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Sox11 as a regulator of neuronal maturation & integration of newborn neurons in the adult hippocampus

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Abstract

New dentate granule (DG) neurons are constantly added to the adult hippocampus (HC). Accumulating evidence indicates a unique contribution of these neurons to the hippocampal network and to hippocampal function. Indeed, adult hippocampal neurogenesis has been linked to hippocampus-dependent learning and memory processes as well as to anxiety and depression. Thus, it is of great importance to decipher the complex genetic programs controlling adult hippocampal neurogenesis. The SoxC transcription factors Sox4 and Sox11 were recently identified to be involved in the transcriptional control of adult hippocampal neurogenesis. Both were found to be expressed in immature neurons of the adult DG and to be required for neuronal fate determination of adult neural stem/progenitor cells. Sox11 expression was found to be transient in the adult hippocampal neurogenic lineage and to be downregulated in mature DG neurons.

The aim of this study was to assess the currently unknown functional relevance of Sox11 downregulation for neuronal maturation and integration in the adult DG. A further goal was to identify new Sox11 downstream targets and to determine pathological conditions that alter Sox11 expression in the adult DG.

To analyze the relevance of Sox11 downregulation in the adult hippocampal neurogenic lineage, Sox11 was ectopically expressed in adult-born DG neurons. This was achieved by retroviral and/or adeno-associated viral injections into the DG of adult mice. Sox11 overexpression resulted in various alterations of marker expression (e.g. prolonged immature marker expression), morphological (e.g. reduced size) as well as electrophysiological characteristics (e.g. age-inappropriate input resistance), and migration (e.g. mispositioning). Thus, persistent Sox11 expression disrupted neuronal maturation and integration of DG neurons. Three factors, i.e., *Delta like ligand 1*, *Nuclear factor IA*, and *Stathmin 1*, were identified by *in vitro* and *in vivo* conditional knockout of Sox4/11 and Sox11 overexpression as putative Sox11 downstream targets. Intriguingly, previous studies implicated these factors in neurogenic processes. Finally, aberrant neuronal activity was found to induce Sox11 expression in mature DG neurons. This raises the possibility that Sox11 is involved in the regulation of transcriptional programs following pathological neuronal activity in the adult DG.

In summary, the present study demonstrates for the first time a crucial role for Sox11 in the control of maturation and integration of adult-born DG neurons and suggests that Sox11-dysregulation may be involved in distinct DG-associated pathologies.

Zusammenfassung

Neue Körnerzellen des Gyrus dentatus werden dem adulten Hippocampus fortlaufend hinzugefügt. Neuere Erkenntnisse deuten darauf hin, dass diese Zellen einen einzigartigen Beitrag innerhalb des hippocampalen Netzwerks und für die hippocampale Funktion leisten. In der Tat wurde adulte hippocampale Neurogenese mit Hippocampus-abhängigen Lern- und Gedächtnisprozessen sowie mit Angst und Depression in Verbindung gebracht. Es ist also von großer Bedeutung die komplexen genetischen Programme zu entschlüsseln, die die adulte hippocampale Neurogenese regulieren. Kürzlich wurde gezeigt, dass die SoxC Transkriptionsfaktoren Sox4 und Sox11 in der transkriptionellen Kontrolle der adulten hippocampalen Neurogenese involviert sind. Beide Proteine sind in unreifen Körnerzellen des adulten DG exprimiert und werden für die Festlegung einer neuronalen Identität von adulten neuralen Stamm-/Vorläuferzellen benötigt. Interessanterweise wird Sox11 nur transient während der adulten hippocampalen neurogenen Entwicklung exprimiert und in reifen Körnerzellen herunterreguliert.

Das Ziel dieser Studie war die Relevanz der Sox11-Herunterregulierung für die neuronale Reifung und Integration im adulten Gyrus dentatus zu bestimmen. Ein weiteres Ziel war die Identifizierung von neuen Sox11 Zielgenen und die Bestimmung von pathologischen Umständen unter denen die Expression von Sox11 im adulten Gyrus dentatus verändert ist.

Um die Relevanz der Sox11-Herunterregulierung während der adulten hippocampalen neurogenen Entwicklung zu analysieren, wurde Sox11 ektopisch in Körnerzellen exprimiert, die im Erwachsenenalter generiert wurden. Dies wurde durch Injektionen von Retroviren oder von Adeno-assoziierten Viren in den Gyrus dentatus adulter Mäuse erreicht. Sox11-Überexpression führte zu diversen Veränderungen im Hinblick auf Markerexpression (z.B. verlängerte Expression unreifer Markerproteine), morphologischer (z.B. verringerte Größe) sowie elektrophysiologischer Charakteristika (z.B. dem Alter nicht entsprechendem Eingangswiderstand) und Migration (z.B. falsche Positionierung). Folglich behindert andauernde Sox11-Expression die neuronale Reifung und Integration von Körnerzellen des Gyrus dentatus. Drei potentielle Sox11 Zielgene, nämlich *Delta like ligand 1*, *Nuclear factor IA*, und *Stathmin 1*, wurden durch konditionalen Knockout von Sox4/11 und Sox11-Überexpression *in vitro* und *in vivo* identifiziert.

Interessanterweise wurden diese Faktoren in vorhergehenden Studien bereits mit neurogenen Prozessen in Verbindung gebracht. Schließlich wurde festgestellt, dass die Expression von Sox11 in reifen Körnerzellen des Gyurs dentatus nach aberranter neuronaler Aktivität induziert wurde. Dies wirft die Möglichkeit auf, dass Sox11 in die Regulation transkriptioneller Programme nach pathologischer neuronaler Aktivität im adulten Gyrus dentatus involviert ist.

Zusammenfassend weist die vorliegende Studie Sox11 erstmalig eine entscheidende Rolle in der Kontrolle der Reifung und Integration von Körnerzellen zu, die im adulten Gyrus dentatus generiert wurden. Weiterhin deuten die Ergebnisses dieser Arbeit darauf hin, dass eine Dysregulation von Sox11 ursächlich in Gyrus dentatus-assoziierten Pathologien involviert sein könnte.

Abbreviations

A	Ampere
AAV	Adeno-associated virus
AD	Alzheimer's disease
AHP	Adult hippocampal progenitor cells
Amp	Ampicillin
ANOVA	Analysis of variance
APS	Ammonium persulfate
Arc	Activity-regulated cytoskeleton-associated protein
Ascl1 (also Mash1)	Achaete-scute complex-like 1
BCA	Bicinchoninic acid
BDNF	Brain-derived neurogenic factor
bio	Biotinylated
BLBP	Brain lipid-binding protein
BMP	Bone morphogenetic protein
bp	Base pair
BrdU	5-Bromo-2-deoxyuridine
BSA	Bovine serum albumine
c	Concentration or centi
CAG	CMV early enhancer/chicken β actin
cDNA	Complementary deoxyribonucleic acid
cfu	Colony-forming unit
CGN	Cerebellar granule neuron
ch	Chicken
ChIP	Chromatin immunoprecipitation
ChIP-Seq	ChIP-sequencing
cKO	Conditional knockout
cm	Centimeter
CMV	Cytomegalovirus
CNS	Central nervous system
C_T	Cycle threshold
Cy3	Carbocyanine
Cy5	Indodicarbocyanine

d	Day
DAPI	4’,6-Diamidino-2-phenylindole
DBN1	Drebrin
DCX	Doublecortin
DG	Dentate gyrus or dentate granule
DISC1	Disrupted in Schizophrenia 1
Dll1	Delta-like 1
DMEM	Dulbecco’s modified eagle medium
dn	Dominant negative
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleotides triphosphate
DOC	Deoxycholate
dpi	Days post injection
E. coli	Escherichia coli
EC	Entorhinal cortex
ECL	Enhanced chemiluminescence
ECS	Electroconvulsive shock
EDTA	Ethylene diamine tetraacetic acid
EGF	Epidermal growth factor
ERT2	Estrogen receptor T2
EtBr	Ethidium Bromide
EtOH	Ethanol
FBS	Fetal bovine serum
FGF	Fibroblast growth factor
FGF	Fibroblast growth factor
FITC	Fluorescein isothiocyanate
FL	Full length
Fmrp	Fragile X mental retardation protein
fwd	Forward
FXS	Fragile X syndrome
G	Giga
g	Gram
GABA	Gamma-aminobutyric acid
GABAR	GABA receptor

gag	Group-specific antigen
gal	Galactosidase
GCL	Granule cell layer
GF	Growth factor
GFAP	Glial fibrillary acidic protein
GFAP	Glial fibrillary acidic protein
GFP	Green fluorescent protein
gt	Goat
H	His-tag
h	Hour
HBS	Hepes buffered saline
HC	Hippocampus
HD	Huntington’s disease
HDAC	Histone deacetylase
HEK	Human embryonic kidney
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
His	Histidine
HMG	High mobility group
HMGU	Helmholtz Zentrum München
HRP	Horseradish peroxidase
Hz	Hertz
IEG	Immediate early gene
IGF-1	Insulin-like growth factor 1
IHC	Immunohistochemistry
ip	Intraperitoneal
IRES	Internal ribosome entry site
JAG1	Jagged 1
kb	Kilo base pair
kDa	Kilo dalton
L	Liter
LB	Lysogeny broth or Luria-Bertani broth
LTR	Long terminal repeats
m	Milli
M	Molar mass

MCL	Molecular cell layer
Mecp2	Methyl-CpG-binding protein 2
MeOH	Methanol
min	Minute
MoMLV	Moloney murine leukemia virus
ms	Mouse
mTLE	Medial temporal lobe epilepsy
MW	Molecular weight
n	Nano
N	Nuclear localization sequence
NaAc	Sodium acetate
Nedd8	Neural-precursor-cell-expressed developmentally down-regulated 8
NeuN	Neuronal nuclei
NeuroD	Neurogenic differentiation
NF	Nuclear factor
Ngn2	Neurogenin 2
NICD	Notch intracellular domain
NPC	Neural precursor cell
NT	Neurotransmitter
o/n	Over night
OB	Olfactory bulb
Oct4	Octamer binding transcription factor 4
p	Pico-
PAGE	Polyacrylamide gel electrophoresis
Pax5	Paired box 5
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline Triton X-100
PC3	Pheochromocytoma cell 3
PCR	Polymerase chain reaction
PD	Parkinson’s disease
PFA	Paraformaldehyde
PMSF	Phenylmethylsulphonyl fluoride
pol	Polymerase

Prox	Prospero homeobox
PSA-NCAM	Polysialylated-neural cell adhesion molecule
PSF	Penicillin-streptomycine-fungizone
PTSD	Post-traumatic stress disorder
PVDF	Polyvinylidene difluoride
qRT-PCR	Quantitative real time PCR
rb	Rabbit
rcf	Relative centrifugal force
RE	Restriction endonuclease
rev	Reverse
RIPA	Radioimmunoprecipitation assay
RMS	Rostral migratory stream
RNA	Ribonucleic acid
rpm	Revolutions per minute
RT	Room temperature or reverse transcriptase
rtTA	Reverse tetracycline-dependent transactivator
SD	Standard deviation
SDS	Sodium dodecyl sulfate
sec	Second
SGZ	Subgranular zone
sh	Sheep
Shh	Sonic hedgehog
Sox	Sry-related HMG box
Sprr1a	Small proline-rich protein 1A
Srp14	Signal recognition particle 14
Sry	Sex-determining region Y
STMN1	Stathmin 1
strep	Streptavidin
SVZ	Subventricular zone
T	Transduction domain
Tbr2	T-box brain gene 2
TAD	Transactivation domain
TAE	Tris-acetate-EDTA buffer
TBB3	β-Tubulin 3

TBS	Tris buffered saline
TBST	Tris buffered saline Triton X-100
TEMED	Tetramethylethylenediamine
TF	Transcription factor
Tis21	Tetradecanoyl phorbol acetate-inducible sequence 21
TK	Thymidinde kinase
TNF	Tumor necrosis factor
TRAF	TNF receptor associated factor
Tris	Tris(hydroxymethyl)aminomethane
U	Unit
UV	Ultraviolet
V	Volume or Volt
vsv-g	Vesicular stomatitis virus G-protein
w	Week
WB	Western blot
wpi	Weeks post injection
wt	Wild type
β	Beta
Δ	Delta
λ	Wavelength
µ	Mikro
Ψ	Psi
Ω	Ohm

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1 Introduction

"The brain is a world consisting of a number of unexplored continents and great stretches of unknown territory." (Santiago Ramon y Cajal, 1852-1934)

At the time of this statement one of the continents still to be explored represented the area of neurogenesis, in particular adult neurogenesis. Despite his great merits in the field of neurosciences, Santiago Ramon y Cajal turned out to be wrong with his assumption that in the adult brain "everything may die, nothing may be regenerated" (1928). But it was not before 1965 that dividing cells were detected in the hippocampus of postnatal rats (Altman and Das, 1965). Later on, cells of the subventricular zone (SVZ) were shown to possess a proliferative potential and to be able to differentiate into neurons and glial cells (Lois and Alvarez-Buylla, 1993). The existence of newborn neurons in the hippocampal dentate gyrus (DG) of adult rats was convincingly demonstrated by bromodeoxyuridine (BrdU) labeling and phenotyping of dividing cells (Kuhn et al., 1996). Dividing cells were also detected post mortem in the human hippocampus of cancer patients treated with BrdU (Eriksson et al., 1998).

Nowadays, it is well accepted that cells exist in the adult mammalian brain which have stem cell like characteristics (Simons and Clevers, 2011): they are capable of self-renewal next to differentiation into all three cell types of the neural lineage – neurons, astrocytes and oligodendrocytes (Ma et al., 2009a). In particular two neurogenic regions have been consistently identified so far: the SVZ adjacent to the lateral ventricles (Alvarez-Buylla and Garcia-Verdugo, 2002), from where newborn neurons migrate via the rostral migratory stream (RMS) towards the olfactory bulb (OB), and the subgranular zone (SGZ) of the hippocampal DG (Zhao et al., 2008). Adult neural stem cells (NSCs) within these regions ensure the capacity for neurogenesis after birth and throughout life. There is still ongoing debate about the extent and functional relevance of adult neurogenesis in humans (Goritz and Frisen, 2012). Especially concerning neurogenesis in the SVZ this is a highly controversial topic since most recent data suggest that human SVZ neurogenesis may be very limited if existent at all (Bergmann et al., 2012; Wang et al., 2011a). With regard to the DG, several studies found correlations between measures of human hippocampi, e.g., hippocampal volume, obtained by non-invasive *in vivo* neuroimaging and levels of stimuli that are known to enhance or diminish adult hippocampal neurogenesis in

animal models suggesting that neurogenesis occurs also in the human adult HC (Ho et al., 2013). Moreover, signals were detected in the human brain by magnetic resonance spectroscopy that correlated in animals with neurogenesis (Manganas et al., 2007). The confirmation of the existence of adult hippocampal neurogenesis in humans will be highly important as hippocampal neurogenesis may add an additional layer of complexity to plasticity and cognition in humans and may further represent a target process for therapeutic interventions in human neuropsychiatric diseases.

