayer culture. Recombination of the conditional loci was induced by transduction with the CAG-GFP-IRES-Cre retrovirus. This retrovirus bicistronically encodes for Cre recombinase and GFP. Parallel cultures that were transduced with a retroviral vector expressing only GFP served as controls. Transduced cultures were differentiated by growth factor withdrawal and were analyzed 4 days post transduction (Figure 6A). Transduced cells were identified based on the expression of GFP. Transduction efficiency reached approximately 80% for both retroviruses. In RT-qPCR analysis (Figure 6C), mRNA expression of Sox4 and Sox11 was strongly reduced in Cre retrovirus transduced cultures, demonstrating that Sox4 and Sox11 loci were efficiently recombined by Cre recombinase. The vast majority of GFP positive cells in CAG-GFP-IRES-Cre transduced cultures did not display a typical neuronal appearance with multiple thin neuritic processes but had large nuclei and cell bodies with thick processes compared with controls (Figure 6B). This morphological appearance suggested that the neurogenesis in Sox4/Sox11-deficient NSCs was impaired. Indeed, mRNA expression of the neuron-specific markers DCX and β-tubulin III was strongly decreased in Sox4/Sox11-deficient NSC culture (Figure 6C). Moreover, Sox4/Sox11-deficient cells showed an approximately 90% reduction in the generation of DCX-immunoreactive neurons (Figure 6B). In contrast to the pronounced decrease in the generation of neurons, the production of GFAP-positive astrocytes in Sox4/Sox11-deficient NSCs compared with control appeared unaffected (Figure 6B, 6C), suggesting that

SoxC transcription factors Sox4 and Sox11 are essential for neurogenesis but not for the astrogliogenesis from adult NSCs.

Decreased cell density of Sox4/Sox11-deficient NSCs raised the possibility that Sox4/Sox11 ablation influenced the regulation of survival and proliferation in adult NSCs. To investigate this presumption, apoptosis and proliferation were analyzed through the activity of caspase 3 and the incorporation of the thymidine analog BrdU in retrovirally-transduced NSCs undergoing *in vitro* differentiation conditions. Caspase 3 activity was similar between Sox4/Sox11-deficient NSCs indicating that Sox4/Sox11 ablation did not significantly alter survival of differentiating NSCs (Figure 6E). On the other hand, Sox4/Sox11 ablation significantly reduced the fraction of cells that had incorporated the BrdU following a 1 hour BrdU pulse prior to fixation among Sox4/Sox11 deficient cells (Figure 6D). This indicated that Sox4/Sox11 ablation decreased proliferation of differentiating adult NSCs.

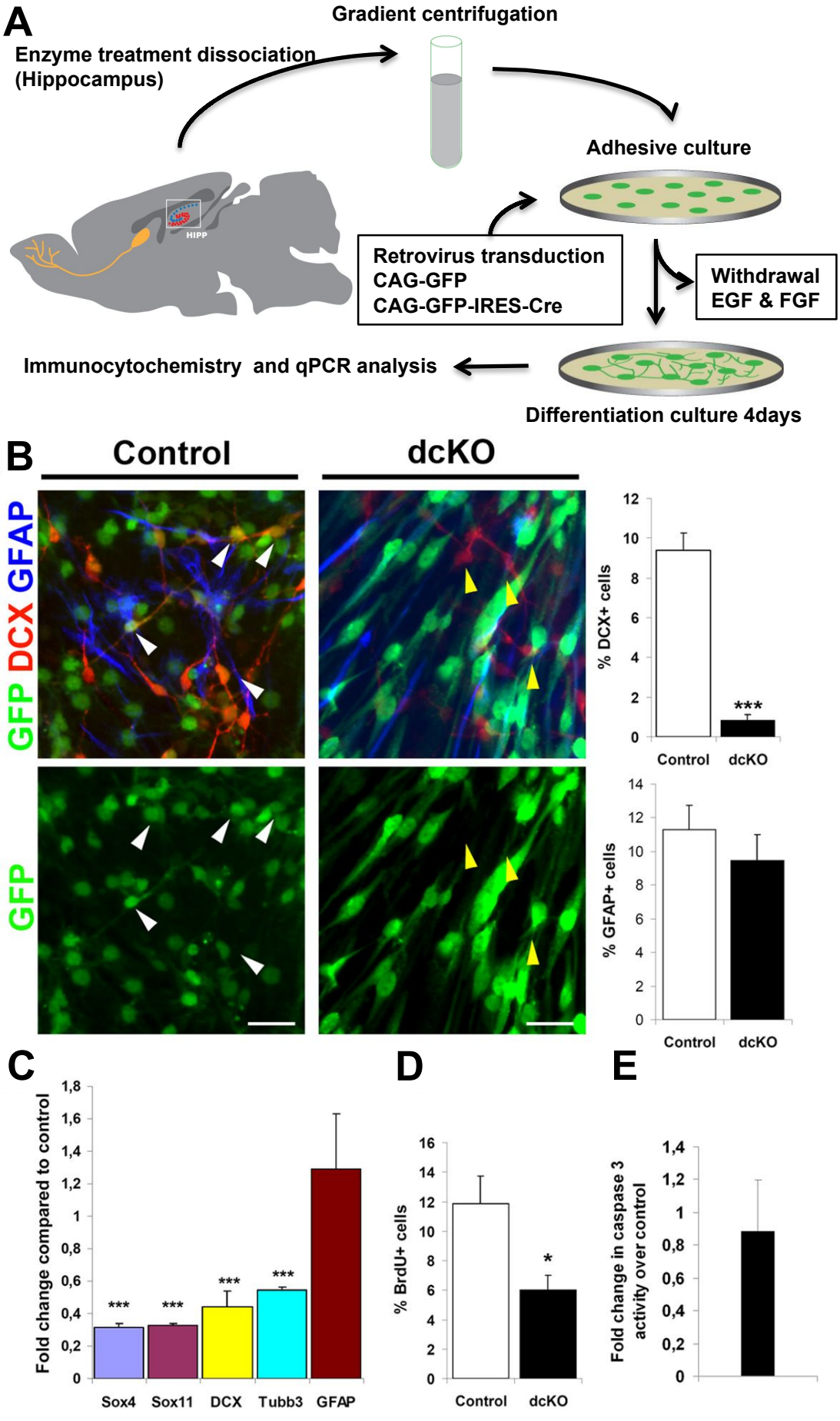
A
Gradient centrifugation
Enzyme treatment dissociation
(Hippocampus)
Adhesive culture
HIPP
Retrovirus transduction
CAG-GFP
CAG-GFP-IRES-Cre
Withdrawal
EGF & FGF
Immunocytochemistry and qPCR analysis
Differentiation culture 4days
B
Control
dcKO
GFP DCX GFAP
GFP
% DCX+ cells
% GFAP+ cells
Control
dcKO
C
Fold change compared to control
Sox4
Sox11
DCX
Tubb3
GFAP
D
% BrdU+ cells
E
Fold change in caspase 3 activity over control

Figure 6. SoxC transcription factors are essential for *in vitro* neurogenesis from the adult NSCs

(A) Methodology for analysis of SoxC transcription factor in adult neurogenesis *in vitro*. (B) In contrast to Cre-transduced *Sox4*$^{flox/flox}$; *Sox11*$^{flox/flox}$ NSCs (green, dcKO), control vector-transduced NSCs (green, control) generated DCX-positive neurons (red) (left, white arrowheads). Note that DCX-positive cells (yellow arrowheads) in the dcKO condition are not transduced with the GFP-IRES-Cre vector. Scale bar: 30μm. (C) qPCR analysis reveals strongly decreased expresssion of SoxC factors and the neuron-specific genes DCX and β-tubulin III (Tubb3) in Cre-transduced *Sox4*$^{flox/flox}$; *Sox11*$^{flox/flox}$ NSC cultures. (D) Cre-transduced *Sox4*$^{flox/flox}$; *Sox11*$^{flox/flox}$ NSC incorporate less BrdU after a 1 hour BrdU pulse before analysis. (E) Sox4/Sox11 ablation does not alter caspase 3 activity.

To further analyze the function of SoxC proteins on adult neurogenesis *in vitro*, Sox4/Sox11 gain-of-function experiments were established. NSCs were derived from hippocampus in the brain of 8-weeks-old C57BL/6 wildtype mice, and grown as monolayers under proliferating culture conditions in the presence of growth factors EGF and FGF2 (Figure 7A). These NSC cultures were transduced with retroviruses bicistronically encoding for GFP and either Sox11 (CAG-Sox11-IRES-GFP) or Sox4 (CAG-Sox4-IRES-GFP). Control cultures were transduced with retroviruses encoding for GFP (CAG-GFP) (Figure 7A). 48 hours after transduction with these viruses, cultures were fixed for analysis. Transduced cells were identified by GFP expression. Transduction efficiencies were approximately 70% for Sox11 retrovirus transduced cultures and 50-55% in NSC cultures transduced by Sox4 retrovirus. Overexpression of the Sox4 and Sox11 transgene was confirmed by RT-qPCR analysis (Figure 7G). Interestingly, the percentages of transduced cells that expressed the immature neuronal structural protein DCX were approximately 5-fold higher among Sox4-transduced cells and about 8-fold higher among Sox11-transduced cells than among control GFP-transduced cells (Figure 7B, 7D). Moreover, Sox4 and Sox11 overexpression promoted the expression of mRNAs for DCX and β-tubulin III (Figure 7G). In contrast to the pronounced increase in the generation of neurons, no significant differences were observed when transduced cells were analysed for generation of astrocytes, which were identified by the astroglia-specific protein GFAP (Figure 7C, 7D). Consistent with this finding, overexpression of either Sox4 or Sox11 did not alter the expression of mRNA for GFAP (Figure 7G). Neither Sox4 nor Sox11 overexpression in transduced cells altered caspase 3 activity (Figure 7F) and BrdU incorporation (Figure 7E) compared to control transduced culture, suggesting that overexpression of SoxC protein neither enhanced neuronal survival nor increased neurogenesis through promoting proliferation of DCX-positive neuroblasts. These observations indicated that SoxC proteins promoted neurogenesis but not astrogenesis from adult neural stem cells grown *in vitro*.

Taken together, gain- and loss-of-function experiments of SoxC proteins Sox4 and Sox11 showed that they regulated neuronal differentiation of adult NSCs

in vitro. Moreover, SoxC transcription factors were potent inducers of neuronal differentiation since their overexpression promoted generation of immature neuronal structural protein DCX even under culture conditions that favored the expension of stem cells.

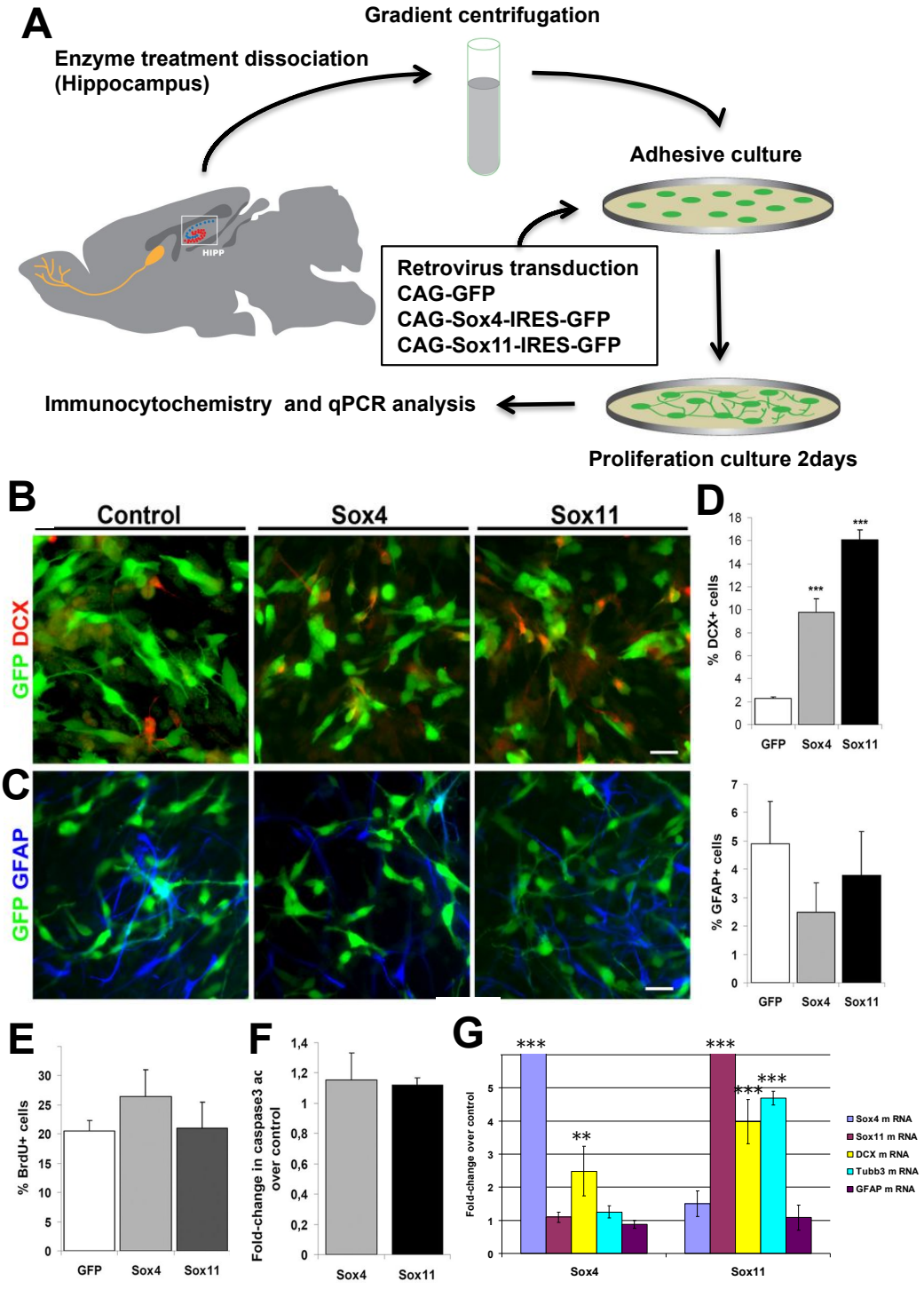

Figure 7. SoxC transcription factors enhance in vitro neurogenesis from the adult NSCs

(A) Methodology for analysis of the effect of SoxC transcription factor gain-of-function on adult neurogenesis *in vitro*. (B) Sox4 (middle) and Sox11 (right) overexpressing NSCs generate more DCX-positive (red) neurons. Transduced cells are identified by GFP expression (green). (C) Sox4 (middle) and Sox11 (right) overexpression does not alter the generation of GFAP-positive (blue) astrocytes. Scale bar: 30μm. (D) Quantification for DCX- and GFAP-positive cells in Sox4 and Sox11 overexpressing NSCs. (E) Sox4 and

Sox11 transduced NSCs do not incorporate BrdU to a higher percentage. (F) Sox4 and Sox11 overexpression do not alter caspase 3 activity. (G) qPCR analysis of Sox4 and Sox11 transduced cultures. Sox11 transduced cultures express significantly higher levels of DCX and β-tubulin III (Tubb3) mRNA. Sox4 overexpressing cultures express significantly higher levels of DCX and show a trend toward higher levels of β-tubulin III.