1.1 Adult hippocampal neurogenesis

The hippocampal formation is anatomically divided into four primary subregions, the DG, the CA1, CA2 and CA3 region, each of which harbors distinct cell types: granule cells accounting for the major cellular fraction of the DG and pyramidal cells being the predominant cell type in the three CA regions. These cells form the trisynaptic circuitry, which processes sensory information from specific cortical areas converging onto the entorhinal cortex (EC). Information flows from the EC to granule cells of the DG through the medial and lateral perforant pathway. Granule cells connect via their axons (mossy fibers) to the pyramidal cells of the CA3 region that in turn project their axons (Schaffer collateral axons) to the CA1 region from where the output signal is transmitted back to the EC (Andersen et al., 2006). GABAergic (gamma-aminobutyric acid) inhibitory interneurons represent another important cell type of the HC, e.g., in context of adult hippocampal neurogenesis, by building up a local neuronal circuitry that can influence adult NSCs of the DG (Song et al., 2012b).

The DG is subdivided in the SGZ, the granular cell layer (GCL), the molecular cell layer (MCL) and the hilus. Cell bodies of granule cells reside in the GCL. Their dendritic trees extend towards the MCL receiving input from the EC, while their axons project across the hilus to the CA3 region (Gage et al., 2007). Adult hippocampal NSCs, which share high similarity to radial glia (RG) cells (Kriegstein and Alvarez-Buylla, 2009), are located in the SGZ, a narrow region located between the hilus and the GCL. This cell population, capable of quiescence, self-renewal, and differentiation into neurons and astrocytes (Bonaguidi et al., 2011), represents the cellular basis for adult hippocampal neurogenesis.

The neurogenic process can be divided into distinct phases each characterized by individual morphological features, electrophysiological properties, and the expression of stage-specific marker proteins (Ge et al., 2008; Zhao et al., 2008). First, RG-like

stem cells classified as Type I cells, which constitute the primary NSCs in the adult brain (Bonaguidi et al., 2011; Dhaliwal and Lagace, 2011), have to be activated followed by expansion of intermediate progenitors (Type II cells) that undergo fate specification and generate neuroblasts (Type III cells). After migration and initial pruning newborn neurons have to undergo maturation and synaptic integration (Song et al., 2012a) (Fig. 1.1 A). The formation of a mature DG neuron from the time of stem cell division takes approximately seven to eight weeks in adult mice (Zhao et al., 2006).

Type I cells are morphologically characterized by a radial process, which extends and branches into the inner MCL. Biochemically, the expression of glial fibrillary protein (GFAP), brain lipid-binding protein (BLBP), Nestin, and the sex-determining region Y (Sry) related high mobility group (HMG) box (Sox) transcription factor Sox2 represents a distinct feature of these cells (Steiner et al., 2006). Type II cells possess horizontal processes (Fukuda et al., 2003) and express the achaete-scute complex-like 1 (Ascl1, also Mash1) and T-box brain gene 2 (Tbr2). With the progression of Type II to III cells and further to immature neurons the expression of proteins of the neuronal lineage including Doublecortin (DCX), polysialylated-neural cell adhesion molecule (PSA-NCAM), neurogenic differentiation (NeuroD), prospero homeobox 1 (Prox1), and finally Calretinin becomes a prominent biochemical feature (Song et al., 2012a). Fully matured adult-born dentate granule (DG) neurons are biochemically and morphologically indistinguishable from granule cells born during the early postnatal period: they express mature neuronal proteins such as Prox1, neuronal nuclei (NeuN), extend a complex dendritic tree into the MCL, and project to the CA3 region (Gage et al., 2007; Kempermann et al., 2004) (Fig. 1.1 B).

In the course of maturation newborn DG neurons become fully integrated into the hippocampal circuitry gradually establishing functional synapses with target cells (Toni et al., 2008) and receiving input from axo-somatic, axo-dendritic, and axo-spinous synapses (Toni et al., 2007). Morphologically, first spines can be observed 16 days after neuronal birth. Thereafter, spine density strongly increases between day 21 and 28 before reaching a plateau 8 weeks after neuronal birth (Zhao et al., 2006). Electrophysiologically, GABA depolarizes young neurons during the first two to three weeks of their development. After this period, they receive hyperpolarizing GABAergic and finally excitatory glutamatergic inputs (Ge et al., 2006; Ge et al., 2008). During late maturation steps (4-6 weeks) newborn neurons exhibit enhanced

synaptic plasticity characterized by increased long-term potentiation (Ge et al., 2007b) and lower activation threshold (Marin-Burgin et al., 2012) (Fig. 1.1 C).

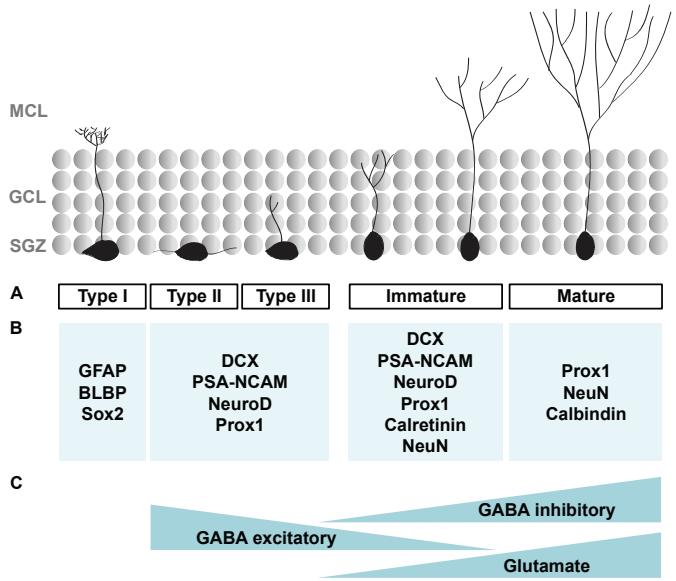


Figure 1.1 Neurogenic process in the adult dentate gyrus

A Schematic illustration of the neurogenic process in the adult DG divided into subgranular zone (SGZ), granule cell layer (GCL), and molecular cell layer (MCL) depicting the development of Type I NSC via transient amplifying Type II and III progenitor cells and immature neuronal precursors into fully mature DG neurons. **B** Assignment of characteristic marker proteins to appropriate developmental stage of newborn DG neurons. **C** Development of electrophysiological properties along with the neuronal maturation process.

1.1.1 Functional implications in the healthy and diseased brain

It is well accepted that newborn DG neurons have a significant impact on hippocampal function. However, it is still a matter of extensive research, which specific hippocampal functions are dependent on hippocampal neurogenesis.

Several experimental studies have shown a correlation between changes in hippocampal neurogenesis and performance in learning and memory (Deng et al., 2010; Song et al., 2012a). For instance, deficits in hippocampal-dependent spatial learning tasks, contextual fear conditioning, and spatial pattern separation were detected after disrupting hippocampal neurogenesis by application of antimitotic drugs, such as methylazoxymethanol acetate and colchicine, or by X-irradiation (Clelland et al., 2009; Gilbert et al., 2001; Kim and Lee, 2011; Rola et al., 2004; Saxe et al., 2006; Shors et al., 2001; Snyder et al., 2005; Winocur et al., 2006). But since those experimental approaches are not restricted to the population of newborn neurons, behavioral impairments might be also due to effects on other cell populations. More recent experimental paradigms make use of the transient

expression of marker proteins with relative specificity to the hippocampal neurogenic subpopulation of the HC such as GFAP and Nestin to selectively ablate this very population. Specific and inducible manipulation is achieved by using GFAP or Nestin promoter elements driving the expression of proteins as thymidine kinase (TK), reverse tetracycline-dependent transactivator (rtTA), or a fusion protein of the Cre recombinase and the estrogen receptor T2 (CreERT2) that can be controlled by application of the appropriate drugs, ganciclovir, doxycyclin, or tamoxifen, respectively (Deng et al., 2009; Dupret et al., 2008; Guo et al., 2011; Imayoshi et al., 2008; Kheirbek et al., 2012b; Nakashiba et al., 2012; Sahay et al., 2011; Saxe et al., 2006; Tronel et al., 2012). These studies reveal that disrupted hippocampal neurogenesis correlates with impaired long-term spatial memory, acquisition and extinction of contextual fear memory, and spatial pattern separation. Interestingly, Sahay and colleagues could also demonstrate that expansion of the newborn neuronal population resulted in enhanced pattern separation (Sahay et al., 2011). Computational models of adult neurogenesis that assign roles to newborn neurons further support experimental evidence (Aimone and Gage, 2011).

Several studies demonstrated a correlation of reduced neurogenesis with increased anxiety thereby functionally implicating adult hippocampal neurogenesis in anxiety-related behavior (Perez et al., 2009; Revest et al., 2009; Sahay et al., 2011). It was hypothesized that impaired pattern separation is causally linked to the pathogenesis of anxiety and that impaired pattern separation may link adult hippocampal neurogenesis with psychiatric disorders such as post-traumatic stress disorder (PTSD) and anxiety disorders (Kheirbek et al., 2012a). Another interesting observation was that adult hippocampal neurogenesis is required for the efficacy of specific classes of antidepressants (Sahay and Hen, 2007). If hippocampal neurogenesis was impaired by X-irradiation, the ameliorative effect of the antidepressant fluoxetine, a selective serotonin reuptake inhibitor, was blocked (Santarelli et al., 2003). However, there are also neurogenesis-independent effects of antidepressants reported and not all antidepressants require neurogenesis for their actions in animal models (David et al., 2009). Hence, whether adult hippocampal neurogenesis is linked to antidepressant treatment or might even be an etiological factor for depression is still controversially debated (Eisch and Petrik, 2012; Sahay and Hen, 2007).

The correlation between adult hippocampal neurogenesis and neurological and neurodegenerative diseases remains unclear. Transgenic animal models for Alzheimer's disease (AD) show dysfunctional hippocampal neurogenesis, both increased and decreased (Lazarov and Marr, 2010), while those for Parkinson's disease (PD) (Marxreiter et al., 2012) and Huntington disease (HD) exhibit a negative impact on adult hippocampal neurogenesis (Winner et al., 2011).

Several risk genes for neuropsychiatric disorders, such as disrupted-in-schizophrenia 1 (DISC1) implicated in major mental disorders or methyl-CpG-binding protein 2 (Mecp2), which is mutated in the Rett syndrome, were shown to be involved in the regulation of adult neurogenesis (Ming and Song, 2011; Winner et al., 2011). In a mouse model for fragile X syndrome, loss of the fragile X mental retardation protein (Fmrp) resulted in altered adult hippocampal neurogenesis (e.g. increased neural progenitor proliferation and altered fate specification) (Luo et al., 2010). Fmrp-null mice exhibited impaired adult hippocampal neurogenesis. Selective restoration of Fmrp expression in NSCs rescued impaired neurogenesis and, most interestingly, also impaired hippocampus-dependent learning in these mice (Guo et al., 2011). Finally, adult hippocampal neurogenesis, more precisely its reduction, was shown to correlate with a decline of cognitive performance during aging since aged animals displayed impairments in learning and memory (Drapeau et al., 2003) (Driscoll et al., 2006) but the causative relationship underlying this observation remains to be proven.

In summary, cumulative evidence suggests an involvement of adult hippocampal neurogenesis in neurological disorders. Further investigation of the precise functional impact of adult neurogenesis on disease onset, progression, and symptoms will provide important insight with respect to possible therapeutic starting points.

1.1.2 Extrinsic and intrinsic regulatory mechanisms

Not only does adult hippocampal neurogenesis modulate brain function, it is itself subjected to environmental changes that modulate the neurogenic process at various phases (s. 1.1.1). Identified extrinsic regulators comprise macro-environmental factors such as complex behavioral modifications and the special microenvironment surrounding neural stem cells, the so-called neurogenic niche, which provides signals that precisely control the rate and timing of neurogenesis. Together with various intrinsic mechanisms, a complex regulatory network emerges comprising

environmental stimuli, niche factors and receptors, cytoplasmic and transcriptional factors, and epigenetic regulators (Faigle and Song, 2012; Kempermann, 2011; Mu et al., 2010; Sun et al., 2011; Zhao et al., 2008).

Adult hippocampal neurogenesis was shown to be regulated by environmental stimuli such as activity under several physiological and also pathological conditions (Ma et al., 2009b). Physical exercise, e.g., voluntary wheel running, exerts a positive neurogenic effect (Vivar et al., 2012), which is mediated by increasing proliferation (van Praag et al., 1999). Exposure to an enriched environment promotes neurogenesis by facilitating neuronal survival (Kempermann et al., 1997) (Rossi et al., 2006). Furthermore, enhanced adult hippocampal neurogenesis was observed when hippocampal-dependent learning tasks were applied (Leuner et al., 2006), such as Morris water maze (MWM) (Drapeau et al., 2007; Sisti et al., 2007) and associative learning tasks (Gould et al., 1999). Pathological behavioral stimuli such as social isolation and stress were found to decrease neurogenesis (Dranovsky et al., 2011; Duman, 2004; Warner-Schmidt and Duman, 2006). For instance, it was recently shown that social isolation resulted in an accumulation of stem cells at the expense of neurons in the adult HC indicating that behavioral stimuli and experience may dictate stem cell fate (Dranovsky et al., 2011). Finally, increased proliferation of precursor cells in the DG could be observed in several studies after seizure induced by either electrical stimulation or chemoconvulsant treatment (Jessberger et al., 2007b; Overstreet-Wadiche et al., 2006; Parent and Kron, 2012).

Changes in the level of adult hippocampal neurogenesis could also be observed in case of antidepressant treatment. Several studies revealed that antidepressant treatment increases neuronal proliferation and survival and accelerates dendritic development (Sahay and Hen, 2007; Warner-Schmidt and Duman, 2006).

How macro-environmental changes mechanistically modify the process of adult hippocampal neurogenesis remains an intriguing question.

It is currently assumed that macro-environmental stimuli modulate the neurogenic niche. The neurogenic niche comprises a multitude of different cell types including mature and immature neurons, astrocytes, and the surrounding vasculature (Zhao et al., 2008). One powerful regulator of adult hippocampal neurogenesis appears to be the modification of hippocampal network activity and the release of neurotransmitters (Song et al., 2012a). Indeed, it was revealed that GABA activates neural progenitors

as well as immature neurons and regulates proliferation and neuronal maturation (Ge et al., 2007a; Song et al., 2012b; Tozuka et al., 2005) and that glutamate can promote the proliferation and survival of newborn neurons (Bruel-Jungerman et al., 2006; Tashiro et al., 2006a).

Growth factors and signaling molecules released by the neurogenic niche also play a prominent role in the regulation of adult neurogenesis (Zhao et al., 2008). For example, it was revealed that astrocytes could promote neuronal differentiation through cell-cell contact dependent signaling via Ephrin-B2 (Ashton et al., 2012). Furthermore growth factors (GFs,), e.g. brain-derived neurogenic factor (BDNF) or insulin-like growth factor 1 (IGF-1), and morphogens including Notch, Sonic hedgehog (Shh), Wnts, and bone morphogenetic proteins (BMPs) were shown to regulate adult hippocampal neurogenesis (Faigle and Song, 2012). For instance, a pro-neurogenic effect could be assigned to the activation of the canonical Wnt signaling pathway by Wnt3 (Lie et al., 2005). Several studies further revealed a crucial role for Notch signaling in various neurogenic steps (Ables et al., 2011). Thus, Notch signaling, which is mediated by binding of different membrane-bound ligands (e.g. jagged 1 (JAG1) or delta-like 1 (Dll1)) to the Notch receptor, was shown to be required for stem cell maintenance (Ables et al., 2010; Ehm et al., 2010; Lugert et al., 2010).

Extrinsic signals arising from the neurogenic niche may converge onto distinct sets of cell intrinsic signaling molecules such as TFs and epigenetic regulators, which control developmental stage specific genetic programs in adult hippocampal neurogenesis (Faigle and Song, 2012; Schwarz et al., 2012; Zhao et al., 2008). For instance, it was revealed that Wnt signaling triggers the expression of the TFs Prox1 (Karalay et al., 2011) and NeuroD (Kuwabara et al., 2009) in the adult HC, which were reported to control neuronal differentiation (Karalay et al., 2011) as well as survival and maturation of adult-born DG neurons (Gao et al., 2009; Kuwabara et al., 2009).

1.2 Sox transcription factor family

One very prominent TF family throughout the field of stem cell research is the Sox family of TFs (Sarkar and Hochedlinger, 2013), the members of which are also important intrinsic regulators of adult hippocampal neurogenesis. Named after the first discovered Sox gene *Sry* (*sex determining region on the Y chromosome*)

(Sinclair et al., 1990) Sox proteins have been the subject of intensive research and implicated as central modulators in developmental and stem cell biology (Sarkar and Hochedlinger, 2013). It is now a well-accepted concept that Sox proteins control stem cell maintenance, cell fate, and differentiation in many key developmental and physiological processes (Lefebvre et al., 2007).

1.2.1 Characteristics and implications in stem cell biology

By now, 20 Sox genes have been identified in humans and mice (Schepers et al., 2002). Sox genes are subdivided into eight groups, A to H, with two B subgroups (B1 and B2) (Bowles et al., 2000). Members of the Sox transcription factor family characteristically feature a high mobility group (HMG) box domain, which has evolved from the canonical HMG domain. HMG domains mediate DNA binding, DNA bending, protein interactions, and subcellular localization (Lefebvre et al., 2007). The HMG box domain of Sox proteins retains the ability of the canonical HMG domain to bend DNA thereby altering DNA conformation (Murphy et al., 1999). In contrast to the canonical HMG domain, which has little if any DNA sequence specificity, the HMG box domain of Sox proteins confers a preference for the hexameric core sequence XXCAAX (X stands for A or T). However, it retained the ability of the canonical HMG domain to bend DNA thereby altering DNA conformation. Next to the HMG box domain Sox proteins can exhibit several other functional domains including transactivation, transrepression, and dimerization domains (Dy et al., 2008). Sox proteins, which belong to different groups, vary with respect to those domains and are only approximately 50 % identical concerning the HMG box domain. Within each Sox group, however, the HMG box domain and other domains share a high degree of identity (Lefebvre et al., 2007). This can on the one hand explain the partial functional redundancy of many Sox proteins of the same group, and on the other hand explain to some extent the different impact of Sox proteins belonging to different groups (Guth and Wegner, 2008).

Sox proteins bind DNA with relative low affinity and specificity. Thus, other mechanisms are required to increase the binding strength and to refine target gene selection of Sox proteins in different cellular contexts. So far several mechanisms have been described: variation of nucleotides directly adjacent to the core sequence, homodimerization, interaction with and recruitment of other DNA binding proteins, e.g. TFs, factors of the transcriptional machinery, or epigenetic modulatory proteins,

posttranscriptional modifications, and possibly facilitation of enhanceosome assembly (Lefebvre et al., 2007; Sarkar and Hochedlinger, 2013). The latter might be achieved by bending of the DNA upon binding of Sox factors to the minor groove which in turn increases the accessibility of and changes the distance to DNA for other DNA binding proteins (Ferrari et al., 1992). The preference of Sox proteins, which belong to different groups, for particular nucleotides flanking the consensus sequence is a well accepted concept to achieve higher DNA sequence specificity (Mertin et al., 1999). Homodimerization by leucin-zipper coiled-coil domains of Sox proteins resulting in more efficient binding to pairs of adjacent recognition sites can represent a further mechanism to increase DNA affinity (Lefebvre et al., 1998). Furthermore, the fact that Sox proteins regulate targets genes by binding to other TFs has been revealed in several studies (Kamachi et al., 2000), e.g. various types of interacting TFs such as homeodomain, zinc finger, basic helix-loop-helix, and leucine zipper proteins were identified as partners for SoxE group members (Wissmuller et al., 2006). Recently, this concept was further confirmed by a study demonstrating that the ability of Sox2 to induce pluripotency is dependent on the functional interaction with the octamer binding transcription factor 4 (Oct4), since no induction was achieved if amino acids responsible for binding to Oct4 were mutated (Jauch et al., 2011). Vice versa, Sox17 could only mimic the function of Sox2 if a mutation in the protein interaction domain of Sox17 was induced that facilitated Oct4 binding. Furthermore, the recruitment of other DNA binding proteins can specify the function of Sox proteins: recruitment of the co-activator p300 by Sox2 causes transcriptional activation (Chen et al., 2008) whereas interaction with the repressive polycomb complex results in gene silencing (Boyer et al., 2006). Finally, posttranslational modifications including phosphorylation, sumoylation, and ubiquitination were suggested to modulate Sox protein function (Lefebvre et al., 2007). The DNA binding efficiency of Sox9 for instance was shown to be increased after phosphorylation (Huang et al., 2000).

Considering the broad spectrum of regulatory capabilities of Sox proteins it is not surprising that they have been associated with multiple processes of developmental and stem cell biology (Lefebvre et al., 2007; Sarkar and Hochedlinger, 2013).

The early embryonic lethality of Sox2 deficient mice already highlights the importance of one of the most prominent and well studied SoxB1 proteins. Its role and also the role of other Sox proteins during fetal development ranges from proliferation, lineage

specification and differentiation to morphogenesis (Sarkar and Hochedlinger, 2013). Sox2 was for example reported to be required for repression of mesendodermal differentiation in favor of an early neuroectodermal fate (Thomson et al., 2011; Wang et al., 2012).

Sox9 (SoxE group) was implicated in proper retinogenesis, since its deletion resulted in a loss of the stem cell potential to differentiate into Muller glial cells (Poche et al., 2008), and was further shown to be crucial for pancreatic development. Pancreatic progenitors were lost in the absence of Sox9 and its requirement was not restricted to maintenance of embryonic but also of adult pancreatic progenitors (Furuyama et al., 2011; Seymour et al., 2007).

Multiple other Sox proteins are involved in adult stem cell biology, such as Sox6 of the SoxD group for which a crucial function in erythropoiesis in adult mice was demonstrated (Dumitriu et al., 2010). Considering more precisely (adult) neural stem cell biology, Sox proteins are once more critical regulators and modifiers of neurogenic processes (Lefebvre et al., 2007; Pevny and Nicolis, 2010; Wegner, 2011; Wegner and Stolt, 2005). The maintenance of neural stem cell identity was assigned to Sox2 first during embryonic development (Avilion et al., 2003; Graham et al., 2003) and later also during adult neurogenesis (Favaro et al., 2009; Ferri et al., 2004). A crucial role of Sox9 in cooperation with nuclear factor IA (NFIA) for the initiation of gliogenesis in the developing central nervous system (CNS) was recently revealed (Kang et al., 2012), which can explain the finding, that Sox9 downregulation is crucial for neuronal differentiation in the adult SVZ (Cheng et al., 2009). A recent study could further demonstrate that Sox2, Sox3 (SoxB1 group) and Sox11 (SoxC group) are required in a sequential manner to coordinate neural gene expression from early lineage specification of pluripotent stem cells to their later neuronal development (Bergsland et al., 2011). This study moreover revealed an intriguing mechanism of action: genes that were bound by Sox factors but were not yet transcribed, i.e. genes poised for activation, were associated with a bivalent chromatin signature harboring both repressive and activating histone modifications. Thus, Sox proteins might be able to function as pioneer factors and to prepare transcriptional programs by binding to and epigenetically predisposing genes to be activated at later developmental steps. Considering the ability of Sox proteins to also act as architectural proteins by bending and unwinding of chromatin, this finding might provide a first indication that the facilitation of enhanceosome assembly is

indeed one mechanism of action of Sox proteins (as discussed above). One particular Sox protein being part of this sequentially acting team is Sox11, which plays together with the other SoxC proteins a decisive role in stem cell biology (Penzo-Mendez, 2010).

1.2.2 Sox11

The SoxC group consists of three members: Sox4, Sox11, and Sox12. SoxC proteins, which are single-exon genes, feature next to the characteristic N-terminal HMG box domain, a C-terminal transactivation domain (TAD). The SoxC TFs share a high degree of identity in both domains (84 % and 95 %, respectively). Together with the observation that SoxC proteins are co-expressed at high levels in neuronal and mesenchymal tissue as well as at lower and more variable levels in other tissues, such as heart, pancreas, and thymus, the structural resemblance suggests that SoxC proteins exhibit similar functional properties (Dy et al., 2008). Nevertheless, several studies demonstrated that Sox11 is the most potent activator followed by Sox4 and Sox12 (Dy et al., 2008; Hoser et al., 2008; Kuhlbrodt et al., 1998). Sox12 deficient mice (*Sox12* $^{-/-}$) that develop normally without any obvious defects further confirmed the relatively low potency of Sox12 and suggest that Sox4 and Sox11 function redundantly thereby compensating for the loss of Sox12 (Bhattaram et al., 2010; Hoser et al., 2008). Contrary, *Sox4* $^{-/-}$ and *Sox11* $^{-/-}$ mice exhibit a severe phenotype: mice die during embryogenesis or immediately after birth, respectively, due to heart defects (Schilham et al., 1996; Sock et al., 2004). *Sox11* $^{-/-}$ are further characterized by many hypoplastic and malformed organs. *Sox4/11/12* triple knockout mice displayed an even more drastic phenotype dying already at midgestation (Bhattaram et al., 2010). By analysis of mouse embryos lacking increasing numbers of *SoxC* alleles, Bhattaram and colleagues could further confirm that SoxC protein largely work in redundancy to ensure proper organogenesis primarily through regulation of neural and mesenchymal progenitor cell survival. Further studies confirmed the decisive role of Sox4 and Sox11 for instance in nephrogenesis (Murugan et al., 2012), retinogenesis (Usui et al., 2013b), and neural crest development (Hong and Saint-Jeannet, 2005). Specifying the impact of Sox4 and Sox11 on neural development, both proteins were for example shown to exert a prosurvival effect on neural cell types during spinal cord development (Thein et al., 2010) as well as to be crucial for the specification of corticospinal neurons and the accurate cortical layering

(Shim et al., 2012). Moreover, they seem to negatively regulate gliogenesis, since their downregulation is required for gliogenic differentiation at the expense of a neuronal fate (Potzner et al., 2007; Swiss et al., 2011). Finally, studies implicated Sox11 in peripheral nerve regeneration after injury (Jankowski et al., 2009; Jing et al., 2012) and in sensory neuron development (Lin et al., 2011). The importance of Sox11 for neuronal fate specification during embryonic development was further illustrated by a study that showed that Sox11 was crucial for the induction of neuronal differentiation during neocortical development (Li et al., 2012). Additionally, Bergsland and colleagues demonstrated that Sox11 controlled the establishment of neuronal properties (Bergsland et al., 2006) and promoted neuronal fate and neuronal-specific gene expression (e.g. *Tubb3*) during neuronal development of pluripotent embryonic stem cells (Bergsland et al., 2011). Taken together, these data strongly support the notion that SoxC proteins, in particular Sox4 and Sox11, regulate differentiation in multiple cell lineages and are especially important for neuronal fate specification.

Despite the broad knowledge about the function of SoxC proteins, little is known about their regulation. There is some evidence that the proneural proteins neurogenin 2 (Ngn2) and/or Mash1 regulate Sox4 and/or Sox11 expression in embryonic brain (Bergsland et al., 2006; Castro et al., 2011). Furthermore, during retinal development Sox4 expression was negatively associated with Notch signaling and, since declining levels of histone modifications associated with active transcription accompanied decreased Sox4 and Sox11 expression, a histone-dependent transcriptional regulation of Sox4 and Sox11 was suggested (Usui et al., 2013a). The latter finding was also described in another study of Sox11 in B-cell neoplasms (Vegliante et al., 2011) and in oligodendrocyte differentiation (Swiss et al., 2011). Moreover, the detection of SoxC mRNA in stem cells (Beckervordersandforth et al., 2010) raises the possibility that SoxC protein expression may be regulated on a posttranscriptional level.