4.3 *In vivo* ablation of Sox4/Sox11 in adult hippocampus inhibited neurogenesis

To address whether SoxC factors influence neuronal differentiation during adult neurogenesis *in vivo*, I downregulated Sox4/Sox11 expression in the adult hippocampal neurogenic lineage of adult Sox4/Sox11 double conditional knock out (Sox4/Sox11 dcKO) mice via stereotactic injection of the MMLV CAG-GFP-IRES-Cre retrovirus into dentate gyrus (Tashiro et al., 2006; Jagasia et al., 2009). To increase the number of transduced cells in the adult hippocampus, mice were exposed to voluntary wheel running, known to strongly stimulate proliferation of neural stem cells (Kempermann et al., 1997; van Praag et al, 1999). Hence, all mice were put in a cage with running wheel for 7 days prior to injection. Sox4/Sox11 dcKO mice injected with a GFP-encoding retrovirus were used as control. All mice were analyzed 5 days after retroviral injection (Figure 8A). Transduced cells were identified on the basis of GFP expression. With immunohistochemistry analysis, I found that approximately 80% of GFP transduced cells expressed Sox4 and Sox11 in the control group (Figure 8B, 8C). In contrast, no CAG-GFP-IRES-Cre transduced cells expressed Sox4 and Sox11, indicating that recombination of the conditional Sox4 and Sox11 alleles was very efficient (Figure 8B', 8C').

Morphology

Sox4/11-ablated cells showed different morphology than control cells. The morphology of newly generated neurons in GFP-transduced control mice resembled that described in a previous time-course study of hippocampal neuron development in adult mouse brain (Zhao et al., 2006). At this 5 days time point, the GFP-transduced cells showed a polarized morphology: the majority of them bore neurites parallel to the granule cell layer and some of them had already formed a primary dendrite that extended toward the molecular layer (Figure 8B-F). In striking contrast, the majority of Sox4- and Sox11-deficient transduced cells showed no typical polarized morphology. Instead, many of them had multiple short processes or a star-like morphology and their neurites grew parallel to the granule cell layer or even towards the hilus (Figure 8B'-F'). SoxC-ablated cells did not show morphological

characteristics of apoptotic cell death, such as membrane blebbing or pyknotic nuclei (Figure 8B'-F'), indicating that SoxC deficiency did not affect survival identity at 5 days time point.

Expression of DCX, NeuroD1, Prox1

Sox4/11 ablation decreased the expression of early neuronal markers. GFP-transduced cells in the control group frequently expressed the early neuronal marker DCX (71.0±16.2% among transduced cells, Figure 8B, 8G) and dentate granule neuron-associated transcription factors NeuroD1 (88.7±10.0% of transduced cells, Figure 8C, 8G) and Prox1 (33.9±16.7% of transduced cells, Figure 8D, 8G). This indicated that the majority of transduced cells had differentiated into neurons and adopted a dentate granule neuron identity. On the other hand, Sox4/Sox11-ablated newborn cells showed strongly impaired expression of neuron-specific markers. Only a minor proportion of Sox4/Sox11-ablated newborn cells expressed DCX (0.7±1.1%, Figure 8B', 8G). Moreover, the expression of NeuroD1 (17.8±8.9%, Figure 8C', 8G) and Prox1 (18.7±10.2%, Figure 8D', 8G) were greatly reduced. These findings indicated that Sox4/Sox11-deficient cells failed to start or to maintain a complete neuronal differentiation program.

Astrogenesis

To test whether the loss of SoxC affected astrocyte differentiation, immunostaining for GFAP, which is commonly used as a marker for astrocytes, was performed. No differences were observed in the expression of GFAP in Sox4/Sox11 ablated cells compared to control (0.4±0.7% vs 0% in control, Figure 8E, 8E', 8G). The expression of the Type 1 and Type 2 a/b/c cells marker Sox2 was significantly increased in Sox4/Sox11-deficient cells (62.0±8.7% vs 30.0±8.2% in control, Figure 8D-D', 8E-E', 8F-F', 8G).

Proliferation

No difference in the expression of the Type 2b/c marker Tbr2 and the proliferation marker Ki67, which is expressed by all precursor cells, was observed between Sox4/Sox11 ablated cells and control cells (14.3±10.4% vs

10.3±4.2% for Tbr2 and 6.8±4.9% vs 4.6±2.3% for Ki67 among SoxC-ablated cells and control, Figure 8F-F', 8G).

In summary, *in-vivo* ablation demonstrated that Sox4/Sox11 were required for neuronal differentiation in the adult hippocampus but dispensable for astrocyte differentiation and for adoption of Type 1 or Type 2 a/b/c precursor cell identity.

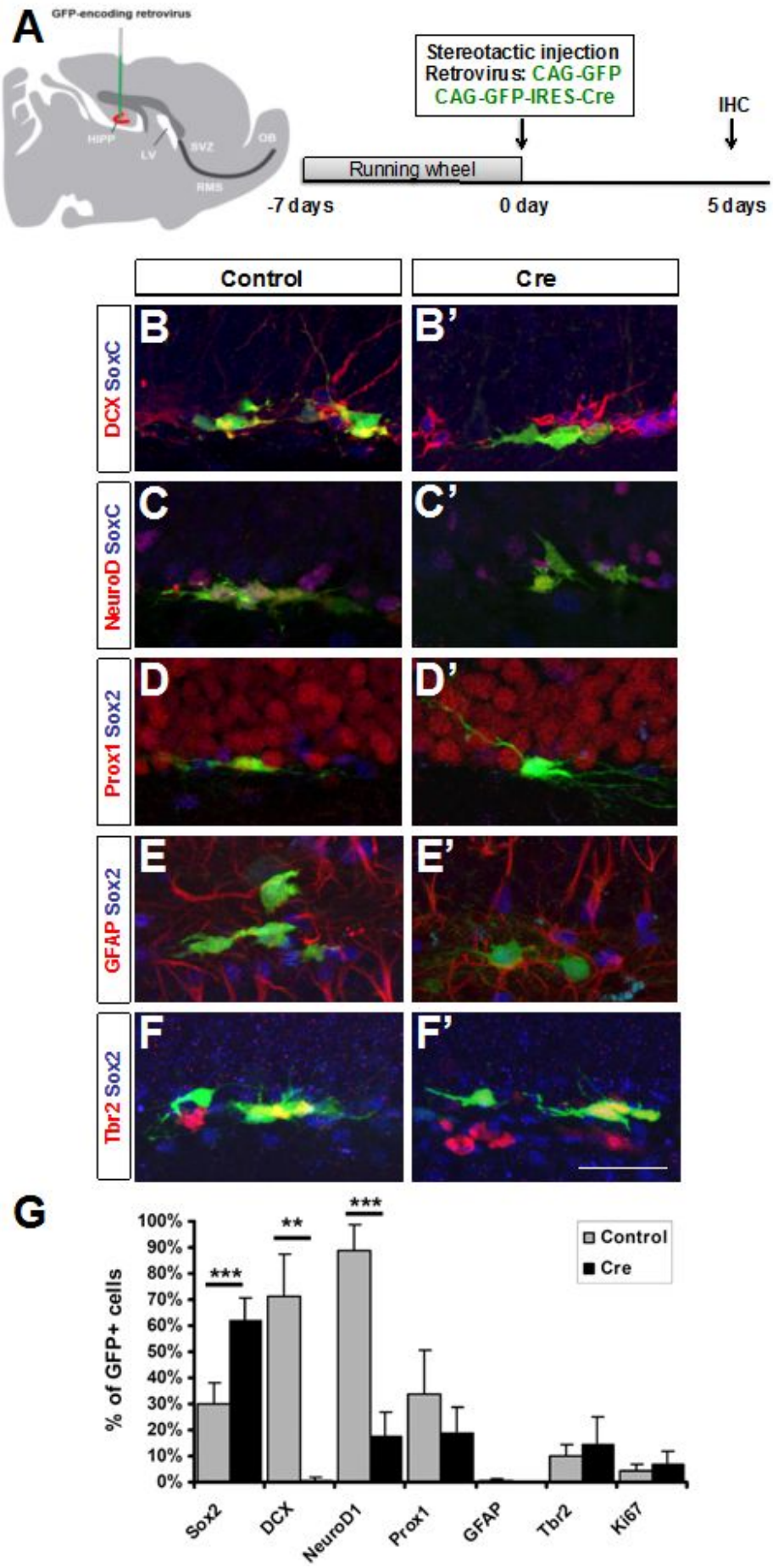
A
GFP-encoding retrovirus
HIPP
LV
SVZ
OB
RMS
Stereotactic injection
Retrovirus: CAG-GFP
CAG-GFP-IRES-Cre
IHC
Running wheel
-7 days
0 day
5 days
Control
Cre
DCX SoxC
NeuroD SoxC
Prox1 Sox2
GFAP Sox2
Tbr2 Sox2
B
B'
C
C'
D
D'
E
E'
F
F'
G
% of GFP+ cells
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
***
**
***
Control
Cre
Sox2
DCX
NeuroD1
Prox1
GFAP
Tbr2
Ki67

Figure 8. SoxC transcription factors are essential for adult hippocampal neurogenesis *in vivo*

(A) Retrovirus encoding for Cre and GFP was stereotactically injected into the dentate gyrus of hippocampus (HIPP) in adult Sox4/Sox11 dcKO mice. Adult Sox4/Sox11 dcKO mice injected with a GFP-encoding retrovirus served as control. Mice were subjected to voluntary wheel running for 7 days before operation, and analyzed 5 days after retroviral injection. LV, lateral ventricle; SVZ, subventricular zone; RMS, rostral migratory stream; OB, olfactory bulb. (B-F) Left column shows transduced cells from dcKO mice injected with the control vector, and the right column shows transduced cells from dcKO mice injected with Cre recombinase. Transduced cells were identified by expression of GFP (green). SoxC expression (blue in B, C) was detected using Sox4 and Sox11 antibodies generated in the same species. SoxC expression is not detected in Cre recombinase transduced cells. (B) Control cells were frequently positive for DCX (red). Moreover, some control cells had developed a primary dendrite that extended toward the molecular cells layer. (B') Sox4/Sox11 ablated cells did not express DCX. In contrast to control cells, most Sox4/Sox11-ablated cells did not express NeuroD1 (red, C, C') or Prox1 (red, D, D'). Control cells (F) and Sox4/Sox11-ablated cells (F') express Tbr2 (red) at comparable frequencies. In both cases, Tbr2 positive cells were mainly found in clusters. Sox4/Sox11-ablated cells frequently expressed Sox2 (blue, D', E', F') but were negative for GFAP (E'). Scale bar: 30μm. (G) Quantification of marker expression by transduced cells.

4.4 *In vivo* overexpression of Sox11 in adult hippocampus prolonged DCX expression but did not affect dendritic branching

In order to determine the effect of SoxC overexpression in the adult neurogenic niche *in vivo,* I used MMLV-based retroviruses transduction. C57BL/6 mice (8 weeks old) were stereotactically injected into the hippocampal dentate gyrus with retroviruses bicistronically encoding for Sox11 and GFP (CAG-Sox11-IRES-GFP). GFP (CAG-GFP)-expressing retrovirus served as control. To increase the number of transduced cells in the adult hippocampus, mice were exposed to voluntary wheel running, shown to provide a strong proliferative stimulus to neural stem cells in the adult hippocampus (Kempermann et al., 1997; van Praag et al, 1999). Hence, all mice were put in a cage with running wheel for 7 days prior to stereotactic injection, and were analyzed 42 days after retrovirus injection (Figure 9A, 9B).

With immunohistochemistry analysis, I found almost no control virus transduced GFP-positive cells expressing Sox11 42 days post infection. In contrast, most (75.8±14.6% among transduced cells) GFP-positive cells in the CAG-Sox11-IRES-GFP injected group expressed Sox11 (Figure 9C). This indicated that the retroviral strategy for Sox11 overexpression *in vivo* was efficient and that it maintained Sox11 expression longer than it occurs in physiological conditions. Retroviral transduced cells displayed the typical morphology of dentate granule neurons with a complex dendritic tree in the molecular layer of the dentate gyrus. Interestingly, Sox11-overexpressing cells assumed different positions in the dentate granule cell layer compared to control cells. Sox11-overexpressing cells were less numerous in the innermost layer of granular cell layer of dentate gyrus but more abundant in the outmost layer (Figure 9E, 9F).

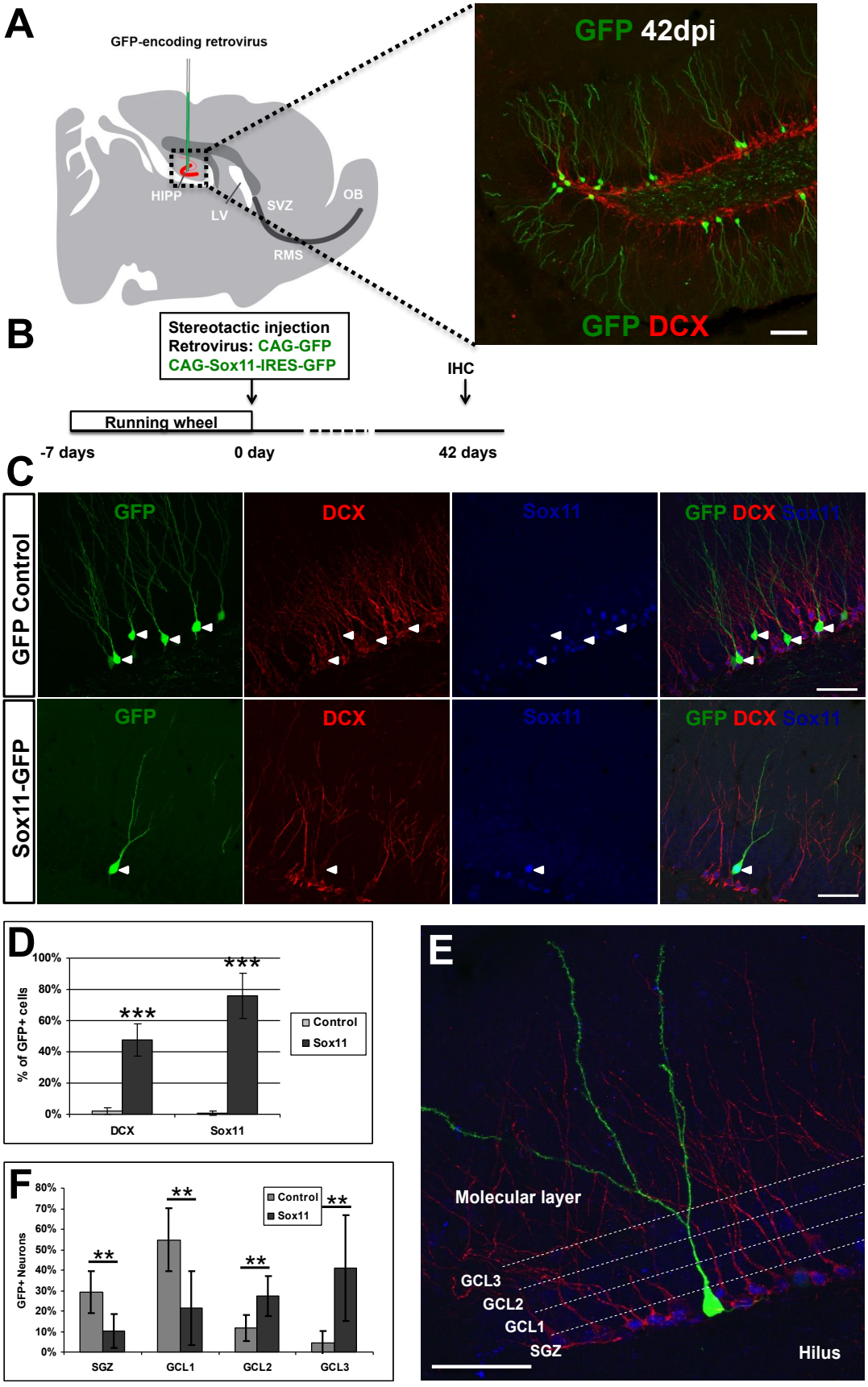
A
GFP-encoding retrovirus
HIPP
LV
SVZ
OB
RMS
GFP 42dpi
GFP DCX
B
Stereotactic injection
Retrovirus: CAG-GFP
CAG-Sox11-IRES-GFP
IHC
Running wheel
-7 days
0 day
42 days
C
GFP Control
Sox11-GFP
GFP
DCX
Sox11
GFP DCX Sox11
D
% of GFP+ cells
100%
80%
60%
40%
20%
0%
***
***
Control
Sox11
DCX
Sox11
E
Molecular layer
GCL3
GCL2
GCL1
SGZ
Hilus
F
GFP+ Neurons
80%
70%
60%
50%
40%
30%
20%
10%
0%
**
**
**
**
Control
Sox11
SGZ
GCL1
GCL2
GCL3

Figure 9. 42days-lasting *in vivo* overexpression of SoxC prolongs the expression of immature neuron-specific protein DCX
(A) Sox11 retrovirus encoding GFP was stereotactic injected into the dentate gyrus of hippocampus (HIPP) in adult BL6 wild type mice. GFP-encoding retrovirus was used as control. LV, lateral ventricle; SVZ, subventricular zone; RMS, rostral migratory stream; OB, olfactory bulb. Scale bar: 30μm. (B) Mice were housed with ad libitum access to running wheels for 7 days before operation, and analyzed 42 days after retroviral injection. (C) The upper column shows transduced cells (white arrowheads) from BL6 wild type mice injected with the control vector, and the lower column shows transduced cells from BL6 wild type mice injected with retrovirus CAG-Sox11-IRES-GFP (white arrowheads). Transduced cells were identified by expression of GFP (green). Sox11 was in blue. Control cells were rarely positive for DCX (red), but with Sox11 overexpressing cells, the proportion of DCX expression increased strongly. Scale bar: 30μm. (D) Quantification for DCX expression in dentate gyrus of hippocampus *in vivo* with Sox11 overexpressed cells. (E) Methodology for position analysis in dentate gyrus of hippocampus in adult brain *in vivo*. Granule cell layer of dentate gyrus was equally divided into four part (from innermost to outermost layer): SGZ, GCL1, GCL2, GCL3. SGZ, subgranular zone; GCL, granule cell layer. Scale bar: 30μm. (F) Quantification for position analysis in dentate gyrus in Sox11 overexpressed cells *in vivo*. In compare to control cells, Sox11 overexpressed cells were decreased in innermost layer SGZ and GCL1, and increased in outmost layer GCL2 and GCL3.

DCX expression

Only 2.1±2.2% of control cells 42 days post-injection were DCX-positive, which was consistent with previous reports showing that DCX expression persists for approximately 3 weeks and is down-regulated upon maturation (Jagasia et al., 2009; Piatti et al., 2011) (Figure 9D). In contrast, 47.7±10.6% of Sox11-overexpression cells were DCX-positive (Figure 9D). Hence, overexpression of Sox11 prolonged the expression of DCX in adult neurogenic niche *in vivo*, strongly supporting the notion that Sox11 operates upstream of DCX.

Dendritic morphology

Prolonged expression of DCX at 42 days post-infection in Sox11-transduced neurons suggest that they may had been held in immature state. To investigate whether overexpression of Sox11 at 42 days interfered with maturation and morphological development of newborn neurons, I compared the dendritic complexity of newly generated neurons from control and Sox11 group using following parameters: total dendritic length, number of branches and dendritic branch points, and *Sholl* analysis.

CAG-Sox11-IRES-GFP stereotactic injected into dentate gyrus of hippocampus in C57BL/6 mice, and CAG-GFP served as control. In addition, RFP encoding MMLV retrovirus was co-injected with the CAG-Sox11-IRES-GFP or GFP viruses (Figure 10A). In analogy to the previous experiments, all mice were trained on running wheels for 7days before injection to increase the amount of proliferating cells in the neurogenic niche of adult hippocampus and sacrificed 42 days after injection (Figure 10B). Only the cells that were double infected with CAG-GFP/CAG-Sox11-IRES-GFP and internal control RFP viruses were used for morphology analysis (Figure 10C, 10C', 10C'').

The morphology of those cells was traced using RFP channel, which was strongly expressed and equally distributed along the dendrites in both groups. This approach helped to avoid potential bias due to the weak expression of GFP in CAG-Sox11-IRES-GFP virus group. Imaris software was used for analysis the whole dendritic morphology of newborn neurons and for

quantification of morphological differences in the total dendritic length and branching complexity (Figure 10D). No statistically significant difference in the total dendritic length and number of branching points was found between Sox11 overexpressing and control neurons (Figure 10E, 10F). Moreover, *Sholl* quantification of branching complexity was comparable between both groups (Figure 10G).

To summarize, these preliminary findings indicated that the overexpression of Sox11 did not alter the overall morphological development of adult-born dentate granule neurons with regard to dendritic length and dendritic branching complexity in the hippocampus of adult mice in vivo.

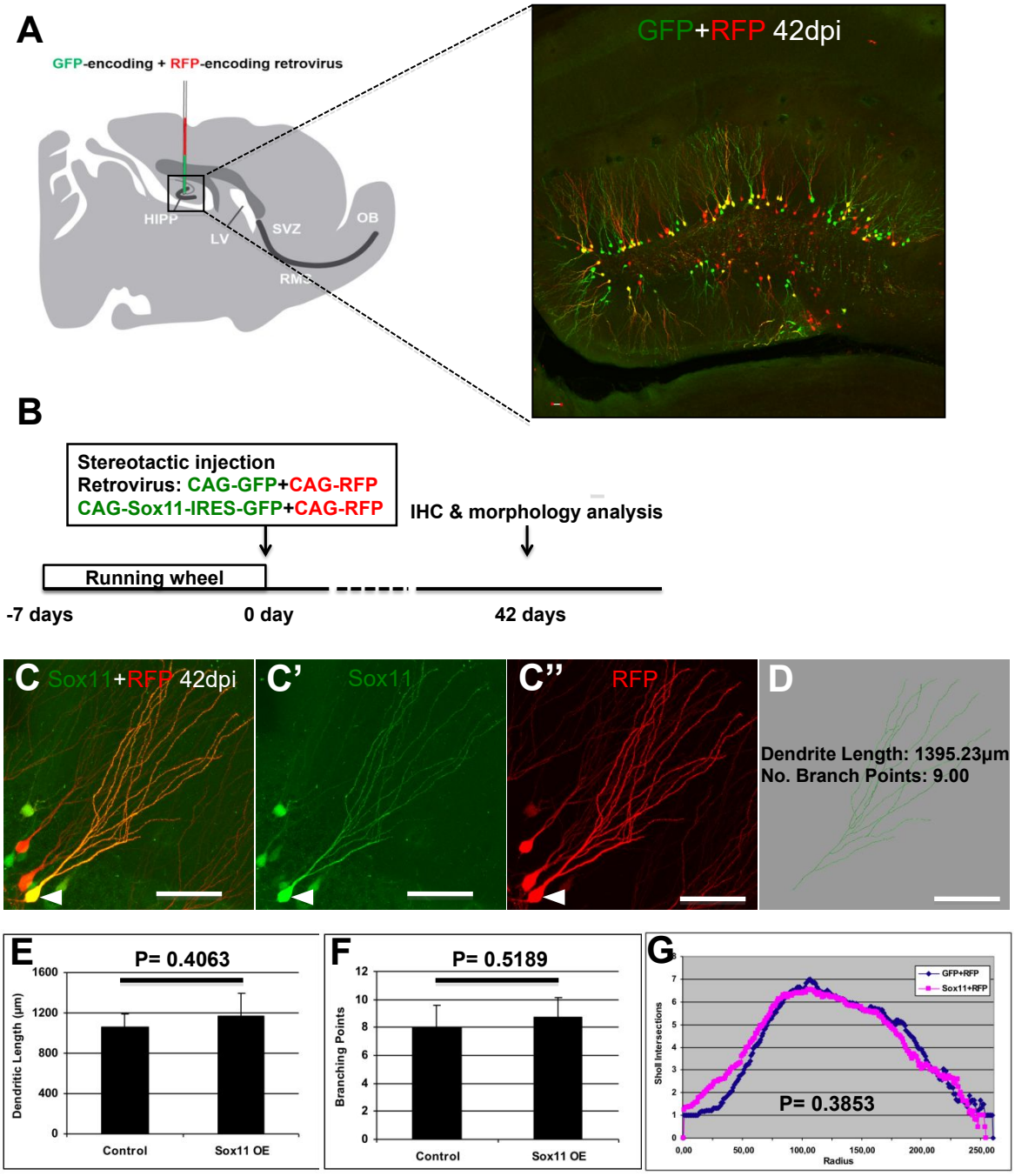

Figure 10. 42days-lasting *in vivo* overexpression of SoxC does not alter the dendritic length and branching complexity

(A) Sox11-GFP and internal control RFP virus were stereotactically injected into the dentate gyrus of hippocampus in adult wild type mice. GFP and RFP retrovirus served as controls. (B) Mice were supplied with running wheels for 7 days before injection, and analyzed 42 days after. (C) Transduced cells (white arrowheads) from wild type mice injected with Sox11-GFP (green) plus internal control RFP (red) virus. (D) 3D-morphology analysis traced by RFP. (E-G) Quantification for total dendritic length, branching points and *Sholl* analysis revealed no statistic difference between Sox11 overexpressing and control GFP expressing newborn neurons. HIPP, hippocampus; LV, lateral ventricle; SVZ, subventricular zone; RMS, rostral migratory stream; OB, olfactory bulb. Scale bar: 30μm.

4.5 Sox11 transcription may be directly regulated by Sox2

Above-described results demonstrated the essential role of SoxC proteins for neuronal differentiation in adult hippocampus. In order to determine how is Sox4/Sox11 expression regulated, I concentrated on Sox2 transcription factor. The Sox2 protein expression in adult hippocampal neurogentic niche appears to occur earlier than the expression of Sox4/Sox11 proteins: the majority of Sox2-positive cells were morphologically younger than SoxC-positive immature neurons and only 8.7±4.1% of Sox4/Sox11-positive cells co-expressed Sox2 (Figure 3B, 3G). Moreover, *In silico* analysis (Genematrix software) revealed putative Sox-binding sites in the 2.3kb proximal promoter of Sox11 gene (Figure 11A).