There is also only sparse knowledge about downstream targets of Sox4 and Sox11. *Pax5* (paired box protein 5), was suggested as a Sox11 target in mantle cell lymphoma (Vegliante et al., 2013) as well as *TRAF* (TNF receptor associated factor) and *Sprr1a* (small proline-rich protein 1A) during nerve regeneration (Jing et al., 2012; Salerno et al., 2012). A genome-wide binding study of neurons derived from pluripotent embryonic stem cells revealed an enrichment of Sox11 on neuron-specific

genes (Bergsland et al., 2011). Furthermore, *Tubb3* (β-tubulin 3) and *DBN1* (drebrin), both genes, which are expressed in immature neurons, were identified as potential SoxC targets (Bergsland et al., 2006; Wang et al., 2010).

Another gene characteristically expressed in immature neurons, *DCX*, was recently identified by our laboratory as a direct target of Sox11 (Mu et al., 2012). The focus of this study was the role SoxC proteins, in particular of Sox4 and Sox11, in adult hippocampal neurogenesis. Interestingly, it was revealed that Sox4 and Sox11 expression is strictly confined to neuronally committed progenitor cells and immature neurons whereas no expression could be detected in non-committed precursors and mature neurons of the adult hippocampal neurogenic lineage (Haslinger et al., 2009). Sox4 and Sox11 overexpression promoted the generation of immature neurons from adult neural stem cells *in vitro* (Haslinger et al., 2009; Mu et al., 2012) raising the hypothesis that SoxC proteins are crucial regulators of neuronal fate commitment in adult hippocampal neurogenesis. To investigate this hypothesis Sox4 and Sox11 double conditional knockout mice (cKO Sox4/11) were used to specifically delete Sox4 and Sox11 either in adult hippocampus-derived NSC cultures *in vitro* or in neural progenitors *in vivo* by retroviral Cre-transduction (Mu et al., 2012). Both *in vitro and in vivo* conditional ablation of Sox4 and Sox11 resulted in fewer neuronally committed progenitor cells. Finally, an enhancement or an impairment of Ngn2-induced reprogramming of astroglia into neurons (Berninger et al., 2007) was detected in case of Sox4 and Sox11 overexpression or knockout, respectively.

In summary, the observations that Sox4 and Sox11 can promote neuronal reprogramming and induce neurogenesis *in vitro* as well as that their expression is initiated upon neuronal fate commitment and is crucial for initiation of neuron-specific gene expression programs *in vivo* identify Sox4 and Sox11 as essential regulators of adult hippocampal neurogenesis (Mu et al., 2012).

1.3 Aim of this study

Sox11 is crucial for neuronal fate determination in the adult hippocampal neurogenic lineage. Interestingly, its expression is confined to immature neurons. This raises the question whether not only its upregulation during early steps of granule cell development but also its downregulation during the further progression of hippocampal neurogenesis is required for accurate neuronal maturation and integration. This becomes an even more significant issue considering the fact that Sox11 seems to be expressed in mature neurons under certain pathophysiological conditions. The aim of this study was to answer this question by analyzing the effects of prolonged or ectopic Sox11 expression in newborn or mature neurons *in vivo* with respect to marker expression and morphological as well as electrophysiological characteristics. The *in vitro* and *in vivo* identification of possible Sox11 downstream targets, which might serve to explain some phenotypic changes, represented another purpose of this study. Finally, Sox11 expression was analyzed in the context of aberrant neuronal network activity.

2 Results

2.1 Phenotypical changes after Sox11 overexpression

Sox11 is a potent inducer of neuronal fate determination of NSCs in the adult DG. Interestingly, Sox11 is transiently expressed during the neurogenic process in the adult DG and downregulated in mature granule neurons (Haslinger et al., 2009). It is unexplored, if and to which extent this downregulation is essential for proper neuronal development. To address this question, the impact of Sox11 expression on newborn DG neurons was examined either at later developmental stages at which Sox11 expression has been downregulated (i.e. 21-day-old neurons (see 2.1.1) or 6-week-old neurons (see 2.1.2)) and after they have fully matured (i.e. 8-week-old neurons (see 2.1.3)).

2.1.1 21-day-old newborn DG neurons

At the age of 21 days, a significant portion of newborn DG neurons have quit the immature developmental stage that is characterized by DCX and Sox11 expression (Haslinger et al., 2009). The first set of experiments was designed to uncover the effect of Sox11 overexpression from neuronal birth until newborn neurons reach an age of 21 days. Sox11 overexpression was achieved by stereotactic injection of a retrovirus named CAG-Sox11-IRES-GFP (+ Sox11) that bicistronically encodes for Sox11 and the green fluorescent protein (GFP). A retrovirus encoding for GFP only, namely CAG-GFP (+ GFP), was injected as control. Mice of both the experimental (+ Sox11) and control (+ GFP) group were perfused 21 days post injection (dpi). GFP expression served as a marker of retrovirally transduced cells.

Immunohistochemical staining for Sox11 was performed to confirm successful Sox11 overexpression (Fig. 2.1 A-B). The number of retrovirally transduced cells identified by GFP expression that additionally expressed Sox11 was quantified and values were given as percentage of all GFP positive cells (Fig. 2.1 G). Only 39.5 ± 12.7 % of transduced neurons in the control group were Sox11 positive. This is in line with the notion that a significant proportion of newborn 21-day-old neurons has exited the immature state characterized by Sox11 expression (Haslinger et al., 2009) and reached a more mature state, characterized by the expression of Calbindin (Jagasia et al., 2009). In contrast, 85.5 ± 5.7 % of CAG-Sox11-IRES-GFP-transduced neurons showed Sox11 immunoreactivity confirming effective Sox11 overexpression ($p = 0.0046$) (Fig. 2.1 G). Immunohistochemical staining for the immature marker

proteins DCX and Calretinin was conducted to further asses maturity status of newborn neurons (Fig. 2.1 C-F). Intriguingly, an elevated number of DCX positive cells in the experimental group was determined (+ Sox11: 91.8 ± 4.0 %, + GFP: 59.0 ± 13.9 %; p = 0.0171) (Fig. 2.1 H). This suggested that newborn neurons of the experimental group were less mature compared to control. Additional evidence for reduced maturity of Sox11 overexpressing cells was provided by the prolonged presence of Calretinin in cells of the experimental group, 53.7 ± 10.1 % of which expressed this immature marker protein compared to only 14.7 ± 10.2 % within the control group (p = 0.0092) (Fig. 2.1 I).

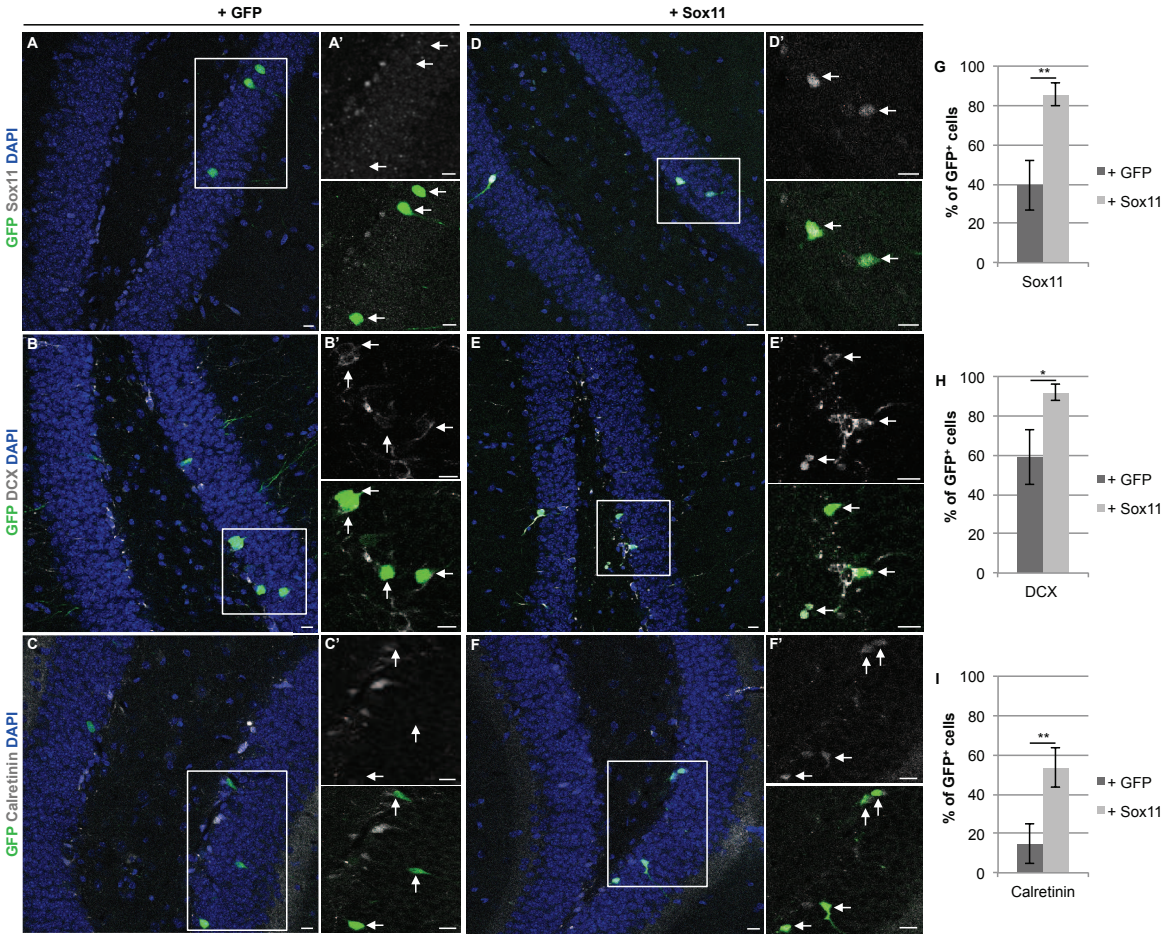


Figure 2.1 Prolonged expression of immature marker proteins 21 days after Sox11 overexpression

A-F Immunohistochemical analysis of mice perfused 21 d after injection of either control virus (+ GFP) (**A**-**C**) or Sox11-overexpressing virus (+ Sox11) (**E**-**F**); retrovirally transduced newborn DG neurons identified by GFP expression were examined with respect to co-expression of Sox11 (**A**,**C**), DCX (**B**,**E**), and Calretinin (**C**,**F**); boxes mark magnified area in **A'**-**F'**. **G-I** Quantification of retrovirally transduced neurons regarding Sox11 (**G**), DCX (**H**), and Calretinin (**I**) expression as percentage of all GFP positive cells depicted effective overexpression of Sox11 and prolonged presence of immature marker proteins DCX and Calretinin. n = 3; scale bar, 10 µm.

Morphology of newborn DG neurons represented the next parameter to be analyzed. For morphological analysis a second retrovirus encoding for the red fluorescent protein (RFP), namely CAG-RFP, was injected together with CAG-Sox11-IRES-RFP or CAG-GFP. This enabled a RFP-based analysis of double-transduced cells excluding methodological errors due to potential different GFP expression level in experimental (+ Sox11) and control (+ GFP) group. Additionally, cells that only expressed RFP served as internal control within the experimental group.

Retroviral birth dating experiments described the presence of two or more basal processes, so-called basal dendrites, as one characteristic of immature DG neurons (Ribak and Shapiro, 2007). Transduced cells of the Sox11 overexpressing group exhibited an average of 1.9 ± 0.1 basal dendrites compared to 1.2 ± 0.2 basal dendrites in the control group ($p = 0.0064$) (Fig. 2.2 D). High resolution images clearly illustrated this phenomenon when comparing either double-transduced cells of experimental to control group (Fig. 2.2 A,A',B,B') or RFP-only- to double-transduced cells within the experimental group (Fig. 2.2 B,B'',C).

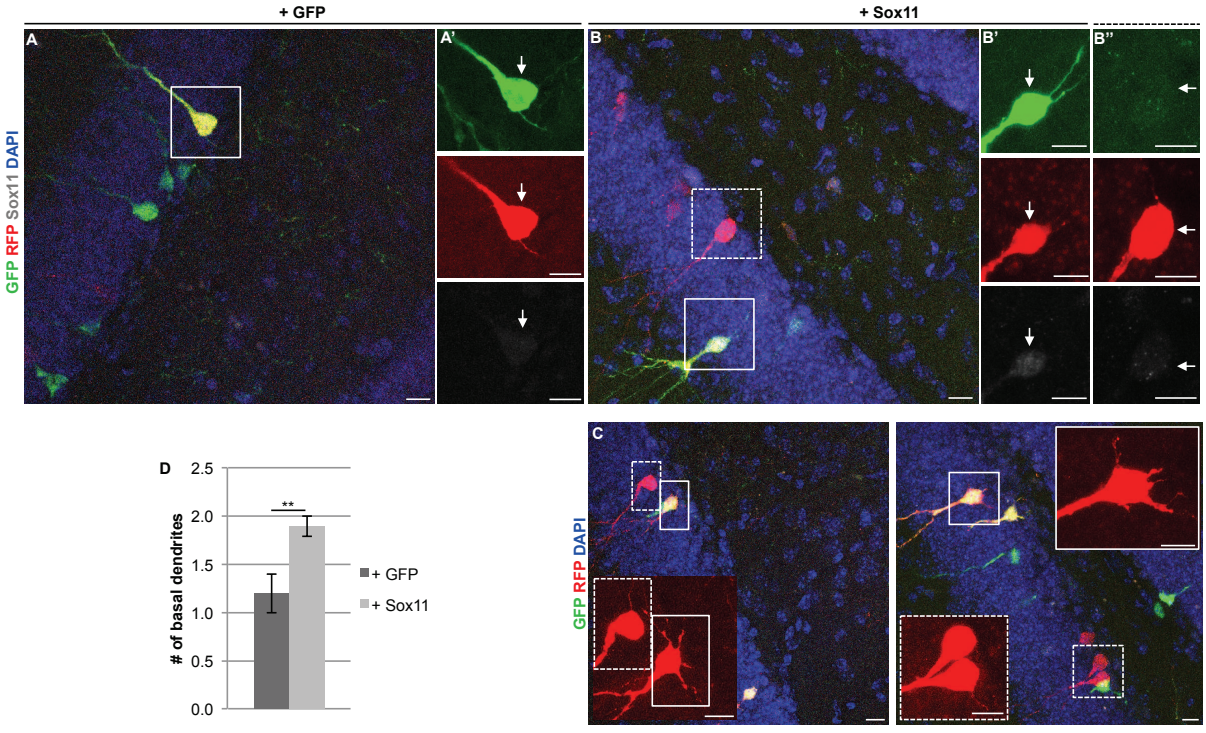


Figure 2.2 Persistence of multiple basal dendrites in 21-day-old newborn DG neurons overexpressing Sox11

A-C High resolution images to detect basal processes of double-transduced Sox11 overexpressing newborn DG neurons (+ Sox11) (**B**,**B'**) compared to either double-transduced control neurons (+ GFP) (**A**,**A'**) or RFP-only-transduced neurons of experimental group (**B''**,**C**). Magnifications of boxes with solid lines for double-transduced cells (**A',B'**) or dashed lines for RFP-only transduced cells (**B'',C**) show more than one basal dendrite in case of Sox11 overexpression. **D** Quantification of number of basal dendrites**.** n = 3; scale bar, 10 µm.