These *in vivo* and in silico data supported the hypothesis that Sox11 could be directly regulated by SoxB transcription factors such as Sox2. To this end, the luciferase reporter plasmid with 2.3kb proximal promoter of Sox11 gene (pGL3-ko Sox11 5') was generated and analyzed in transiently transfection assay. Transfection of HEK 293T cells showed that the Sox11 promoter activity was strongly increased by Sox2 overexpression (Figure 11B).

Together with the expression pattern of Sox2 and Sox11, these luciferase assay data suggest that Sox2 may operate upstream of Sox11 in the adult hippocampal neurogenic lineage.

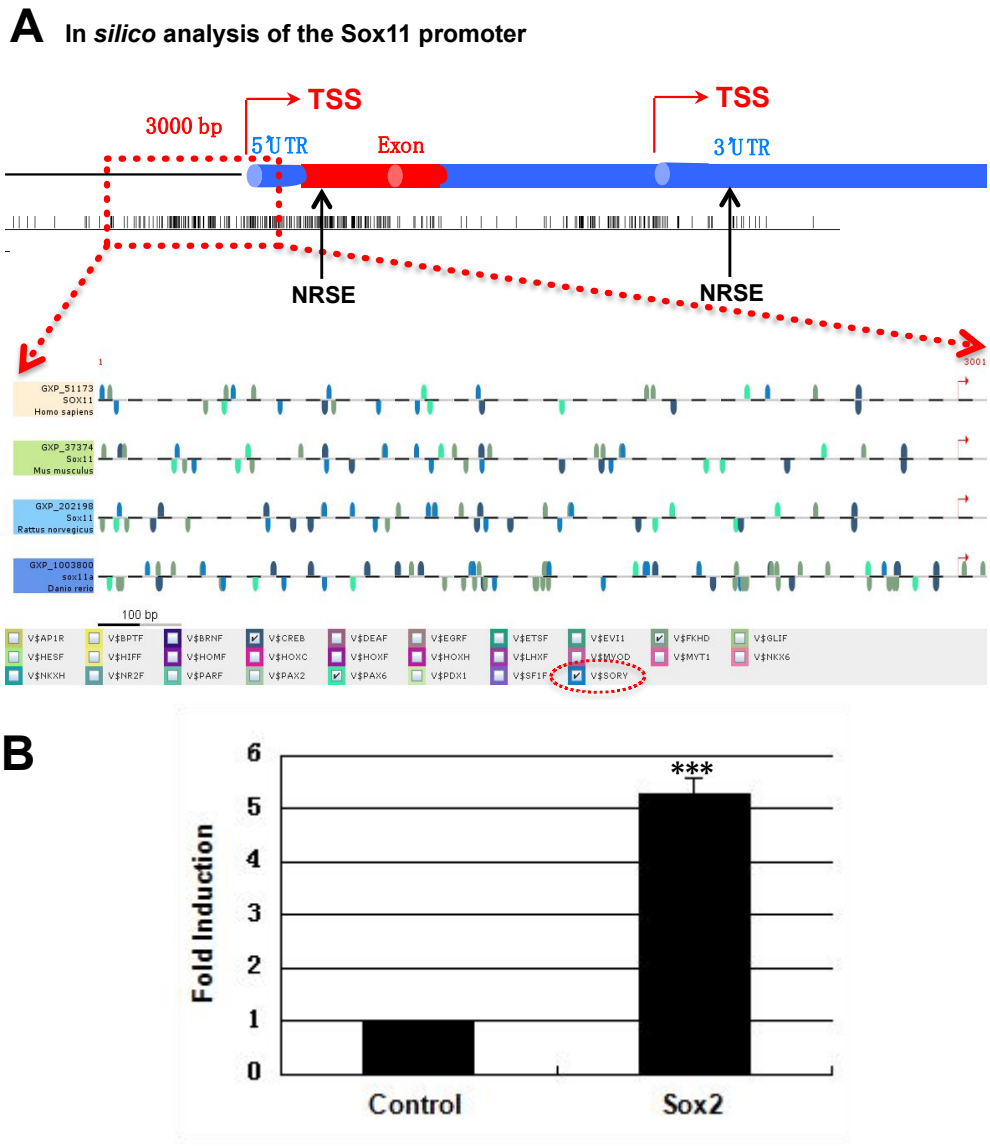

Figure 11. Promoter of Sox11 gene in NSCs is stimulated by SOX2
(A) *In silico* analysis of the Sox11 regulatory regions revealed that presence of putative predicated binding sites for Sox factors in 2.3kb proximal promoter of Sox11 gene. (B) Sox11 promoter-luciferase reporter was transiently transfected in 293T cells with other candidate genes. Luciferase analysis revealed the Sox11 promoter activity was strongly increased by Sox2.

4.6 SoxC proteins directly regulate the expression of immature neuron-specific proteins

In vivo loss-of-function results demonstrated that Sox4 and Sox11 were essential regulators of neuronal differentiation in adult hippocampal neurogenesis. This raised the possibility that Sox4 and Sox11 could directly transcriptionally activate neuron-specific genes during fate determination of adult hippocampal neural stem cells since Sox4 and Sox11 expression almost completely overlap with DCX (Figure 3A, 3G; Haslinger et al., 2009). The microtubule-associated protein DCX, specifically expressed in newly generated immature neurons of the adult brain, is regarded as one of the earliest markers for neuronal lineage (Couillard-Despres et al., 2005,2006; Ming et al., 2011; Merz et al., 2013). Here I screened *in silico* DCX promoter and analyzed it in a transcription assay to focus on the potential regulation of DCX by Sox4 and Sox11.

The region 3.5 kb upstream of the DCX transcriptional start site have previously been shown to contain key regulatory elements and is sufficient to target transgene expression with high specificity to neuronal precursor and immature neurons in adult transgenic mice (Couillard-Despres et al., 2006,2008; Zhang et al., 2010). Therefore, 3.5 kb upstream of the DCX transcriptional start site was screened *in silico* for putative Sox transcription factor binding sites (Figure 12A). I indeed found several of them, however, I could not predict if they were specific for the SoxC family of transcription factors as their exact DNA binding sequence is not known.

Next, HEK 293T cells were transfected with the pGL3 luciferase vector containing all 3.5 kb of the DCX promoter (Karl et al., 2005) together with either expression constructs for Sox2, Sox4 and Sox11 or GFP as a control (see Material and Method). These assays revealed potent activation of the DCX promoter by Sox4 and Sox11 (Figure 12B). In contrast, the stem-cell transcription factor Sox2 did not induce DCX promoter activity (Figure 12B). This indicated that, despite the fact that all Sox proteins recognize a similar DNA sequence motif (Harley et al., 1994), DCX promoter activation may be a specific property of Sox4 and Sox11. Sox2 may downregulate Sox4- and

Sox11-induced DCX activation since cotransfections with increasing amounts of Sox2 inhibited Sox4/11-dependent upregulation of DCX promoter (Figure 12B). These observations raised the hypothesis that Sox2 may compete with Sox4 and Sox11 for Sox-binding sites in the DCX promoter.

Collectively, these data identified DCX as a direct target of SoxC transcription factors in adult NSCs and suggested a functional role of SoxC transcription factors in the control of a neuron-specific gene expression program.

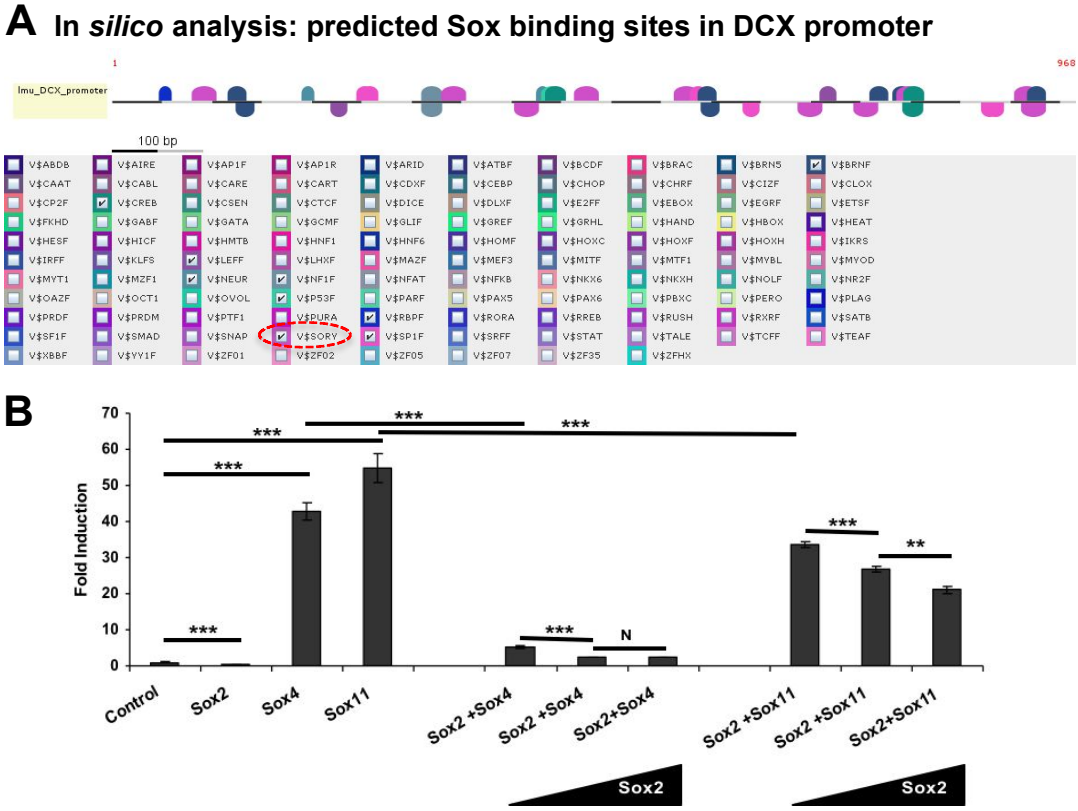

Figure 12. Regulation of the neuron-specific DCX-promoter by SoxC factors
(A) *In silico* analysis for 3.5 kb genomic region upstream of the DCX start codon reveals putative predicted Sox binding sites in this region. (B) Luciferase reporter assays in transiently transfected HEK 293T cells using a DCX-promoter revealed potent activation of the DCX promoter by Sox4 and Sox11. In addition, when I co-transfected Sox4 or Sox11 and increasing amount of Sox2 together with the DCX promoter-luciferase reporter, Sox2 strongly inhibited Sox4 and Sox11 induced DCX-reporter activation.

5 Discussion

In this study I give new evidence about the role and regulation of SoxC transcription factors Sox4 and Sox11 in adult hippocampal neurogenesis. This is supported by the following observations and summarized in Figure 13:

1) Sox4 and Sox11 protein expression are initiated in the adult hippocampal neurogenic lineage upon neuronal fate commitment

2) Sox4 and Sox11 potently induce neuronal differentiation of adult neural stem cells and directly activate the promoter of the neuronal lineage-specific gene DCX

3) Loss of Sox4 and Sox11 *in vivo* abolishes the expression of neuron-specific structural protein DCX in adult mouse hippocampus

4) Sustained overexpression of Sox11 in adult neurogenic niche prolongs DCX expression, shifts the cell position towards the outmost region of granule cell layer, but does not affect dendritic length and branching

5) Sox2 might be one of the activators of Sox11 transcription in the adult hippocampal neurogenic lineage

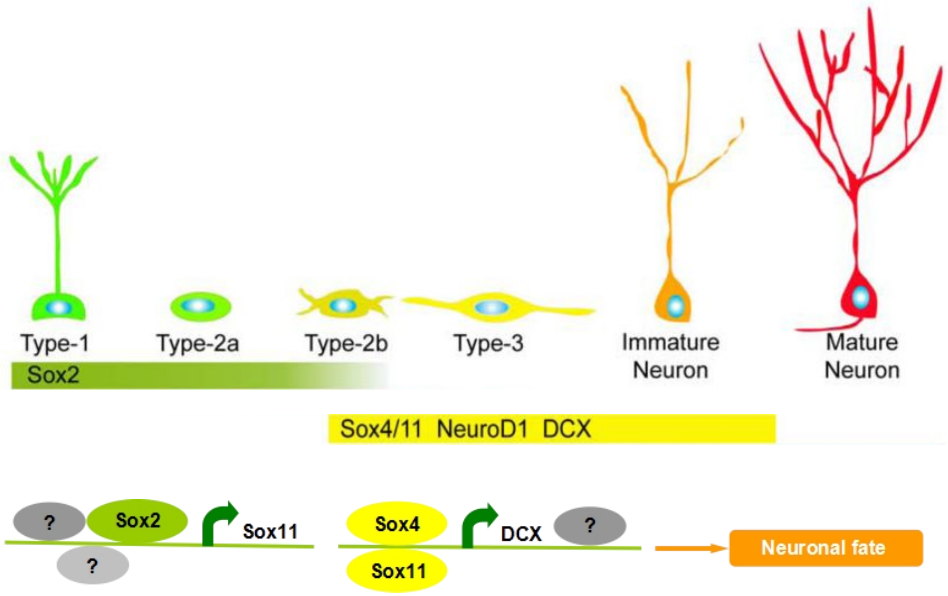

Figure 13. Proposed model for the regulation of adult neurogenesis by Sox4 and Sox11

5.1 What is the role of SoxC proteins in the adult neurogenesis?

5.1.1 SoxC factors initiate and/or maintain neuronal differentiation

Gain- and loss-of-function experiments showed that Sox4/Sox11 proteins regulate neuronal differentiation. Sox4 and Sox11 overexpression in adult NSCs, which normally do not express SoxC proteins under proliferation growth conditions, stimulates neuronal fate determination *in vitro* as evidenced by the strong induction of Dcx and *β*-tubulin III expression (Figure 7). On the other hand, ablation of Sox4/Sox11 impairs neuronal differentiation since loss of Sox4 and Sox11 suppresses the generation of Dcx-positive neurons *in vitro* (Figure 6). Moreover, ablation of Sox4 and Sox11 suppresses the generation of Dcx- and NeuroD1-positive neurons in adult mouse dentate gyrus (Figure 8).

In vivo sustained Sox11 expression in 42 day old newborn neurons of the adult mouse dentate gyrus results in maintenance of expression of Dcx (Figure 9); under physiological conditions this immature neuron marker is down-regulated at this time point (Jagasia et al., 2009; Piatti et al., 2011). Doublecortin (Dcx) is a 40-kDa microtubule associated protein required for proper neuronal precursor migration. This probably occurs through its interactions with the microtubule network, dynein and/or the mitotic spindle apparatus (for review see Couillard-Despres et al. 2001). In the adult brain, DCX expression is restricted to the areas of continuous neurogenesis, i.e. the hippocampus and the subventricular zone/rostral-migratory stream/olfactory bulb axis (Brown et al. 2003) and is rarely detected outside these regions (Nacher et al. 2001). Moreover, DCX is one of the earliest markers of neuronal fate determination of stem cells in the adult neurogenic niches (for review see Kempermann et al., 2004). I found that Sox4 and Sox11 could directly activate the promoter of the neuronal lineage-specific gene DCX by luciferase assay and ChIP analysis. Luciferase assays with a 3.5kb-long upstream regulatory region of Dcx gene showed that Sox4 and Sox11 could potently activate it, up to 50-fold (Figure 12B), and ChIP data suggest that *in silico* predicted SoxC binding sites could indeed contribute to the SoxC-

dependent regulation of the DCX promoter (Mu et al., 2012). This suggested that Dcx is a novel direct target of Sox4 and Sox11.

My finding that SoxC primarily regulate the initiation and maintenance of the immature neuronal differentiation program in the adult hippocampal neurogenic lineage, is consistent with the observation that Sox11 targets neuron-specific promoters during the *in vitro* neuronal differentiation of mouse embryonic stem cells (Bergsland et al., 2011). In 2006, Jonas Muhr's group reported that the induction of two SoxC family members --Sox4 and Sox11-- is a critical step in the acquisition of a neuronal phenotype in the developing chicken spinal cord and identified *β*-tubulin III and Map2 as potential SoxC targets (Bergsland et al., 2006). In 2011, the same group compared the genome-wide DNA binding pattern of SoxB1 and SoxC proteins in embryonic cells, neural precursor cells, and maturing neurons. They demonstrated that Sox factor binding depends on developmental stage-specific constraints and that neuron-specific genes become activated during neural lineage development through sequentially acting Sox2-->Sox3-->Sox11 complexes. In embryonic cells, neural lineage specific genes were initially preselected by Sox2 binding, this was followed by Sox3 in neural precursor cells and then by Sox11 during neuronal differentiation. This observation indicated that a single family of transcription factors may coordinate the whole neural lineage-specific gene expression program, starting from the early pluripotent stem cell stage to the final onset of neuronal and glial gene expression in differentiated cells (Bergsland et al., 2011).

Moreover, Sox4 and Sox11 promote neuronal reprogramming and are essential for reprogramming of astrocytes into neurons (Mu et al., 2012). In combination with Ngn2, transcription factors Sox4 and Sox11 potently enhanced Ngn2-induced reprogramming of astrocytes into neurons with all double-transduced cells displaying a complex neuronal morphology and expression of the neuron-specific markers *β*-tubulin III and DCX (Mu et al., 2012). In contrast, Ngn2-transduced neuronal reprogramming was impaired in Cre-transduced Sox4$^{loxP/loxP}$; Sox11$^{loxP/loxP}$ astrocytes. This observation

provided strong support for the notion of Sox4 and Sox11 as key contributors to the transcriptional network underlying neuronal differentiation.

Strikingly, two studies recently reported that Sox11 could contribute to the conversion of non-neuronal cells into neurons (Liu et al., 2013; Su et al., 2014). Liu and colleagues found that Sox11 efficiently converts postnatal and adult skin fibroblastes from healthy and diseased human patients to cholinergic neurons (Liu M et al., 2013). Su and colleagues showed that Ngn2 synergized with Sox11 in the very efficiently conversion of human glioma cells to terminally differentiated neuron-like cells, both in cell culture and in adult mouse brains (Su Z et al., 2014). In addition, intracranial injection of Ngn2- and Sox11-expressing virus into the tumor mass also curtailed glioma growth and significantly improved survival of tumor-bearing mice (Su Z et al., 2014). These studies suggested that Sox4/Sox11 are not only the key regulators of neuronal fate determination in adult neurogenesis but also of neuronal fate determination in general.

What is the role of SoxC factors in neurodevelopmental disorders? Tischfield and colleagues reported eight heterozygous missense mutations in TUBB3, encoding the neuron-specific *β*-tubulin III, in a series of autosomal dominant disorders of axon guidance called TUBB3 syndromes (Tischfield MA et al., 2010). Each mutation causes the ocular motility disorder congenital fibrosis of the extraocular muscles type 3 (CFEOM3), whereas some of them also result in intellectual and behavioral impairments, facial paralysis, and/or later-onset axonal sensorimotor polyneuropathy (Tischfield MA et al., 2010). This observation rises the question whether also mutations in SoxC genes could cause neurodevelopmental disorders since SoxC factors regulate the expression of genes such as DCX and *β*-tubulin III that are involved in neuronal developmental diseases. Indeed, Sox11 mutations have recently been linked to Coffin-Siris syndrome (Tsurusaki Y et al., 2014). Coffin-Siris syndrome (CSS) is a congenital disorder characterized by growth deficiency, intellectual disability, microcephaly, characteristic facial features and hypoplastic nails of the fifth fingers and/or toes. Tsurusaki and colleagues applied whole-exome sequencing to 92 CSS patients, and identified two *de*

novo Sox11 mutations in two patients with a mild CSS phenotype (Tsurusaki Y et al., 2014). Sox11 knockdown in zebrafish caused brain abnormalities with a smaller head and significant mortality, which were partially rescued by human wild-type Sox11 mRNA, but not by mutant mRNA (Tsurusaki Y et al., 2014), suggesting the importance of Sox11 in human brain development.

5.1.2 SoxC factors exert effect on proliferation

The loss of function experiments in adult NSCs undergoing *in vitro* differentiation showed that the ablation of Sox4/Sox11 correlates with decreased proliferation (Figure 6D, BrdU staining) but did not induce cell death (Figure 6E, Caspase activity). *In vivo* experiments, however, showed that the conditional knockout of Sox4/Sox11 in adult mouse dentate gyrus neurogenic lineage does not decrease the proliferation since the number of Ki67/Tbr2-positive Sox4/Sox11-ablated cells was comparable to the number of control virus-infected cells 5 days post-infection (Figure 8F, 8F', 8G, Tbr2 and Ki67 staining). This difference might be due to different approaches used to assess proliferation. *In vitro* experiments measured BrdU incorporation, which occurs only during S-phase of the cell cycle, while *in vivo* experiments relied on the immunological detection of Ki67, a proliferation marker expressed for the whole duration of cell cycle (Kee N et al., 2002). Hence, the discrepancy between the Ki67-based and BrdU-based proliferation data may reflect an impact of Sox4/Sox11 on the cell cycle length. An additional caveat is the fact that the number of cells transduced by the retrovirus *in vivo* is low (approximately 100-200 cells per stereotactic injection) and is therefore not the ideal method to determine potentially subtle impacts of Sox4/Sox11 on proliferation.

My *in vitro* observations of a function of SoxC factors in proliferation are consistent with the studies by Wang and colleagues (Wang Y et al., 2013). They generated Sox11 null allele in mice and demonstrated that Sox11 null embryos had smaller and disorganized brains, accompanied with *in vivo* proliferation deficits in the neuroepithelium at E11.5 by BrdU staining (Wang Y

et al., 2013). In addition, ablation of Sox11 specifically in adult NPCs using Nestin::CreER^{T2};Sox11 conditional knockout mice after tamoxifen injections caused a significant reduction of proliferation in hippocampus as examined by BrdU and Ki67 immunofluorescence (Wang Y et al., 2013).

To better understand the impacts of SoxC on proliferation in the adult neurogenic lineage, future experiments could included: BrdU or EdU pulse-chase in adult Nestin::CreER^{T2};Sox4/11 conditional knockout mice in order to compare the fraction of BrdU positive cells between knockout and control mice. Moreover, combined labeling for Ki67 would allow to assess if Sox4/11 removal affects cell cycle withdraw (BrdU positive and Ki67 negative) (Castro et al., 2011). Finally, cell-cycle analyses, e.g., using FAC-sorting could further shed light on the impact of Sox4/Sox11 on cell cycle parameters.

5.1.3 SoxC factors may activate Dcx gene through competition with Sox2

Sox2 prevents Sox11-mediated transcription activation of Dcx promoters: co-transfection of Sox2 inhibits Sox4/11-dependent potent activation of Dcx promoter luciferase reporter (Figure 12B). Sox2 inhibition of Sox11 target genes may be caused by the competition for the Sox-binding sites on Dcx promoter (Figure 12). Such exchange between different factors of the Sox family was shown to shift NSCs from the stem cell mode towards the neurogenic mode (Kondoh and Kamachi, 2010) and was as also termed ‘SOXession’ (Wegner 2011).

What would be the reason for Sox2-dependent inhibition of Sox11 target genes? Analysis of Sox2 mutant mice demonstrated a key role for Sox2-dependent regulatory network in the adult hippocampal neurogenesis (Ferri et al., 2004; Favaro et al., 2009). Genome-wide analyses revealed that the half of Sox2-bound genes in embryonic stem cells (ESCs) are repressed lineage-specific genes (Boyer et al., 2005). Genome-wide DNA binding analyses of SoxB1 and SoxC proteins (Bergsland et al., 2011), which have vital functions in embryonic cells, neural precursor cells, and maturing neurons, respectively,

demonstrated that Sox factor binding depended on developmental stage-specific constraints and become activated during neural lineage development through remarkable sequentially acting Sox2-->Sox3-->Sox11 complexes. In embryonic cells, Sox2 preselected the neural lineage specific genes destined to be bound and activated by Sox3 in neural precursor cells and by Sox11 in differentiating neurons. Sox2 binding is associated with a bivalent chromatin signature, which is resolved into a permissive monovalent state upon binding of activating Sox factors such as Sox3 and Sox11 (Bergsland et al., 2011). In another study it was found that Sox4 replaces Sox2 during activation of genes needed for B-lymphocyte differentiation, which led to the proposition of the relay between SoxB and SoxC transcription factors to ensure lineage progression and cell fate decisions (Liber et al., 2010). To summarize, these data indicated that the Sox transcription factor family acts sequentially on the same set of genes and coordinates neural gene expression from the early lineage specification in pluripotent cells to later stages of neuronal development (Bergsland et al., 2011).

My immunohistochemistry analyses of hippocampal sections suggest that the possible relay between Sox2 and Sox4/11 factors may occur in the early stages of neuronal commitment in dentate gyrus since approximately 10% of Sox4-positive cells co-express Sox2 protein (Figure 3B, 3G), suggesting that the Sox2-dependent inhibition of Sox11 target genes may be an important mechanism in adult NSCs helping to prevent precocious expression of neuronal lineage specific genes such as Dcx, Map2 and *β*-tubulin III. Sox2-dependent inhibition may be mediated through: i) direct competition with Sox11 for their DNA-binding sites (Wiebe et al., 2003); ii) setting up a bivalent promoter chromatin conformation.

How does Sox2 expression become repressed during adult neurogenesis? Ehm and colleagues found that the downregulation of Notch/RBPJ$_k$ signaling may reduce Sox2 expression (Ehm et al., 2010). Conditional inactivation of Notch/RBPJ$_k$ signaling in neural stem cells *in vivo* resulted in depletion of the Sox2-positive neural stem cell pool (Ehm et al., 2010). Moreover, RBPJ$_k$-deficient neural stem cells resulted in the loss of Sox2 expression *in vitro*

(Ehm et al., 2010). These observations identified that Notch/RBPJ$_k$ signaling pathways are essential regulators of adult neural stem cell maintenance and suggested that their actions control of Sox2 expression (Ehm et al., 2010). Hence, upon inactivation of Notch signaling in differentiating stem cells, Sox2 levels will decrease paving the way for induction of expression of SoxC target genes.

5.2 How is the expression of SoxC factors during adult neurogenesis regulated?

The expression of SoxC factors in the adult mouse hippocampal neurogenic lineage is confined to approximately the first three weeks of neuronal development. SoxC expression begins around the time of neuronal fate commitment, i.e., in the late Type 2a and Type 2b cells, is maintained in immature neurons, and extinguishes during neuronal maturation (Figure 5). Although there is a lot of evidence about the regulation of SoxC expression in tumors, the regulation of SoxC initiation and downregulation during adult neurogenesis is less investigated. Here I discuss some interesting observations from our laboratory.

Sox2 overexpression strongly activated Sox11-promoter luciferase reporter in HEK 293T cells (Figure 11). Sox2 belongs to SoxB1 transcription factors that control stem cell maintenance and proliferation (Pevny and Placzek, 2005; Favaro et al., 2009; Ehm et al., 2010). It was reported that NSCs express Sox4 and Sox11 mRNA, whereas their proteins were detectable only in neuroblasts (Beckervordersandforth et al., 2010), indicating that posttranscriptional mechanisms play a significant role in the expression of SoxC proteins and SoxC-dependent neuronal differentiation.

Moreover, we also observed that adult NSCs grown in proliferating monolayer or neurosphere cultures express similar levels of Sox2, Sox4 and Sox11 mRNA while Ngn2 and NeuroD1 mRNA were undetectable. This expression pattern correlated with the presence of H3K4me3 marks and elongating form of RNA ploymerase on Sox2, Sox4 and Sox11 but their absence on Ngn2 and

NeuroD1 gene loci (Marcela Covic, unpublished). It thus seems plausible that the transcription of SoxC factors is initiated in adult NSCs through Sox2 and precedes neurogenic bHLH transcriptional initiation.