Next, extension of apical dendrites was analyzed. The more mature a newborn DG neuron gets the farther its apical dendrites reach towards the hippocampal fissure, which marks the spatial limit for dendritic outgrowth (Krzisch et al., 2013). Dendritic extension, more precisely the distance from the neuron's soma to the tip of its longest dendrite, was determined relative to the maximum obtainable dendritic length defined as the distance from the soma to the hippocampal fissure (Fig. 2.3 A,B). Dendrites of Sox11 overexpressing neurons were much shorter than those of control neurons as illustrated by the cumulative plot of the relative dendritic extension (Fig. 2.3 C). For instance, only 39.4 ± 8.9 % of CAG-Sox11-IRES-GFP-transduced cells spread their dendrites farther than 60 % of the potential dendritic extension in contrast to 79.7 ± 6.8 % of cells in the control group ($p = 0.0004$). In-depth morphological analysis confirmed the reduced dendritic length in the experimental group by quantifying total dendritic length (+ Sox11: 437.1 ± 172.4 µm, + GFP: 1133.1 ± 136.7 µm, $p = 0.0007$) (Fig. 2.3 E). A trend towards less branching points of Sox11 overexpressing neurons was further detected but did not reach significance (+ Sox11: 7.3 ± 3.2, + GFP: 11.2 ± 2.6, $p = 0.1122$) (Fig. 2.3 F). Finally, Scholl analysis once more emphasized the reduced morphological extension of Sox11 overexpressing compared to control neurons (Fig. 2.3 D).

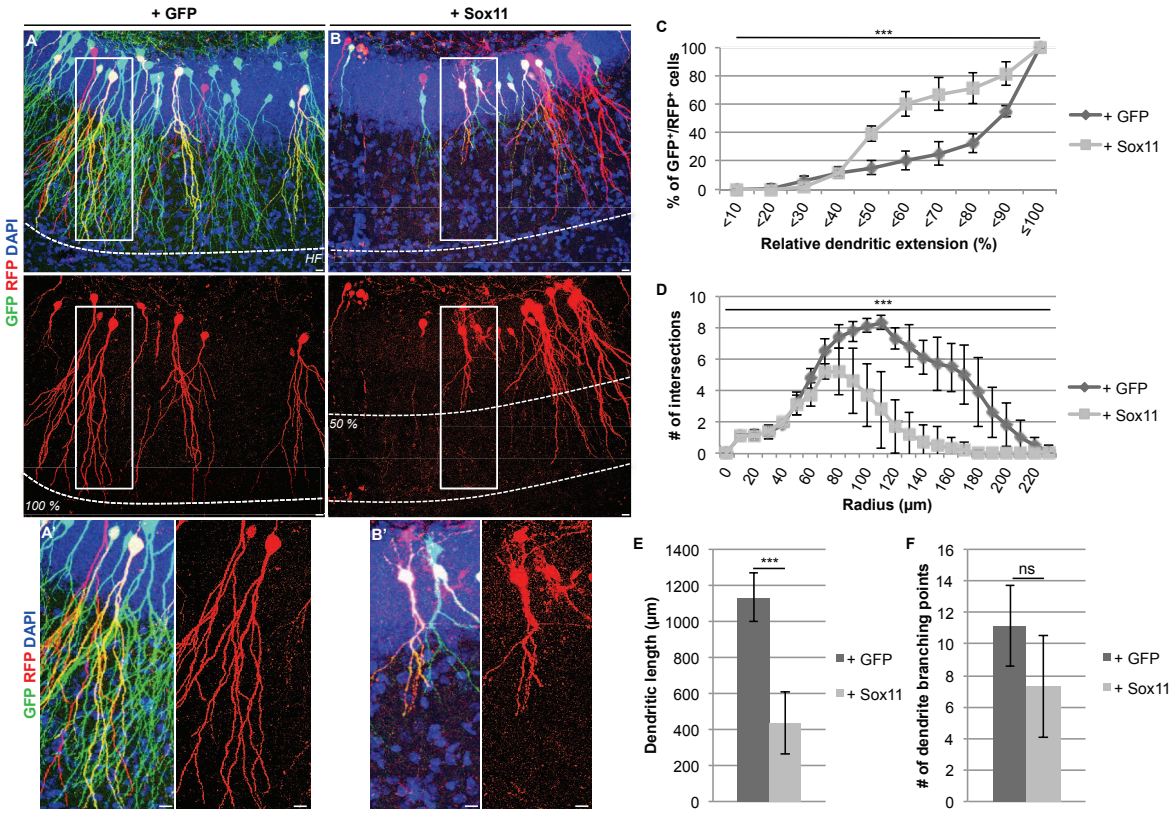


Figure 2.3 Altered morphology of newborn DG neurons following 21-day-lasting Sox11 overexpression

A-B Illustration of morphological analysis of double-transduced newborn DG neurons with CAG-RFP and either CAG-GFP (+ GFP) (**A**) or CAG-Sox11-IRES-GFP (+ Sox11) (**B**) retrovirus 21 dpi; hippocampal fissure (HF) represented maximum achievable dendritic extension; magnified boxes are shown in **A'** and **B'**. **C** Cumulative representation of relative dendritic extension depicted shorter apical dendrites in experimental group (+ Sox11). **D** Scholl analysis after RFP-based cell tracing further illustrated altered morphology due to Sox11 overexpression. **E-F** Quantitative analysis on basis of RFP-based cell tracing revealed reduced dendritic length of Sox11 overexpressing compared to control neurons (**E**) but no significant changes with respect to number of dendritic branching points (**F**). n = 4; scale bar, 10 µm.

Neuronal maturation comprises the establishment of synaptic contacts. Spines developing on the apical dendrites of newborn DG neurons represent the morphological correlate of excitatory glutamatergic synapses. Thus, spine development was used as another morphological indicator for the maturity of newborn neurons and could further provide an indication of their electrophysiological development. Dendrites of Sox11 overexpressing neurons appeared to less frequently have spines (Fig. 2.4 A-C). Indeed, quantification of presence or absence of dendritic spines showed that fewer newborn neurons of the experimental group featured spines 21 days after birth compared to control group (+ Sox11: 34.3 ± 8.7 %, + GFP: 69.8 ± 3.7 %, p = 0.0017) (Fig. 2.4 D).

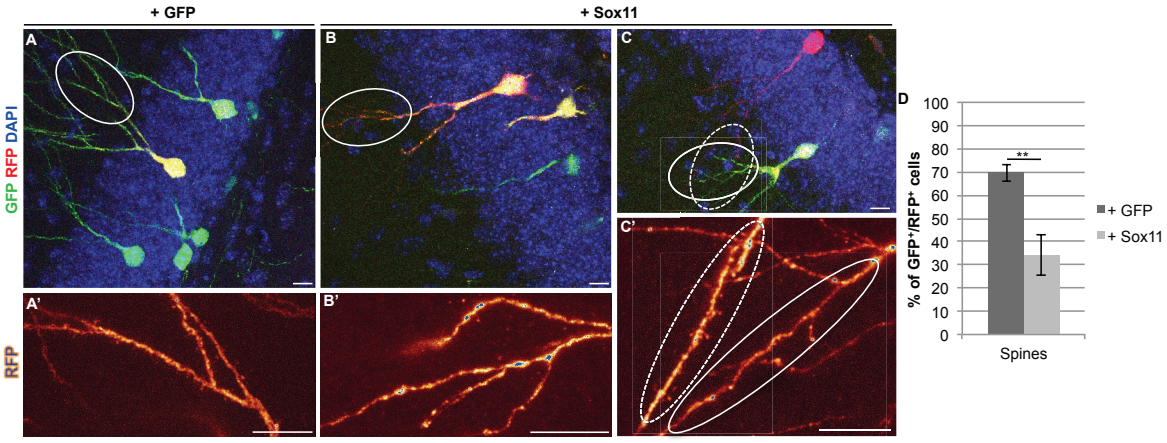


Figure 2.4 Impairment of spine development by Sox11 in 21-day-old newborn DG neurons

A-C Representative illustration of CAG-RFP and either CAG-GFP (+ GFP) (**A**) or CAG-Sox11-IRES-GFP (+ Sox11) (**B**,**C**) double-transduced newborn DG neurons (solid line circle) or RFP-only-transduced neurons (dashed line circle) for qualitative spine analysis; encircled dendrites are magnified in **A'**-**C'**. **D** Quantification of percentage of cells featuring spines in control (+ GFP) versus experimental (+ Sox11) group revealed delayed spine development in Sox11 overexpressing cells. n = 4, heteroscedastic t-test; scale bar, 10 µm.

Data derived from morphological analysis revealed that morphological features of Sox11 overexpressing neurons were different to neurons of control group and resembled those of newborn DG neurons at a more immature stage. Thus, not only prolonged marker protein expression but also altered morphological characteristics of 21-day-old DG neurons conclusively indicated defects in maturation of neurons exposed to Sox11 overexpression .

Since newborn DG neurons migrate during their development, the final parameter to be studied was the cellular positioning of retrovirally transduced neurons within the DG. Quantitative description was achieved by measuring the distance from the soma to the SGZ relative to the total thickness of the GCL. The resulting positioning index can hypothetically encompass a range from ≤ 1.0 to ≥ 0.0 indicating that somata resided in the outer part of the GCL (i.e. close to the MCL) or in the SGZ (i.e. close to the hilus), respectively (Fig. 2.5 A,B). Cumulative representation depicted a tendency towards more Sox11 overexpressing neurons located more distant to the SGZ (Fig. 2.5 C). For example, 16.5 ± 6.7 % of CAG-Sox11-IRES-GFP-transduced cells compared to only 5.5 ± 7.7 % of control cells resided in the outer GCL (positioning index > 0.5) ($p = 0.1359$).

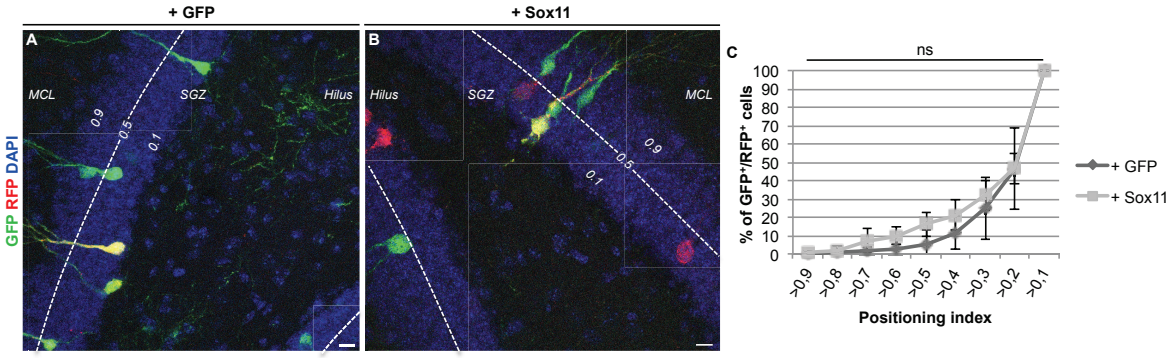


Figure 2.5 Studying the effect of Sox11 overexpression on cellular positioning of newborn neurons 21 days post injection

A-B Illustration of positioning analysis by calculation of positioning index (≤ 1.0 to ≥ 0.0) of double-transduced newborn DG neurons (CAG-RFP + CAG-GFP (**A**) or + CAG-Sox11-IRES-GFP (**B**) retrovirus) depending on relative location of somata between subgranular zone (SGZ) and molecular cell layer (MCL). **C** Cumulative representation of positioning index 21 dpi depicted a trend towards more Sox11 overexpressing neurons residing within the outer part of the GCL. n = 3; scale bar, 10 µm.

In summary, biochemical and morphological data collected for the 21 dpi paradigm suggest that the precise regulation of Sox11 protein expression is crucial for proper maturation of newborn granule neurons in the adult DG. Failed downregulation or increased Sox11 level resulted in a delayed progression of neuronal development including persistence of immature marker protein expression and basal dendrites as well as reduction of morphological extension and impaired spine development.

2.1.2 6-week-old newborn DG neurons

The next question to be addressed was the effect of an even longer Sox11 overexpression lasting for 6 weeks after neuronal birth. The experimental procedure was the same as described in 2.2.1 with the exception that mice were perfused not 21 days but 6 weeks post injection (wpi) or in case of electrophysiological recordings 5 wpi.