The temporally restricted and sequential expression of Sox11 and Sox4 was also reported during retinal development (Usui et al., 2013a) and Notch signalling and histone modification were shown to be implicated in their regulation (Usui et al., 2013b). Similarly to our laboratory evidence, the authors reported that H3-lysine 4 (H3K4) trimethylation at the Sox11 promoter declines during retinal differentiation in exchange with increasing H3-lysine 27 trimethylation (H3K27me3) and did not observe any difference in Sox11 promoter DNA methylation (Usui et al., 2013, Marcela Covic unpublished). Histone 3 lysine 4 trimethylation (H3K4me3) is known to be associated with transcriptionally active or poised genes and required for postnatal neurogenesis within the subventricular zone (SVZ) in the rodent model (Lim et al., 2009) while H3K27 trimethylation is known for shutting down transcription. Trimethylated H3K27 tightly associated with inactive gene promoters and thus acts in opposition to H3K4me3 (Young et al., 2011). It seems therefore that H3K27me3 but not DNA methylation participate in the suppression of Sox4 and Sox11 transcription both in the adult retina and adult hippocampus. In addition to H3K4me3, the chromatin remodeler chromodomain-helicase-DNA-binding protein 7 (CHD7) was recently reported to stimulate the expression of Sox4 and Sox11 during adult neurogenesis (Feng et al., 2013). CHD7 stimulated SoxC expression through remodeling of their promoters to an open chromatin state. In conclusion, H3K4me3 by TrithoraxG (TrxG) and H3K27me3 by PolycombG (PcG) group proteins regulate the timely pattern of SoxC expression during adult neurogenesis.

Sox11 was recently shown to form a transcriptional cross-regulatory network downstream of the Pax6-BAF complex (Ninkovic et al., 2013). Ninkovic and colleagues identified that this network drives neurogenesis and converts postnatal glia into neurons. Brg1 (Smarca4) binds to the Sox11 promoter, and interaction with Pax6 is sufficient to induce Sox11 expression in neurosphere-derived cells in a Brg1-dependent manner. Therefore, the Pax6-BAF complex

activates a cross-regulatory transcriptional network, maintaining high expression of genes involved in neuronal differentiation and execution of cell lineage decision (Ninkovic et al., 2013).

5.3 What remains to be done for complete understanding of the SoxC role in adult neurogenesis?

5.3.1 Do SoxC family members play similar roles in adult neurogenesis?

The expression of Sox4 and Sox11 proteins in both neurogenic niches of adult mouse brain almost completely overlaps (Figure 2, Figure 4). Whether the third member of the SoxC family Sox12 shows the same expression pattern in adult mouse brain could not be examined in this study due to the lack of suitable antibodies. *In situ* hybridization data available from the Allen Brain Atlas Project (http://www.brain-map.org/) indicate that the Sox4, Sox11 and Sox12 mRNAs are co-expressed in the adult hippocampus.

Sox12 is a weak transactivator compared with Sox4 and Sox11 and seems to be dispensable for mouse embryonic development (Hoser et al., 2008; Bhattaram et al., 2010). Similarly, my finding that Sox4/Sox11 deletion was sufficient to abolish neuronal differentiation suggests that Sox12 may not compensate for the loss of Sox4/Sox11 function in adult hippocampal neurogenesis. Therefore, it seems plausible that the role of Sox12 may be different from Sox4 and Sox11 roles. Sox12 single conditional knockout mice studies will allow to answer whether Sox12 participates in adult neurogenesis.

Despite their very similar expression pattern, Sox4 and Sox11 may still have different roles in adult neurogenesis. The Sox4/Sox11-deficient mice display major alterations in the proliferation and survival of sympathoadrenal lineage cells (Potzner et al., 2010). Interestingly, the effects occur during different stages of development: proliferation defects were restricted to earlier times when Sox11 expression was dominant, whereas increased apoptosis occurred only at later times when Sox4 expression dominated. This suggests that Sox11 regulates proliferation of tyrosine hydroxylase-expressing cells in

the developing sympathetic nervous system, while Sox4 regulate their survival (Potzner et al., 2010). Moreover, Sox4 and Sox11 appear to exhibit different activities in carcinogenesis. Glioma stem cells express Sox4 but not Sox11 (de la Rocha et al., 2014). Overexpression of Sox11 blocks tumorigenesis in glioma stem cells, suggesting that its ectopic restoration may prevent tumor development *in vivo* (Hide T et al., 2009). Whether Sox4 and Sox11 have distinct roles in hippocampal neurogenic lineage remains unknown and could be analyzed in single Sox4 and Sox11 conditional knockout mice.

5.3.2 What are downstream targets of SoxC factors?

Genome-wide characterization of the SoxC target genes combined with expression profiling will help to discover their new functions in adult neurogenesis. So far, only one genome-wide profiling of Sox11 DNA binding has been reported (Bergsland et al., 2011). Using mouse ESC-derived NPCs or neurons as cellular sources, Bergsland and colleagues explored genome-wide DNA binding pattern of SoxB1 and SoxC proteins during neurogenesis by employing chromatin immunoprecipitation (ChIP) combined with massive parallel sequencing (ChIP-seq). They found that genes occupied by Sox11 had the highest expression in differentiating neurons, and revealed that the potential target genes of Sox11 included Notch1, Sox2, Lstz2, Tubb3, and Lhx2. Another systematic genome-wide analysis of SoxC downstream targets was performed in prostate cancer. It showed that Sox4 activates genes involved in Egf, Notch, Wnt/*β*-catenin and TGF*β* signalling and genes for miRNA processing such as AGO and Dicer (Scharer et al., 2009). In TGF*β*-dependent malignant glioma, Sox4 activates the transcription of Sox2 (Ikushima et al., 2009). In neurogenesis, Sox4 and Sox11 activate microtubule-associated proteins like *β*-tubulin III (also named Tuj1), neuronal somatodendritic protein Map2ab and Dcx (Bergsland et al., 2006; this study).

Further identification of SoxC target genes would be achieved by Sox4/Sox11 loss of function gene expression analyses. Neural stem cells from double knockout Sox4/Sox11 mice undergoing differentiation *in vitro* could be used to

investigate which mRNAs (genes) are down- or up-regulated upon removal of Sox4/Sox11.

5.3.3 Cross-talk between SoxC and bHLH transcription factors

The basic helix-loop-helix (bHLH) factors have been shown to play key roles in self-renewal of NPCs and fate determination of neurons, oligodendrocytes, and astrocytes (Bertrand et al., 2002; Ross et al., 2003; Meijer et al., 2012; Namihira and Nakashima, 2013). Repressor bHLH factors like Hes1 regulate the self-renewal of NPCs, whereas proneural bHLH factors, such as Mash1 and Ngn2, promote neuronal differentiation. Moreover, SoxC factors cooperate with proneural bHLH factors Ngn2, which is sufficient for neuronal reprogramming of astrocytes (Mu et al., 2012), Sox11/Sox4 and NeuroD1 are expressed in a high overlapping pattern in adult hippocampal neurogenesis (Figure 3) and NeuroD1 is cell-intrinsically required for the survival and maturation of adult-born neurons (Gao et al., 2009).

Further research is necessary to disentangle if SoxC factors work in concert or independently of neurogenic bHLH transcription factors such as Neurogenin 2 (Ngn2) and NeuroD1. Genome-wide chromatin immunoprecipitation assays will help revealing whether bHLH and SoxC-dependent activation of neuronal differentiation is mediated through different targets genes.

5.3.4 How is the expression of SoxC proteins repressed in adult NSCs?

Timely-controlled expression of SoxC proteins is crucial for neuronal maturation in the adult neurogenesis. This raises the question how SoxC expression itself is mechanistically regulated. Regulation of protein expression is not only restricted to transcription level but also includes further modulations on the posttranscriptional level, e.g. micro-RNA mediated reduction of transcribed mRNA. Shi and colleagues brought forth the concept that microRNAs are crucial for the balance between stem cell maintenance

and fate determination (Shi et al., 2010). Shaham and colleagues identified Sox11 as a new target of miR-204 in eye development (Shaham et al., 2013). The hypothesis that SoxC-dependent neuronal differentiation might be posttranscriptionally regulated by microRNA interference is supported by the fact that NSCs express Sox4 and Sox11 mRNA but not their proteins, which become detectable only in neuroblasts (Beckervordersandforth et al., 2010).

Knuckles and colleagues identified that the disrupting the major components in miRNA biogenesis may cause dysfunction or degeneration independent of an effect on miRNAs. Drosha as another component of the microprocessor is reported to regulate neurogenesis by controlling Ngn2 expression independent of its role in miRNA processing (Knuckles et al., 2012). This function entails Drosha binding and cleavage of a hairpin structure in the 3'UTR of Ngn2 mRNA (Knuckles et al., 2012).

Further analysis of SoxC pathway activity would help defining new therapeutic avenues for the treatment of neuropathologies such as epilepsy and age-related cognitive decline. Understanding the molecular mechanisms which are essential for adult neurogenesis and proper hippocampal function is the prerequisite to influence these mechanisms and thereby prevent or slow down age-related cognitive decline.

6 Materials and Methods

6.1 Animals

For all animal experiments, mice were used at the age of 8 weeks old. All mice were group housed under standard laboratory housing conditions with a light/dark cycle of 12 hours each and had *ad libitum* access to food and water. No difference was made between ganders and females as well as males were used for injections, histology and primary cell culture. Wild type C57BL/6 mice which were obtained from Charles River (Wilmington, US-MA) or European Clinical Institute were used for expression analysis and gain-of-function experiment of SoxC transcription factors. For loss-of-function experiments, the inducible conditional Sox4 and Sox11 knockout mice (dcKO Sox4/Sox11) mixed 129SvJ / C57BL6J background carrying the following Sox4 and Sox11 alleles $Sox4^{loxP}$ (Penzo-Mendez et al., 2007) and $Sox11^{loxP}$ (Bhattaram et al., 2010) were performed, and these mice were obtained from collaboration with Dr. Veronique Lefebvre (Dy et al., 2008).

6.2 Retrovirus production

HEK293 GPG-IF8 (IF8) cells were used for retrovirus production in this study. (Ory et at., 1996, Heinrich et al., 2011)

IF8 cell mantaining and passaging

IF8 cells were grown at 37°C with 5% CO_2 in 75cm^2 flasks in the following medium:

IF8 growth medium:

DMEM, High Glucose/Glutamax/Hepes	500 ml
Fetal bovine serum	55 ml
Non-Essential Amino Acids(NEAA, 100 x)	5 ml
Na-Pyruvate(100 x)	5 ml
Puromycin (10 mg / ml)	100 µl

Tetracyclin (1 mg / ml)	1 ml
Geneticin (50 mg / ml)	3 ml

When cells reach confluence about 80%, they were split at a ratio 1:3 to 1:5. For passaging, medium was aspirated. Cells were washed with 5 ml PBS and subsequently treated with diluted Trypsin/EDTA (0.25% Trypsin/EDTA: PBS=1:5), and left at room temperature for 2 minute until the cells started to detach. Cells were resuspended in 1 ml medium and the cell suspension was subsequently seeded into new 75 cm^2 flask with 15 ml IF8 growth medium.

DNA purification

For purification of plasmid for virus production, DNA was separated by phenol-chloroform-extraction. For that, one volume (relative to the sample volume) of phenol / chloroform / isoamylalcohole was added. The mixture was vortexed shortly and centrifuged for 5 minutes at 13000 rpm in a tabletop microcentrifuge. The upper phase was recovered and 1/10 volume of 3M sodium acetate and 0.7 volumes of 100% ethanol (RNase free) were added to precipitate DNA. The sample was centrifuged 10 minutes at room temperature for 13000 rpm. The supernatant was removed and the pellet was washed with 70% ethanol. After air drying, the pellet was resuspended in RNAse free water at a concentration of 1 µg/µl.

Retroviral packaging

IF8 cells were harvested from two confluent 75 cm^2 flasks: Cells were dissociated in 20 ml OPTI-MEM + 10% FCS and were collected in a 50 ml Falcon tube. Then cells were centrifuged at 1000 g for 7 minute. Supernatant was removed and cells were resuspended with 30 ml OPTI-MEM +10%FCS. For transfection, two 50 ml Falcon tubes were filled with 9 ml each OPTI-MEM (GIBCO, Invitrogen) without additives, 360 µl Lipofectamine 2000 was added to one tube and 150 µg retroviral plasmid DNA to the other tube. Following incubation for 10 minute at 22°C, the content of the two tubes were mixed and incubated at 22°C for another 30 minute. Subsequently the cell suspension from two confluent 75 cm^2 flasks was mixed with the Lipofectamin / DNA mix, and seeded into six 10 cm dishes. After 14 hours, medium was aspirated from

the cells. Cells were washed with 4 ml packaging medium. Finally, 6 ml packaging medium was added to each dish.

Packaging medium:

DMEM, High Glucose/Glutamax/Hepes	500 ml
Fetal bovine serum	55 ml

Virus harvest

Following transfection, virus packaging cells were cultured for 2 days. After 2 days, supernatant was collected from six packaging cells dishes into one 50 ml Falcon tube, and was centrifuged at 2200 g for 15 minutes. At the same time, 6 ml packaging medium was added to each dish for second viral harvest. Next, supernatant was filled into 50 ml syringe and was filtered through 0,45 µm low-protein-binding PVDF filter (Millipore Ireland Ltd.) into SW28 ultracentrifuge tubes (Beckman). Then all tubes were centrifuged at 50.000 g for 2 hours at 4°C. Supernatant was removed and 2 ml OPTI-MEM buffer was added to soak the pellet for one hour. Following resuspension, the volume was filled to a total volume of 36 ml and was centrifuged again at 50.000 g for 2 hours at 4°C. After that, supernatant was removed and the pellet was resuspended with 100 µl TNE buffer and the final viral was aliquot and stored at -80°C freezer. Virus packaging cells were cultured for an additional 6 days, the virus harvesting process was repeated on day 2, 4, and 6.

TNE buffer:

Tris-HCl	50 mM
NaCl	130 mM
EDTA	1 mM

Determination of retroviral titres

Viruses were used for injections when titers were above 10^6 cfu / ml. Virus titering was performed with HEK293T cells in 24 well plates containing 12 mm non-coated glass coverslips. Viral aliquot was dilute into HEK293T medium in different dilution 1:1000; 1:10000; 1:50000; 1:100000 and cell suspension was

added to each viral dilution. After 2 days incubation, cells were fixed with 4% PFA for 10 minutes and stained for DAPI (1:10000). Coverslips were washed and mounted onto glass-slides. Fluorescent cells were counted and the titer was calculated.

6.3 Stereotactic injections

All processes for stereotactic injections of retroviruses into the brain of adult mice were approved by the Government of Upper Bavaria. Mice were group housed and kept under a 12 hours light / dark cycle. To increase the number of transduced cells in the adult hippocampus, mice were exposed to voluntary wheel running, which has been repeatedly shown to provide a strong proliferative stimulus from neural stem cells in the adult hippocampus. Hence, all mice have ad libitum access to a running wheel for 7 days prior to stereotactic injection.

Anesthesia:

For viral injections mice were deeply anesthetized with the following mixture sleep / awake solutions (Table 1) according to the mice body weight (350 µl per 20 g mouse for sleep solution; 400 µl per 20 g mouse for awake solution). Insulin needles (U-100, 1ml, BD Micro Fine, PZN: 324870) were used for the injection of the anesthesia.

Sleep solution	name	stock mg/ml	injection ml/kg	injection mg/ml	mix ml
Fentanyl	Fentanyl (Janssen-Cilag)	0.1	10	0.005	0.25
Midazolam	Dormicum (Roche)	5	10	0.5	0.5
Medetomidline	Domitor (Pfizer)	1	10	0.05	0.25
NaCl	NaCl				4

Awake soluton	name	stock mg/ml	injection ml/kg	injection mg/ml	mix ml
Buprenorphine	Temgesic (Essex Pharma)	0.3	10	0.01	0.17
Atipamezol	Antisedan (Pfizer)	5	10	0.25	0.25
Flumazenil	Anexate (Hexal)	0.1	10	0.05	2.5
NaCl	NaCl				2.08

Table 1: sleep and awake solution used for anesthesia in this study.

Injection:

Mice were fixed in the stereotactic apparatus and eyes were kept wet using Bepanthen (Bayer company). The fur on top of the head was sterilized with 70% EtOH and a small incision was performed in the midline. The skull was dried with 0.1% H_2O_2 and the bregma determined. The digital display of the stereotactic apparatus was set to zero after the glass capillary was set onto bregma. The capillary was set to the coordinates, and a drill was used to open the skull cautiously following. The digital display was adjusted to zero again and 1 μl virus suspension ($1x10^8$ cfu/ml) was stereotactically injected at the coordinates into both sides of dentate gyrus of adult mice brain. The coordinates (relative to Bregma) for dentate gyrus were -1.9 anterior/posterior, ±1.6 medial/lateral, -1.9 dorsal/ventral. After finishing the injection, the capillary remained in place for 2 minutes. The skin covering the skull was sewed by filaments (Ethicon Vicry, 4-0, SH-1 plus, 21.8 mm 1/2c, 70 cm filament). For recovery from anesthesia mice were kept in an airing cupboard at 37°C and checked every 5-10 minutes.

6.4 Tissue processing & Immunohistochemistry & Morphology analysis

Tissue processing

Mice were sacrificed using CO_2. They were transcardially perfused with phosphate-buffered saline (PBS, PH7.4) at a speed of 10 ml per minute for 5 minutes, and then followed by 4% paraformaldehyde (PFA) for 5 minutes.

4% PFA solution:

Paraformaldehyde	40 g
0.2 M phosphate buffer	500 ml
NaOH	6-8 tablets
ddH_2O	500 ml

Dissolved by heating 97°C and shaking

Adjust to PH 7.4

0.2M phosphate buffer:

Sodium phosphate monobasic	16.56 g
Sodium phosphate dibasic	65.70 g
ddH_2O	3 L

The brain was taken out, post-fixed in 4% PFA overnight at 4°C, and then subsequently transferred to 30% sucrose in 0.1 M phosphate buffer. Brains were cut on a sliding microtome (Leica Microsystems, Welzlar, Germany).

30% Sucrose solution:

Sucrose	150 g
0.1M phosphate buffer	500 ml

After 2 days of incubation in the sucrose solution, 40 µl thick coronal brain sections were cut for phenotyping studies, and 120 µl thick coronal sections for morphological analysis of dendrites. All brain sections were stored with cryoprotectant solution in 96 well plates at -20°C for further immunohistochemical staining.

Cryoprotectant solution:

Glycerin	250 ml
Ethylene Glycol	250 ml
0.1M Phosphate buffer	500 ml

Immunohistochemistry

Sections were washed three times in 0.1 M Tris-buffered saline (TBS) for 15 minutes each, followed by blocking step in TBS supplemented with 3% normal donkey serum and 0.25% Triton-X 100 (TBS++) for 60 minutes.

TBS (10×) buffer:

Tris	250 mM
NaCl	1.37 M
KCl	26 mM
Adjust to PH 7.5	

TBS++ buffer:

TBS	1×
3% donkey serum	3% (v/v)
Triton-X100	0.25% (v/v)

Brain sections were incubated in blocking solution containing the primary antibodies (Table 2) with appropriate dilutions at 4°C for 48 hours. Primary antibodies against the following antigens were used:

Antigen	Species	Dilution	Reference
Sox4	guinea pig	1:1000	Hoser et al., 2008
Sox11	guinea pig	1:500	Hoser et al., 2008
Sox11	goat	1.500	Santa Cruz Biotechnology, Heidelberg
Sox2	rabbit	1:1000	Millipore, Schwalbach, Germany
Sox2	goat	1:1000	Santa Cruz Biotechnology, Heidelberg
GFAP	rabbit	1:500	Dako, Hamburg, Germany
DCX	goat	1:250	Santa Cruz Biotechnology, Heidelberg
Calretinin	rabbit	1:1000	Swant
Calbindin	rabbit	1:1000	Swant
NeuN	mouse	1:50	Millipore, Schwalbach, Germany
GFP	chicken	1:500	Aves Labs, Tigard, US-OR
RFP	rat	1:50	Rottach et al., 2008
NeuroD1	goat	1:200	Santa Cruz Biotechnology, Heidelberg
Tbr2	rabbit	1:500	Abcam
Ki67	rabbit	1:1000	Novocastra Laboratories

Table 2: Primary antibodies used for immunohistochemistry in this study.

After three washes in TBS, brain sections were blocked in blocking solution (TBS++) for 30 minutes, and then incubated in blocking solution containing subclass specific secondary antibody conjugated to Cy3, Cy5, FITC, Alex633 or Alex488 for 2 hours at room temperature with shaking. Prior to this step, secondary antibodies (Jackson immunoresearch, US) were diluted to 1:250 after resuspension in 200µl H_2O and 200µl Glycerol. Sections were washed three times with TBS for 15 minutes, and incubated in 10 mg/ml 4',6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich Chemie GmbH, 1:10000) for 15 minutes, and washed again two times with TBS for 15 minutes. Brain sections were mounted by the use of brushes on Superforst glass slides (Menzel-Gläser, Braunschweig) and dried shortly and embedded with Aqua PolyMount (Polysciences Inc., Eppelheim, Germany). Mounted sections were kept overnight at room temperature to harden and then stored at 4°C. DAPI staining was used for tracing the granule cell layer. Equidistant brain sections containing the dentate gyrus were selected and stained to characterize the phenotype of the retrovirally transduced cells. Selected sections from different animals were similar with regards to their position along the dorsoventral axis for dentate gyrus analysis. Transduced cellls were identified based on the expression of GFP and / or RFP. All transduced cells were analyzed by confocal microscopy for immunoreactivity for the respective marker (n>50 cells per mouse and marker). Confocal single-plane images and Z-stacks were obtained on the Olympus FluoView 1000 (Olympus, Hamburg, Germany) confocal microscope or Leica SP5 confocal microscope (Leica Microsystems). Distance between single planes were 0.3 µm for dendritic morphology, and 1.0 µm for all other analysis. All experiments were performed in at least three different mice.

Morphology analysis

To determine the effects of prolonged expression of Sox11 *in vivo*, RFP encoding MMLV retrovirus was injected together with either CAG-Sox11-IRES-GFP or GFP viruses. To identify the dendritic morphology of transduced newborn neurons in the Sox11 overexpression experiment, 120 µm thick sections were used to ensure that the section comprised the entire dendritic

tree. Only the cells that were double infected with CAG-GFP/CAG-Sox11-IRES-GFP and internal control RFP viruses were used for morphology analysis. Morphology of those cells was traced in RFP channel, which was strongly expressed and equally distributed along the dendrites in both groups, and this approach could avoid potential biases due to the weak expression of GFP in CAG-Sox11-IRES-GFP virus group. Images were made from Z-series stacks of confocal images. In addition, for analysis of the dendritic complexity (Sholl analysis), three-dimensional reconstructions of GFP and RFP-transduced neurons were performed with Z-series stacks of confocal images. Imaris software (BITPLANE Scientific Solution) was used to analysis the whole dendritic morphology of newborn neurons and to quantify the morphology differences on total dendritic length and branching complexity.

6.5 Neural stem cell culture & Immunocytochemistry & Caspase 3 activity assay

Neural stem cell culture

Neural stem cells were isolated from the hippocampal dentate gyrus of 8 weeks old adult mouse brain. Mice were sacrificed by cervical dislocation and the brains were removed and immediately stored in ice cold PBS. The hippocampus was removed under a binocular microscope (WILD MZ8, Leica microsystems) and was subsequently cut into approximately 1mm^3 pieces and resuspended in 2.5 ml dissociation medium at 37°C for 30 minutes.

Dissociation medium: (Solution I plus Trypsin and Hyaluronidase)

Solution I:

HBSS (Life Tech., 10×)	50 ml
D-Glucose (Sigma, stock 300 mg / ml)	9 ml
HEPES (Life Tech., 1M)	7.5 ml
ddH_2O to final volume 500 ml	
Adjust to PH 7.5	

Trypsin (Sigma T4665)	6.7 mg / 5ml total volume
Hyaluronidase (Sigma H3884)	3.5 mg / 5ml total volume

The dissociation reaction was stopped by the application of 3 ml ice-cold solution III. Cells were passed through 70 µm strainer (Falcon Cat.: 352350), and then centrifuged at 800 g for 5 minutes.

Solution III:

BSA (Sigma A4503)	20 g
HEPES (Life Tech., 1M)	10 ml
EBSS (Life Tech., 1×)	fill to final volume 500 ml
Adjust to PH 7.5	

Supernatant was removed and cells were resuspended in 5 ml ice-cold solution II. Cells were centrifuged at 1333 g for 20 minutes, and then removed supernatant and resuspended in 0.5 ml ice-cold solution III. The 0.5 ml cell solution was gently added on top of 3 ml ice-cold solution III in new tube, and centrifuged at 800 g for 12 minutes.

Solution II:

HBSS (Life Tech., 10×)	25 ml
Sucrose (Sigma)	154 g
ddH_2O	fill to total volume 500 ml
Adjust to PH 7.5	

Supernatant was removed and cells were resuspended in 1ml medium. Cells were cultured on Poly-D Lysine (PDL; 10 µg / ml, Sigma) and Laminin (5 µg / ml, Invitrogen) coated plates in monolayer growth medium at 37°C and 5% CO_2, and were passaged every 5-7 day when they reached approximately 90% confluency. Cultures were supplemented with growth factors (EGF + FGF2) every other day.

Monolayer growth medium:

DMEM/F12+Glutamax (Life Tech. 31331-028)	500 ml
B27 supplement (Life Tech. 17504-044)	10 ml
Penicillin/ Streptomycin /fungizone (Life Tech. 15140-114, 100×)	5 ml
HEPES buffer (Life Tech. 15630-056, 1M)	8 mM

Epidermal growth factor (EGF, Roche)	20 ng / ml
Basic fibroblast growth factor (FGF2, Roche)	20 ng / ml

Viral transduction of neural stem cells

1.5 x 10^5 cells/well were seeded on PDL/Laminin coated 24 well plates in the deletion and overexpression experiments. For Sox4/Sox11 deletion experiments, cells derived from Sox4/Sox11 dcKO mice were transduced with CAG-GFP-IRES-Cre retrovirus (moi~1). Transduction with CAG-GFP virus served as control. Differentiation was induced by growth factor withdrawal after 48 hour post transduction. For overexpression experiments, cellls were transduced with CAG-Sox4-IRES-GFP or CAG-Sox11-IRES-GFP retrovirus (moi~1). Cells transduced with CAG-GFP served as control.

Immunocytochemistry

To determine the impact of Sox4/Sox11 on differentiation, virally transduced cells were fixed in 4% PFA for 10 minutes at 22°C at 4 days (overexpression experiments) or 6 days (deletion experiments) after viral transduction. For BrdU incorporation experiment, in order to determine proliferation cells, cells were treated at 2 days post transduction with 5 µM BrdU. Cell were fixed using 4% PFA one hour after the incubation with BrdU. Fixed culture were treated for 30 minutes in 2N HCl, and then rinsed three times with TBS and blocked with in TBS supplemented with 3% normal donkey serum and 0.25% Triton-X 100 for 60 minutes. Cells were incubated with primary antibodies at 4°C for 24 hour. The following primary antibodies were used:

Target	Species	Dilution	Reference
GFP	chicken	1:500	Aves Labs
GFAP	mouse	1:1000	Sigma-Aldrich
DCX	goat	1:250	Santa Cruz Biotechnology
BrdU	rat	1:500	AbD Serotec/Morhoosys, Oxford, UK

Table 3: Primary antibodies used for immunocytochemistry in this study

In the second day, cells were rinsed three times gently in TBS, and blocked 30 minutes with blocking solution as above. After blocking, cells were incubated with secondary antibodies coupled to Cy3, Cy5, FITC, or Alex488 at a dilution 1:250 in blocking solution for 2 hours at 22°C. Cells were rinsed in TBS supplemented with DAPI (1:10000) for 10 minutes, and then washed two times in TBS. Coverslips were mounted on Superforst slides (Menzel-Gläser, Braunschweig) in Aqua PolyMount (Polysciences Inc., Eppelheim, Germany). The percentage of marker positive cells among the total GFP positive population was determined for 3 wells within three biological replicates. Approximately 1500 transduced cells from 5-7 randomly selected fields were evaluated for each condition in each experiment. Single plane images were taken on a Leica DMI 6000B Fluorescence microscope.