First, newborn DG neurons were biochemically characterized by evaluation of the expression of Sox11 and immature marker proteins including DCX, Calretinin, NeuroD, and PSA-NCAM (Fig. 2.6). Effective Sox11 overexpression was confirmed: 74.8 ± 2.8 % of cells transduced with the Sox11-encoding retrovirus CAG-Sox11-IRES-GFP (+ Sox11) were Sox11 immunoreactive in contrast to only 2.5 ± 1.7 % of control cells transduced with the CAG-GFP retrovirus (+ GFP) ($p = 0.0000$) (Fig. 2.6 A,F,K). The fraction of DCX positive neurons was strongly

increased in the Sox11 overexpressing group (+ Sox11: 63.3 ± 4.5 %, + GFP: 3.3 ± 5.8 %, p = 0.0001) (Fig. 2.6 B,G,L). In contrast, none of the other immature marker proteins was upregulated (Fig. 2.6 C-E,H-J,M-O).

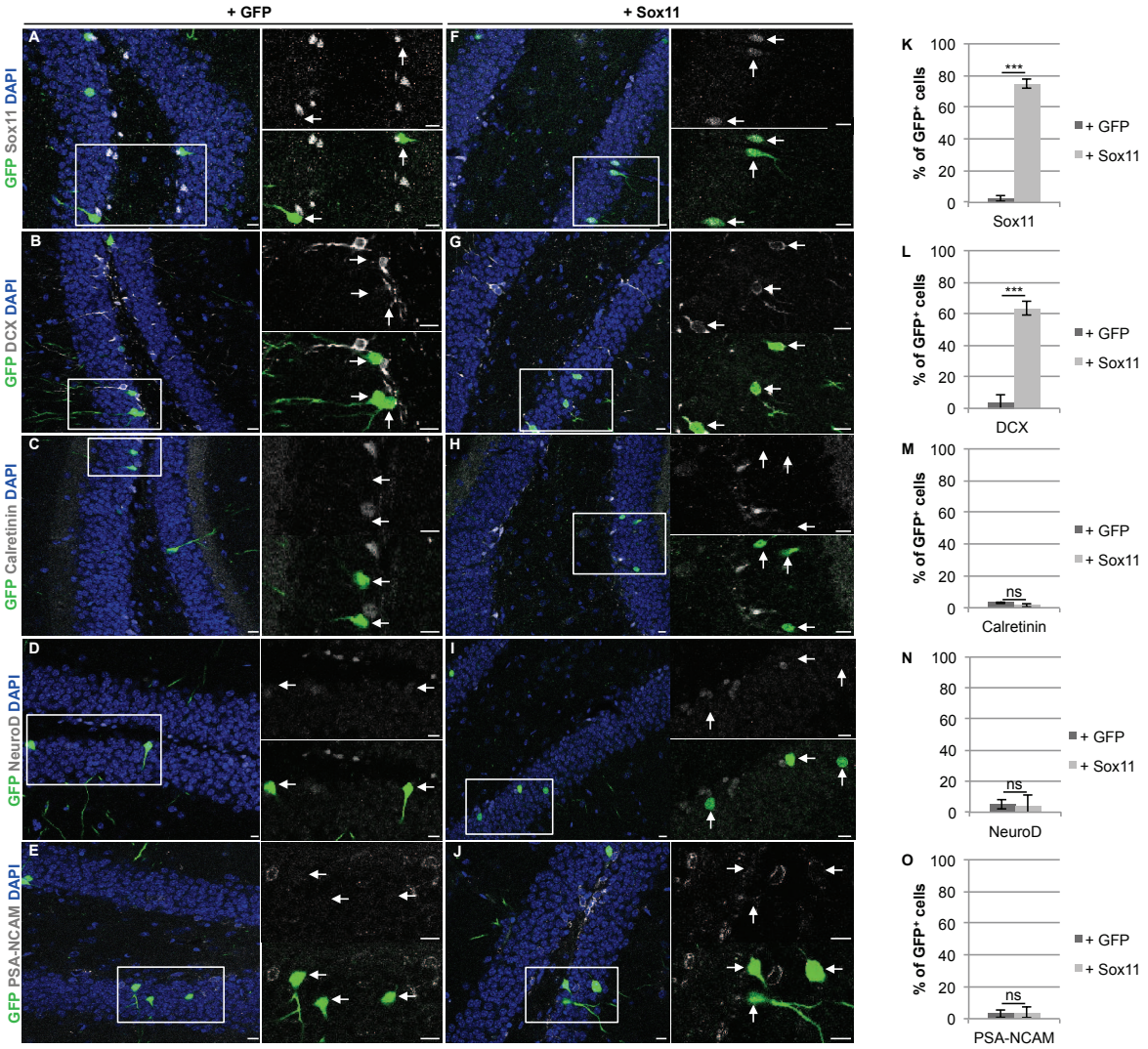


Figure 2.6 Effective Sox11 overexpression 6 weeks post injection along with increased DCX but not other immature marker protein expression

A-J Immunohistochemical analysis of mice perfused 6 wpi of either control retrovirus (+ GFP) (**A**-**E**) or Sox11-encoding (+ Sox11) (**F**-**J**); retrovirally transduced newborn DG neurons identified by GFP expression were examined with respect to co-expression of Sox11 (**A**,**F**), DCX (**B**,**G**), Calretinin (**C**,**H**), NeuroD (**D**,**I**), and PSA-NCAM (**E**,**J**); boxes mark magnified area in **A'**-**J'**. **K-O** Quantification of retrovirally transduced neurons regarding Sox11 (**K**), DCX (**L**), Calretinin (**M**), NeuroD (**N**), and PSA-NCAM (**O**) expression as percentage of all GFP positive cells revealed effective overexpression of Sox11 and prolonged presence of DCX but not other immature marker proteins. n = 3; scale bar, 10 µm.

For subsequent morphological analysis mice injected with CAG-RFP retrovirus or CAG-mtdsRed retrovirus, which encodes mitochondrially targeted RFP variant, together with either CAG-Sox11-IRES-GFP (+ Sox11) or CAG-GFP (+ GFP) were used as described in 2.2.1.

No differences with respect to number of basal dendrites were observed: Virtually no neurons of both groups featured additional basal processes (Fig. 2.7 C). Likewise, no significant changes were observed for dendritic length (+ Sox11: 1422.8 ± 331.7 µm, + GFP: 1506.4 ± 146.2, p = 0.6199) and number of branching points (+ Sox11: 10.0 ± 1.4, + GFP: 9.4 ± 2.1, p = 0.5941) (Fig. 2.7 D,E). In case of branching characteristics, which were determined by Scholl analysis, significant changes were detected (Fig. 2.7 F): Sox11 overexpressing neurons featured more primary apical dendrites and dendrites that branched closer to the soma as indicated by the increased number of Scholl intersections at a distance of 0-90 µm from the soma (e.g. at 90 µm + Sox11: 9.4 ± 1.3, + GFP: 6.9 ± 0.6, p = 0.0047). Furthermore, number of Scholl intersections was smaller at greater distances from the soma between both groups (e.g. at 190 µm + Sox11: 3.0 ± 2.8, + GFP: 7.2 ± 1.4, p = 0.0173) suggesting less branching complexity in this region of Sox11 overexpressing neurons.

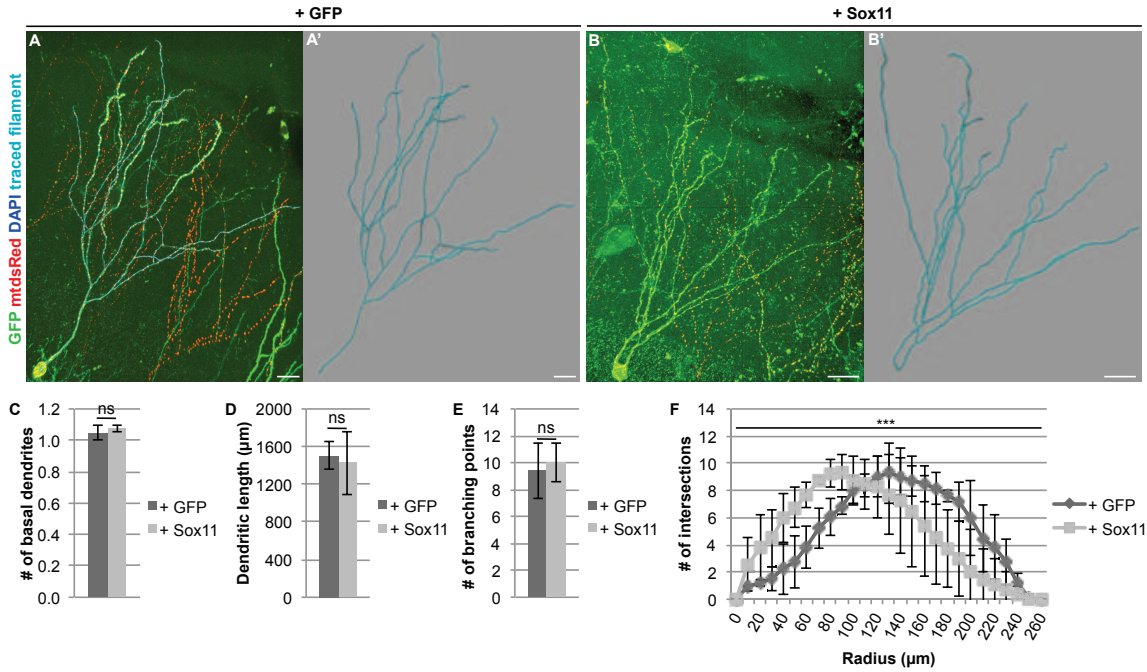


Figure 2.7 Altered branching characteristics of newborn DG neurons due to 6-week-lasting Sox11 overexpression

A-B Exemplary illustration of morphology of 6-week-old newborn DG neurons transduced with CAG-RFP and either CAG-GFP (+ GFP) (**A**) or CAG-Sox11-IRES-GFP (+ Sox11) (**B**) retrovirus together with corresponding traced filament (**A'**,**B'**). **C-E** Quantification of neither number of basal dendrites (**C**), nor dendritic length (**D**), nor number of dendritic branching points (**E**) revealed significant changes. **F** Scholl analysis showed altered branching characteristic of Sox11 overexpressing neurons featuring more intersections near to and less intersections farther away from the soma. n = 5 for all experiments except for quantification of number of basal dendrites, n = 3; scale bar, 10 µm.

Unfortunately, the expression of both GFP and RFP negatively correlated with Sox11 overexpression, i.e. neurons with high Sox11 protein level exhibited only very low level of fluorophores resulting in a very weak signal at the microscope. Neither immunohistochemical staining nor higher laser intensity could compensate this phenomenon. Thus, detailed spine analysis of number and type of spines was not feasible. The only statement that can be made so far is that, if visible, both filopodia-like and mushroom spines could be detected on dendrites of transduced cells of the experimental and the control group. Especially spine characteristics such as the number of mushroom spines were supposed to provide a hint if Sox11 could influence synaptic connectivity and integration newborn neurons. To determine the development of electrophysiological properties, recordings of retrovirally transduced cells were performed by our collaborators G. D. Sala and Dr. A. Schinder, Buenos Aires. Different electrophysiological parameters were determined including input resistance, action potential half width, peak amplitude of the first elicited spike, and number of action potentials elicited by injected current steps (Fig. 2.8 A-D). In contrast to CAG-GFP-transduced neurons, Sox11 overexpressing neurons behaved in all recordings not appropriate to their age of 5 weeks but rather like 22-day-old neurons (cf. (Mongiat et al., 2009)). For instance, input resistance added up to 1065.8 ± 192.5 GΩ in CAG-Sox11-IRES-GFP-transduced cells versus 492.4 ± 42.8 GΩ in control cells (mean ± SEM, $p = 0.0035$) (Fig. 2.8 A) and spike amplitude was reduced to 103.4 ± 1.6 mV in the experimental compared to 113.4 ± 1.0 mV in the control group (mean ± SEM, $p = 0.0000$) (Fig. 2.8 C). Thus, electrophysiological recordings clearly assigned defects in maturation to Sox11 overexpressing neurons.

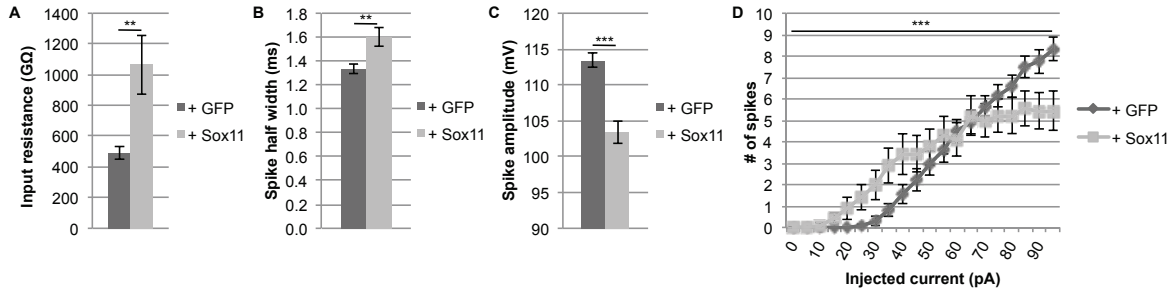


Figure 2.8 Inappropriate electrophysiological development of Sox11 overexpressing newborn DG neurons

A-D Measurement of input resistance (**A**), spike half width (**B**), and spike amplitude (**C**) as well as quantification of number of spikes while stepwise increasing injected current (**D**) revealed an electrophysiological status of 5-week-old newborn DG neurons in the experimental group injected with CAG-Sox11-IRES-GFP retrovirus (+ Sox11) that resembled the status of approximately 3-week-old neurons; contrary, age-adequate electrophysiological behavior of control neurons retrovirally transduced with CAG-GFP (+ GFP) was detected (cf. (Mongiat et al., 2009)). n = 13 (+ GFP) and n = 10 (+ Sox11) for input resistance measurement, n = 12 (+ GFP) and n = 9 (+ Sox11) for spike half width and amplitude measurements, n = 12 (+ GFP) and n = 9 (+ Sox11) for quantification of number of spikes; values are given as mean ± SEM.

Finally, cellular positioning was investigated analogously to the 21 dpi paradigm (see 2.1.1). Already during preceding analyses the impression arose that newborn DG neurons of the experimental group (+ Sox11) were mispositioned within in the GCL, since somata of these cells seemed to have migrated farther into the GCL towards the MCL. To quantitatively comprehend this notion the positioning index, which ranged between ≤ 1.0 to ≥ 0.0 with higher indices indicating that somata were located closer to the MCL, was evaluated (Fig. 2.9 A,B) as described in 2.2.1. The mispositioning of Sox11 overexpressing neurons towards the MCL was highly significant and clearly depicted by the cumulative representation of the positioning index (Fig. 2.9 C). For instance, 51.4 ± 2.5 % of CAG-Sox11-IRES-GFP-transduced cells resided in the outer GCL (positioning index > 0.5) whereas only 5.6 ± 4.8 % of control cells were detected within this region ($p = 0.0001$).

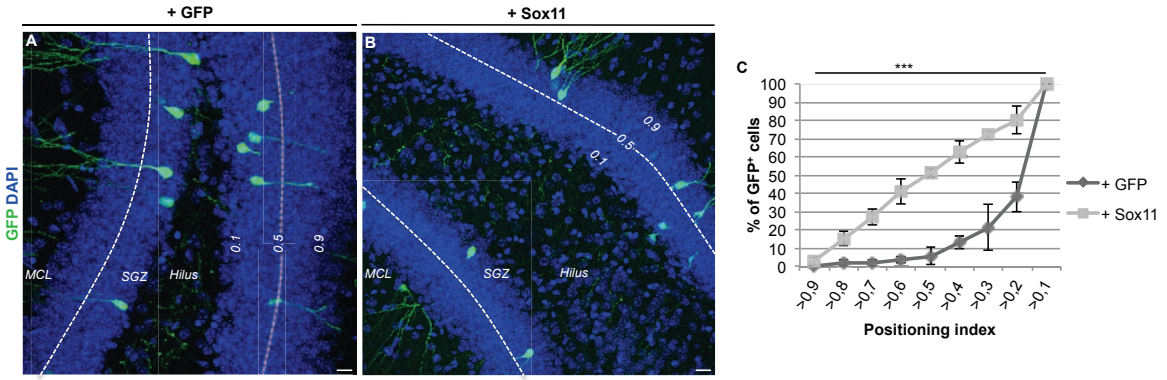


Figure 2.9 Cellular mispositioning induced by overexpression of Sox11 in 6-week-old newborn DG neurons

A-B Illustration of positioning analysis: Positioning index (≤ 1.0 to ≥ 0.0) of retrovirally transduced newborn DG neurons of control group injected with CAG-GFP retrovirus (+ GFP) (**A**) and experimental group injected with CAG-Sox11-IRES-GFP retrovirus (+ Sox11) (**B**) was calculated depending on relative location of somata between subgranular zone (SGZ) and molecular cell layer (MCL). **C** Cumulative representation of determined positioning indices 6 wpi revealed that Sox11 overexpressing neurons resided in closer vicinity to the MCL. n = 3; scale bar, 10 µm.

In summary, data collected for the 6 wpi paradigm indicate that persistent Sox11 expression negatively influences maturation and integration of newborn granule neurons in the adult DG. Sox11 caused misexpression of the immature marker protein DCX, morphological alterations concerning branching characteristics, delayed development with respect to electrophysiological parameters, and migratory abnormalities as displayed by cellular mispositioning.

2.1.3 Mature adult-born DG neurons

So far, experimental paradigms were designed in such a way that Sox11 overexpression lasted from neuronal birth until the age of 21 days (2.2.1) or 6 weeks (2.2.2). Thus, phenotypes might partially be due to early developmental modifications of newborn DG neurons by Sox11 biasing later outcomes. The purpose of the following experiments was to determine the effects of Sox11 expression specifically in mature adult-born DG neurons. To this end, a combined retrovirus / adeno-associated virus (AAV) strategy was pursued (Fig. 2.10 C): CAG-RFP retrovirus was injected to birth-date and mark newborn neurons that were subsequently allowed to fully mature for 8 weeks. 8 weeks after retroviral labeling, AAVs encoding either for Sox11 (AAV-Sox11) or for GFP (AAV-GFP) were stereotactically injected. Mice were perfused 10 days later (8 wpi + 10 dpi). Double-transduced cells of either experimental group (+ Sox11) or control group (+ GFP) that featured RFP together

with Sox11 or GFP expression were chosen for analysis. Those cells represented adult-born DG neurons that overexpressed Sox11 or GFP not until they had reached a fully mature state.

Prior to double injections the efficacy of adeno-associated viral Sox11 overexpression was determined by single injections of either AAV-Sox11 or empty adeno-associated viral particles (AAV-empty). An increase in Sox11 immunoreactivity in the GCL of AAV-Sox11- (+ Sox11) but not AAV-empty- (+ empty) injected mice could be visualized after immunohistochemical treatment. Some cells with a strong Sox11 signal could be detected in the GCL. The majority of DG cells featured lower levels compared to Sox11 expression in the SGZ (Fig. 2.10 A,B). Thus, a fair amount of granule neurons in the GCL expressed Sox11 following stereotactic injection of AAV-Sox11. Further confirmation of adeno-associated viral transduction was provided by the fact that Sox11 immunoreactive cells were detected in the hilus of AAV-Sox11- but not AAV-empty-injected mice, a region, which is normally devoid of Sox11 expression.

Analysis of DCX expression in double-transduced cells revealed only a minor impact of AAV-mediated Sox11 overexpression on the number of DCX expressing cells: Slightly, though not significantly, more DCX positive RFP-labeled adult-born neurons could be found in the experimental compared to control group (+ Sox11: 27.7 ± 13.5 %, + GFP: 8.5 ± 9.3 %, $p = 0.1122$) (Fig. 2.10 D,E). But due to extremely strong RFP signal, which resulted in bleed-through into the channel used to visualize DCX, this analysis may have included some false positives. Thus, Sox11 expression in mature adult-born DG neurons appears not to be sufficient to efficiently reactivate DCX expression. Whether this failure to activate DCX is the result of low level expression of Sox11 or of modifications to the DCX locus in mature DG neurons, which prevent its activation, cannot be distinguished in this experiment.

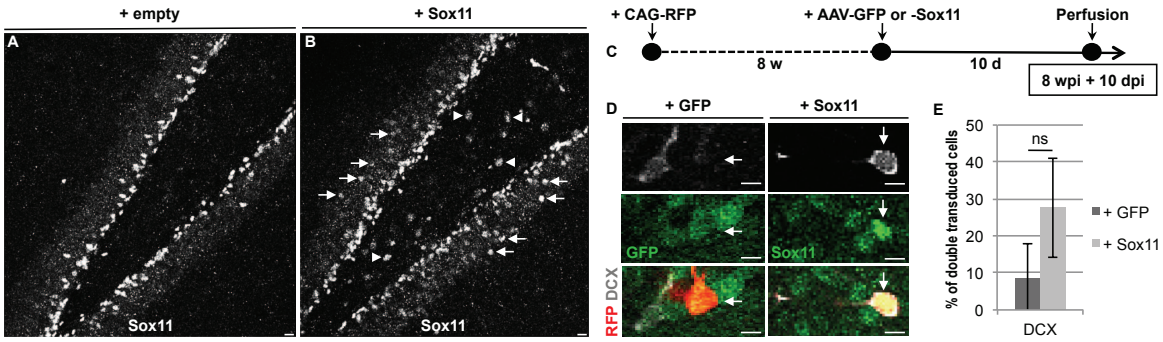


Figure 2.10 Sufficient ectopic Sox11 expression by adeno-associated viral transduction in DG neurons

A-B Immunohistochemical analysis of Sox11 expression 10 days after injection of AAV-empty (+ empty) (**A**) or AAV-Sox11 (+ Sox11) (**B**) demonstrated that ectopic expression of Sox11 in the GCL is, even though at low level, feasible by adeno-associated viral transduction (arrows exemplarily point out DG neurons featuring high Sox11 immunoreactivity outside of the SGZ); note also Sox11 positive cells in the hilus of AAV-Sox11-injected mice (arrowheads). **C** Paradigm of combined retro- and adeno-associated viral approach to ectopically overexpress Sox11 in mature adult-born DG neurons. **D** Determination of DCX expression revealed by trend more CAG-RFP- and AAV-Sox11-double-transduced (+ Sox11) cells being DCX positive compared to control cells (+ GFP), which was quantified as percentage of all double-transduced cells in **E**. n = 3; scale bar, 10 µm.

In addition, analysis of morphological parameters including dendritic length, number of dendritic branching points, and Scholl intersections was performed. No significant changes were detected for any investigated parameter (Fig. 2.11). The same prove true for number and type of spines, both of which were unaffected by adeno-associated viral-mediated Sox11 overexpression (Fig. 2.11 H,I). Since morphological and spine analysis relied on a strong RFP signal, cells were chosen according to this criterion. But the problem that strong Sox11 expression correlated with low RFP levels, as described above (see 2.1.2), possibly biased the analysis towards cells with low Sox11 protein level thereby potentially missing less pronounced phenotypes. Thus, these data should be considered preliminary. Further analysis will be required to finally answer the question whether Sox11 can influence morphology and spine architecture in mature adult-born DG neurons.

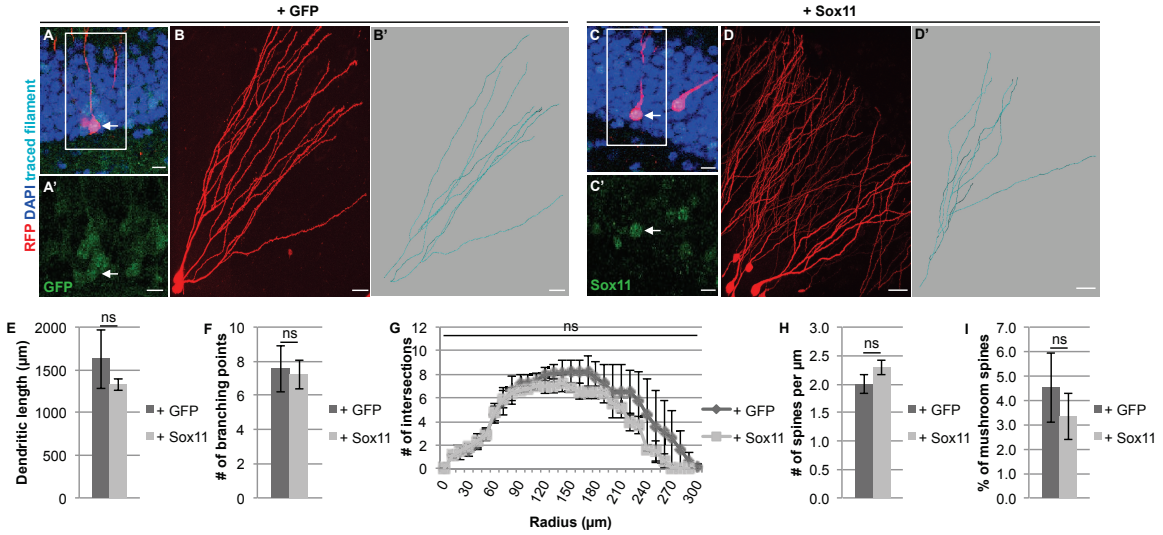


Figure 2.11 No impact of Sox11 overexpression on morphology and spines of mature adult-born DG neurons

A-B Example of mature adult-born DG neurons double-transduced with CAG-RFP retrovirus and either AAV-GFP (+ GFP) (**A**,**B**) or AAV-Sox11 (+ Sox11) (**C**,**D**) adeno-associated virus together with corresponding traced filaments (**B'**,**D'**) used for RFP-based morphological and spine analysis. **E-I** Quantitative analysis revealed no significant differences between neurons ectopically expressing Sox11 and control neurons with respect to dendritic length (**E**), number of branching points (**F**), branching characteristics determined by Scholl analysis (**G**), number of spines (**H**), and fraction of mushroom spine (**I**). n = 3; scale bar, 10 µm.

Quantification and cumulative representation of the positioning index (procedure described in 2.2.1) revealed that Sox11 overexpressing mature adult-born DG neurons were located further outside in the GCL than control neurons (Fig. 2.12 A,B,C). For instance, 38.8 ± 4.1 % of CAG-Sox11-IRES-GFP-transduced cells resided in the outer two thirds of the GCL (positioning index > 0.3) compared to only 21.2 ± 6.9 % of control cells ($p = 0.0192$). Strikingly, some transduced cells that expressed Sox11 could even be found outside the GCL in the MCL (Fig. 2.12 D,E). The morphology of those cells indicated a non-glial but neuronal nature (Fig. 2.12 D), which was further confirmed by the co-expression of Prox1, a characteristic neuronal marker protein of the DG (Fig. 2.12 E).