Caspase 3 activity assay

Caspase activity was measured using the Caspase-Glo 3/7 Assay (Promega). Cells were lysed in 1% (v/v) NP-40 and 0.1% (v/v) SDS in PBS containing phosphatase and protease inhibitors (PhosphoSTOP and Complete EDTA-free, respectively; Roche). After 20 minute on ice, cell lysates were centrifuged at 14,000 g for 10 minutes at 4°C. Supernatants were then collected for caspase activity measurements. Ten micrograms of protein of each sample in 50 µl of lysis buffer were mixed with 50 µl Caspase-Glo 3/7 reagent in a 96-well plate and incubated under slow shaking for 30 minutes. Luciferase activity was measured using a Luminometer Centro LB 960 (Berthold Technologies). Luminescence was proportional to the amount of caspase activity present in the samples. Results are the average of three biological replicates.

6.6 Cloning of Sox11 reporter construct & Luciferase reporter assay

The Sox 11 promoter (pGL3-ko Sox11 5') was constructed from the plasmid pBKS-ko Sox11 5' (gift from collaboration Dr. Elisabeth Sock, Erlangen) to cut the 2.3 kb fragment ko Sox11 5' from shuttle vector pBKS, and subsequently cloned into the pGL3-basic vector. The pGL3-basic luciferase vector was

bought from Invitrogen company. In cloning procedures, restriction endonucleases and the respective restriction enzyme buffers, alkalic phosphatase (Promega) treatment and T4-ligations (New England Biolabs) were performed according to standard procedures. Recognition sites for restriction endonuclease were visualized and the appropriate plasmid maps were generated using the program Vector NTI (Invitrogen).

pGL3 vector digest and dephosphorylation

Restriction digest of the pGL3-basic plasmid was performed in 30 µl total volume with 2 µg DNA, and 1 µl SmaI enzyme together with 3 µl Tango buffer for 2 hours at 30°C. After another incubation period of 2 hours at 37°C with 1 µl XhoI enzyme, the mixture was incubated at 65°C for 20 minutes to inactivate restriction enzymes. The dephosphorylation of digested vectors usually decrease religation of the vector. The dephosphorylation procedure was performed with 30 µl heat inactivated digested vector. 1 µl antarctic phosphatase plus 3 µl antarctic phosphatase reaction buffer were added to a total volume of 30 µl. After an incubation period of 15 minutes at 37°C, the mixture was incubated at 65°C for 5 minutes to inactivate enzyme following incubation on ice for 10 minutes. Gel purification and extraction were performed subsequently.

pBKS-ko Sox11 restriction digest

Digests were performed in 30 µl total volume with 2 µg DNA of pBKS-ko Sox11 5’ plasmid, and 1 µl BstXI enzyme (Fermentas) together with 3 µl reaction buffer O (Fermentas) for 3 hours at 37°C. The mixture was incubated at 65°C for 20 minutes to inactivate enzyme. Next, 2 unit T4 DNA polymerase (3000U/ml) and NEB buffer 3 were added and incubated for 15 minutes at 12°C to fill in 5’ overhangs to form blunt ends. The reaction was stopped by EDTA (10mM), and enzyme was inactivated at 75°C for 20 minutes subsequently. 1 µl XhoI and 3.1µl buffer R were added to mixture, and incubated for 3 hours at 37°C. Gel purification and extraction were performed subsequently.

Ligation

Ligations were performed with a 2:1 molar ratio insert / vector. The samples were incubated at 4°C overnight together with T4 ligase and ligase buffer and transformed to TOP10 competent cells the next day.

Transformation into TOP10 competent E. coli

100 µl of TOP10 chemically competent *E. coli* were incubated with approximately 10 µl ligation mix for 30 minutes on ice. After a 45 seconds heat shock at 42°C, the bacteria were chilled on ice for at least 2 minutes, and 1 ml LB medium was added subsequently. The cells were incubated for 1 hour at 37°C on a shaker, and subsequently centrifuged at 6000 rpm for 1 minute, and plated on LB agar with Ampicillin plates to incubate overnight at 37°C. Colonies were selected in the next day, and inoculated in LB medium with Ampicillin at 37°C overnight. DNA mini-prep was performed in the next day using the Pure Yield Plasmid midiprep system (Promega, Germany). To confirm the correct insertion of the insert into the vector, digestion was performed at 37°C for 2 hours with 2 µg DNA, 1 µl KnpI, 1 µl XhoI and 3 µl NEB buffer 3, and subsequently the resulting products were analyzed on a 1% agarose gel.

LB medium:

Bacto-Trypton	10 g/l
Bacto-yeast extract	5 g/l
NaCl	10 g/l

Ph 7.0; autoclave (120°C, 20 minutes)

LB agar:

Bacto-Trypton	10 g/l
Bacto-yeast extract	5 g/l
NaCl	10 g/l
Bacto-agar	15 g/l

Ph 7.0; autoclave (120°C, 20 minutes)

LB^{amp} agar plates:

LB agar + Ampicillin	0.1 mg/ml

Luciferase reporter assay

To identify the effect of specific proteins on the activity of gene promoters, the Luciferase reporter assay was performed. In my work, the promoters of gene DCX and Sox11 were studied. The gene promoter was cloned into the pGL3 luciferase backbone, in which the firefly luciferase is under the control of the gene promoter. The firefly catalyzes the ATP-dependent oxidation of D-luciferin to oxyluciferin, which results in emission of light that can be measured by Centro LB 960 luminometer (Berthold Technologies GmbH & Co. KG, Bad Wildbad, Germany). Genomatix software (Genomatix Software GmbH, Germany) was used to predict putative potential transcription factor binding sites on the promoter of DCX gene and promoter of Sox11 gene. Human embryonic kidney 293T (HEK 293T) cells were cultured in DMEM high Glucose/L-Glutamine/Pyruvate medium supplemented with 1 % PSF and 10 % FBS at 37 °C with 5 % CO2. One day before transient transfection, cells were split by Trypsin-EDTA, and incubated in 24-well plate (1ml / well) with dilution 50.000 cell / ml. HEK 293T cells were transfected using the $CaCl_2$ method with equal molar amounts (500 fmol) of the pCAG-GFP, pCAG-Sox4-IRES-GFP, or pCAG-Sox11-IRES-GFP plasmids together with DCX promoter Luciferase reporter constructs (Karl et al., 2005) and a *Renilla*-luciferase under the control of the human elongation factor 1 promoter (Lie et al., 2005). Before transfection, a transfection mix was prepared consisting of 547.5 µl water with 2 µg of the pCAG-GFP, pCAG-Sox4-IRES-GFP, or pCAG-Sox11-IRES-GFP plasmid together with 1.5 µg DCX promoter Luciferase reporter construct and 0.1 µg Renilla-TK. 77.5 µl 2 M $CaCl_2$ and 625 µl 2×HBS were added to this mixture under continuous shaking.

HBS (2×) solution:

NaCl		8 g
KCl		0.37 g
$Na_2HPO_4\,7H_2O$		201mg
Glucose		1 g
HEPES	5 g	per total volume 500 ml
NaOH		fill to PH 7.05

The transfection mix was incubated for 20-30 minutes at 22°C. 100 µl of the transfection mix were added to each well. Three technical replicates were performed. Cells were cultured 48 hours after transfection and lysed using 5x passive lysis buffer (Promega) (150 µl of 1x passive lysis buffer per well) for 10 minutes with shaking at 22°C. Afterward 5 µl of the cell lysate were transferred into a 96 well plate (Nunc, via Thermo Electron LED GmbH, Langenselbold, Germany). Dual luciferase kit (Promega GmbH) was used for luciferase assay, and luciferase activity was measured using a Centro LB 960 luminometer. Luciferase assays were performed from three biological replicates.

6.7 RNA Isolation & cDNA synthesis & Quantitative real time PCR

RNA Isolation

Total RNA was isolated from monolayer cells using 1 ml Trizol reagent (Invitrogen) per $5x10^5$ cells. Cells were incubated for 5 minutes at 22°C, and then mixed with 0.2 ml Chloroform (Invitrogen) per 1 ml Trizol. Mixed aliquots were incubated for 2-3 minutes after vigorously vortexing for 15 seconds, and then centrifuged for 15 minutes with 12.000 g at 4°C. Afterward, the aqueous phase was transferred to a fresh tube, and precipitated with 0.5 ml Isopropanol (Invitrogen) per 1 ml Trizol. The mixture was incubated for 10 minutes at 22°C, and centrifuged for 30 minutes with 12.000 g at 4°C. The supernatant was removed and the pellet was washed with 1 ml 75% EtOH, and subsequently centrifuged for 5 minutes with 7.500 g at 4°C. The supernatant was discarded and the pellet was dried for 15 minutes at 22°C, and then dissolved in RNAse free water at 60°C for 10 minutes in a thermal shaker. RNA concentration was measured with NanoDrop 1000 Spectrophotometer (Thermo Scientific). Isolated RNA was treated with DNAse (Promega) according to the protocol of the manufacturer. 2 µg isolated RNA was incubated with 1 µl 10 × buffer, 1 µl DNAse Q, and H_2O to achieve 10 µl total volume. The mixture was incubated for 30 minutes at 37°C, and the reaction was stopped with 1 µl Stop solution (Promega) and incubated for 10 minutes at 65°C. RNA was stored at -80°C.

cDNA synthesis & Quantitative real time PCR

cDNA was synthesized from 1.5 µg RNA using 1 µl Random Hexamer primers (Fermentas), 0.5 µl RiboLock RNAse I (Fermentas), and 1 µl RevertAid^{TM} premium Reverse Transcriptase (Fermentas) in a total volume of 20 µl. Quantitative RT-PCR was performed on a StepOne device (Applied Biosystem Deutschland GmbH, Darmstadt, Germany). Power SYBR Green PCR Master Mix (Applied Biosystems) was used for detection. Primer dimer melting temperatures were determined in order to exclude primer dimers from the analysis. The housekeeping gene used was GAPDH for normalization of the target gene's expression. The relative expression was calculated as $E=½^{(-\Delta Ct)}$, where Ct is the difference between the threshold of cycle number of GAPDH and the gene analyzed. qPCR experiments were performed with cDNA from three biological replicates. Primers for qPCR were as follows (Table 4).

Primer

Target gene	Primer sequence
Sox4	Forward: 5'-GAA CGC CTT TAT GGT GTG GT-3' Reverse: 5'-GAA CGG AAT CTT GTC GCT GT-3'
Sox11	Forward: 5'-CCC TGT CGC TGG TGG ATA AG-3' Reverse: 5'-GGT CGG AGA AGT TCG CCT C-3'
DCX	Forward: 5'-TGC TCA AGC CAG AGA GAA CA-3' Reverse: 5'-CTG CTT TCC ATC AAG GGT GT-3'
Beta-tubb III	Forward: 5'-CTG GAA CCA TGG ACA GTG TTC G- Reverse: 5'-CGA CAT CTA GGA CTG AGT CCA C-
GFAP	Forward: 5'-AAC GAC TAT CGC CGC CAA CTG C- Reverse: 5'-ATG GCG CTC TTC CTG TTC GC-3'

Table 4: Primers used for RT-PCR in this study

Plasmid

Name	Properties	Reference
pCAG-Sox4-IRES-GFP	Expression vector, Amp^r	This work
pCAG-Sox11-IRES-GFP	Expression vector, Amp^r	

pCAG-Sox2-IRES-GFP	Expression vector, Amp^r	
pCAG-IRES-GFP	Expression vector, Amp^r	
pCAG-RFP	Expression vector, Amp^r	
pCAG-IRES-dsRed	Expression vector, Amp^r	
pCAG-GFP-IRES-Cre	Expression vector, Amp^r	
pGL3-Basic	Luciferase reporter vector, Amp^r	Invitrogen
pBKS-ko Sox11 5'	Cloning shuttle vector, Amp^r	Elisabeth Sock, Erlangen
pGL3-ko Sox11 5'	Luciferase reporter vector, Amp^r	This work
pKSPS(+)-Sox4	Cloning shuttle vector, Amp^r	

Table 5: Plasmids used in this study

6.8 Software & Statistical analysis

Software

FluoView 1.7	Olympus, Hamburg, Germany
Leica Application Suite AF	Leica Microsystems, Wetzlar, Germany
Vector NTI	Invitrogen, Karlsruhe, Germany
Genomatix	Genomatix Software GmbH, Munich, Germany
StepOne Software	Applied Biosystem GmbH,Darmstadt, Germany

Statistical analysis

All numerical analysis were performed using Excel (Microsoft). Unpaired Student's t-test was used for comparisons. Differences were considered statistically significant at *$p<0.05$, **$p<0.01$ and ***$p<0.001$. All data were presented as mean ± s.e.m.

7 Publication

Part of this study is published as:

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Other publications:

Cao L*, **Mu L***, Qiu Y, Su Z, Zhu Y, Gao L, Yuan Y, Guo D, He C. (2010) Diffusible, membrane-bound, and extracellular matrix factors from olfactory ensheathing cells have different effects on the self-renewing and differentiating properties of neural stem cells. ***Brain Research*** 1359:56-66.

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Cao L, Zhu YL, Su Z, Lv B, Huang Z, **Mu L**, He C. (2007) Olfactory ensheathing cells promote migration of Schwann cells by secreted nerve growth factor. ***Glia*** 55:897-904.

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9 Appendix

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