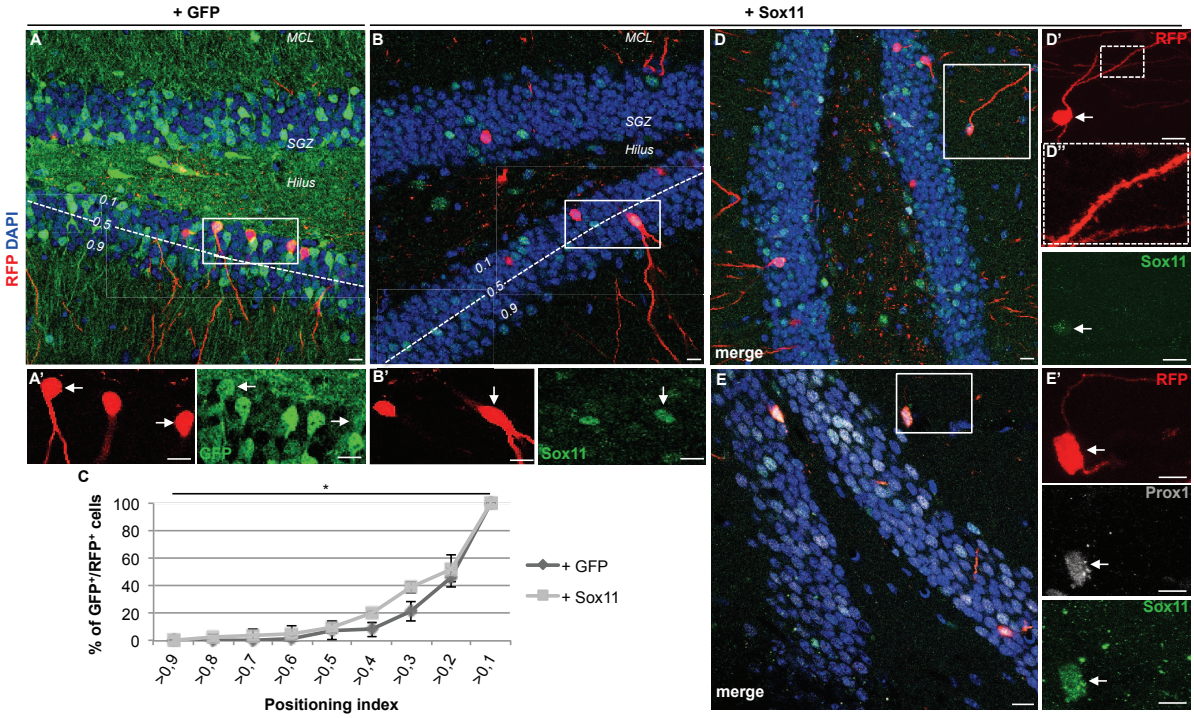


Figure 2.12 Mispositioning of mature adult-born DG neurons induced by Sox11

A-B Illustration of positioning analysis: Positioning index (≤ 1.0 to ≥ 0.0) of mature adult-born DG neurons double-transduced with CAG-RFP retrovirus and either AAV-GFP (+ GFP) (**A**) or AAV-Sox11 (+ Sox11) (**B**,**D**) adeno-associated virus was calculated depending on relative location of somata between subgranular zone (SGZ) and molecular cell layer (MCL); neurons were chosen for analysis on basis of successful double-transduction as depicted in magnified boxes (**A'**,**B'**). **C** Cumulative representation of determined positioning indices 8 wpi + 10 dpi revealed that Sox11 overexpressing neurons resided in closer vicinity to the MCL. **D-E** Example of mature adult-born granule neurons residing in the MCL; neuronal, not glial, nature was asserted either by visualizing morphology and demonstrating the existence of spines (**D**, magnification **D'** and **D''**, respectively) or by detected co-expression of Prox1 (**E**, magnification **E'**). n = 3; scale bar, 10 µm.

In summary, data derived from this combined retroviral and adeno-associated viral approach suggest that Sox11 overexpression can induce migration of mature adult-born DG neurons towards the outer part of the GCL and even beyond into the MCL. To determine the effect of Sox11 on the structure of dendrites and spines of these neurons further detailed analysis is required.

2.2 Potential Sox11 downstream targets

Previous results and results from this study demonstrate a crucial function for Sox11 in adult hippocampal neurogenesis. So far, only few Sox11 target genes are known in this cellular context. Thus, the second part of this study focused on the identification of genes potentially regulated by Sox11. Since Sox proteins can bind DNA at sequences that only partially match the consensus sequence, potential imperfect Sox binding sites are abundant and can be found throughout the genome

at high frequency (Lefebvre et al., 2007). Thus, identification of potential downstream targets cannot be accomplished simply by prediction of Sox binding sites. Genes to be analyzed in this study were therefore chosen on the basis of different facts as described separately in respective chapters. The starting point of each analysis was to determine mRNA level in neural progenitor cell cultures derived from hippocampi of cKO Sox4/11 mice by qRT-PCR after conditional knockout of Sox4/11 (Fig. 2.13 B). Cell cultures derived from conditional knockout mice not only of Sox11 but also of Sox4 were used to prevent compensating effects by potential redundant activity of Sox4 as described in 1.2.2. Treatment of cKO Sox4/11 monolayer with a recombinant Cre protein (HTN-Cre), which is cell permeable and translocates into the nucleus, resulted in a very efficient knockout of Sox11 (see individual experiments). The optimal HTN-Cre concentration (0.5 µM), which displayed minimal toxic or unspecific side effects in wild type (wt) control monolayer and induced sufficient recombination in cKO Sox4/11 monolayer, was initially determined.

2.2.1 Delta like ligand 1

Delta like ligand 1 (Dll1) represents one of several Notch receptor ligands that induce upon binding the cleavage of the Notch receptor thereby releasing the Notch intracellular domain (NICD) that in turn activates Notch target genes (Kadesch, 2004). Since Notch signaling is strongly implicated in neural development (Pierfelice et al., 2011) and, more specifically, in the regulation of adult neurogenesis including stem cell maintenance, neuronal fate decision, and migration (Ables et al., 2011; Imayoshi and Kageyama, 2011), certain Notch ligands might as well play an important role within the neurogenic process for example by providing feedback to neighboring cells. This concept of lateral inhibition assumes that cells featuring Notch ligands can modulate the genetic program of adjacent cells by activation of Notch signaling in these cells. Translating this idea onto neurogenic processes would imply that Notch ligand expressing neuronal progenitors regulate for example the maintenance or fate choice of adjacent neural stem cells (Kiparissides et al., 2011; Wang et al., 2011b). Several studies previously described this mechanism in other developmental contexts: Jag1 and even more strongly Dll1 were shown to control neurogenesis in the mammalian spinal cord (Ramos et al., 2010). Repression of the expression of the proneuronal gene *Mash1* through a Dll1/Notch/Hes1 pathway was further suggested to negatively modulate neuronal differentiation (Kiparissides et al., 2011). Finally, a lateral inhibition-like mechanism, in which neuroblast derived signals

inhibit the proliferative activity of neighboring NSCs, was identified in the adult zebrafish brain, which is potentially mediated by the zebrafish ortholog of mammalian Dll1, DeltaA (Chapouton et al., 2010). Since several studies implicated Dll1-mediated lateral inhibition in neural development, it might be interesting to apply this concept also to adult hippocampal neurogenesis. Furthermore, Notch signaling was reported to be important for proper neuronal maturation in the adult brain including neuronal migration (Hashimoto-Torii et al., 2008) and dendritic growth (Ables et al., 2010). Studies focusing on the adult HC revealed that Notch signaling is required for maintenance of adult hippocampal NSCs (Ehm et al., 2010; Lugert et al., 2010), that it can regulate cell fate and dendrite morphology in the postnatal DG (Breunig et al., 2007), and that it is involved in hippocampal synaptic plasticity (Alberi et al., 2011; Wang et al., 2004b), which makes it very appealing to study the role of Notch signaling and more specifically of Notch ligands, e.g., Dll1, within the process of neuronal development in the adult DG. *Dll1* was shown to be a direct transcriptional target of Sox4 in prostate cancer cells (Scharer et al., 2009) providing an indication that *Dll1* might be regulated by SoxC proteins. Since a transient expression pattern of *Dll1* resembling the transient expression of Sox11 was detected in differentiating neural monolayer cultures *in vitro* (Fig. 2.13 A), *Dll1* was the first candidate target gene of Sox11 to be studied.

cKO Sox4/11 monolayer treated with HTN-Cre (+ Cre) revealed a slight decrease in *Dll1* mRNA level (reduced to 0.7 ± 0.3 fold of control level, p = 0.1475) accompanying a drastic reduction of *Sox11* level (reduced to 0.0 ± 0.1 fold of control level, p = 0.0000) relative to control monolayer (- Cre), which were solely treated with the solvent of HTN-Cre (Fig. 2.13 B,C). Application of HTN-Cre to wt monolayer resulted in no significant decrease of either *Sox11* or *Dll1* level (data not shown) excluding unspecific effects of HTN-Cre on mRNA transcription *per se*. Luciferase assays using a *Dll1*-promoter driven luciferase construct, which features a 4.3 kb DNA fragment upstream of the *Dll1* transcriptional start site (TSS) (Dll1-4.3p::luc, kindly provided by PhD J. Breunig (Los Angeles)), revealed that Sox11 was able to induce an increase of *Dll1*-promoter driven luciferase activity (2.5 ± 0.0 fold, p = 0.0226) whereas Sox2, which served as a negative control, was not (0.8 ± 0.3 fold, p = 0.6349) (Fig. 2.13 D).

The observation that *Dll1* mRNA was only slightly reduced following ablation of Sox4/11 might be due to the fact that additional TFs are required for Dll1 expression. Since several studies implicated Mash1 in the regulation of *Dll1* (Casarosa et al.,

1999; Ito et al., 2000; Nelson and Reh, 2008), a possible synergistic activation of *Dll1* transcription by Sox11 together with Mash1 was examined. *In vitro* evidence for this hypothesis were provided by luciferase assays using the Dll1-4.3p::luc construct. Increasing amounts of Mash1 plasmid together with a luciferase construct resulted in a dose-dependent induction of luciferase activity (Fig. 2.13 E). Luciferase activity induction by Mash1 could be further augmented by co-transfection of Sox11 plasmid (5.6 ± 0.5 fold, p = 0.0001) but not of Sox2 plasmid, which in contrast reduced luciferase activity (0.5 ± 0.0 fold, p = 0.0000) (Fig. 2.13 F). Finally, application of increasing amounts of Sox11 together with Mash1 revealed a dose dependency for the synergistic effect of Sox11 and Mash1 on activation of the *Dll1* promoter (Fig. 2.13 G).

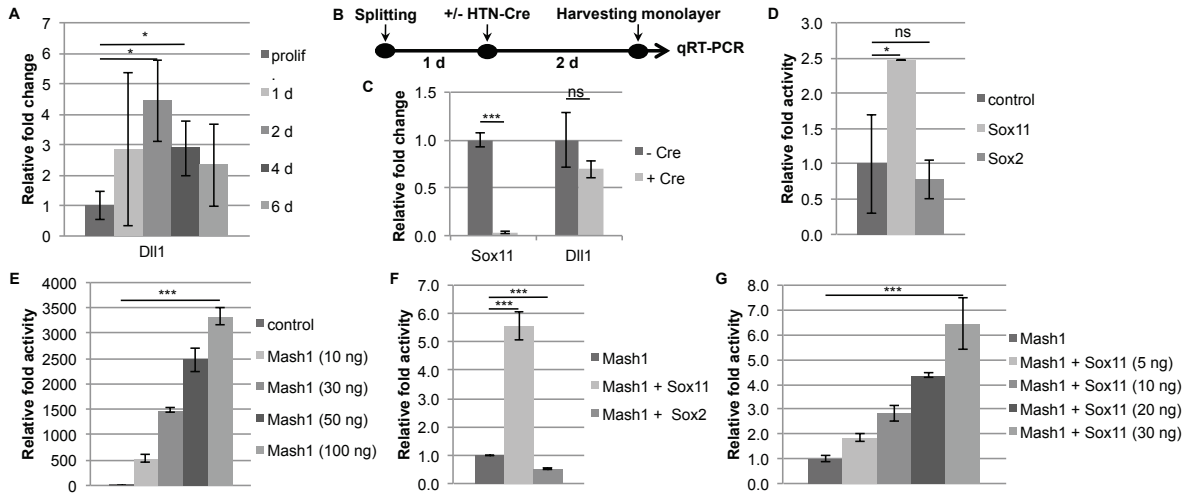


Figure 2.13 *In vitro* regulation of *Dll1* transcription by Sox11

A Analysis of *Dll1* mRNA level in cKO Sox4/11 neural monolayer cultures under proliferating conditions (prolif) and after differentiation for 1, 2, 4, and 6 days (1, 2, 4, 6 d) by qRT-PCR revealed a transient *Dll1* mRNA course. **B** Experimental paradigm illustrating conditional knockout of Sox4 and Sox11 in cKO Sox4/11 neural monolayer cultures with subsequent analysis of mRNA level by qRT-PCR. **C** Quantification of fold change of *Sox11* and *Dll1* mRNA level in HTN-Cre treated (+ Cre) relative to control (- Cre) monolayer depicted drastic reduction of *Sox11* mRNA accompanied by slight but not significant decrease of *Dll1* level. **D-G** Luciferase assays using a *Dll1* promoter driven luc-construct (Dll1-4.3p::luc) evaluated as relative fold activity of luciferase: Significantly increased luciferase activity after Sox11 but not Sox2 transfection revealed an induction of *Dll1* promoter driven luciferase expression by Sox11 (**D**); Dose-dependent activation of Dll1-4.3p::luc by Mash1 (**E**); Synergistic activation of *Dll1* promoter by Mash1 and Sox11 but not Sox2 as depicted by higher luciferase activity in case of co-transfection of Mash1 and Sox11 (**F**,**G**); n = 3.

So far, immunohistochemical analysis of Dll1 has not been feasible since staining with Dll1 antibody did not result in a reproducible and sufficiently strong signal. Biotin-streptavidin mediated signal amplification lead to an unspecific staining. Thus, *in vivo* analysis of Dll1 expression could not be performed within this study.

In summary, first clues for a potential regulation of *Dll1* by Sox11 were provided. Further experiments have to clarify the involvement of Mash1 and whether Sox11 influences Dll1 expression by a direct or indirect mechanism of action.

2.2.2 Nuclear factor IA

Nuclear factor IA (NFIA) belongs to the nuclear factor one (NFI) family of TFs. NFI mRNA and protein are dynamically expressed in the developing and postnatal mouse brain (Chaudhry et al., 1997; Plachez et al., 2008). Knockout mice display a severe brain phenotype, e.g., agenesis of the corpus callosum, enlarged ventricles, or, in case of NFIA knockout, failed formation of the hippocampal commissure (Mason et al., 2009). NFI proteins were implicated in multiple aspects of embryonic neural development (Mason et al., 2009) including progenitor self-renewal (Piper et al., 2010), fate determination (Namihira et al., 2009), gliogenesis (Deneen et al., 2006; Kang et al., 2012), migration (Deneen et al., 2006; Shu et al., 2003; Wang et al., 2007) as well as neuronal maturation (Kilpatrick et al., 2012). For instance, Wang and colleagues applied a NFI dominant repressor to competitively inhibit NFIA activity. This resulted in impaired axonal outgrowth and dendrite formation of cerebellar granule neurons (CGNs) suggesting a delay in postmitotic CGN maturation (Wang et al., 2007). The function of the NFI TF family in adult neurogenesis has not been investigated so far. Lately, the expression of NFIA was detected in progenitor cells, migrating neuroblasts, and astrocytes in the adult SVZ, RMS and OB (Plachez et al., 2012). The expression pattern of NFIA in the second neurogenic niche of the adult brain, the DG, was recently determined in our laboratory (Schwarz, 2013). NFIA expression in the adult DG is restricted to the SGZ, mainly to DCX expressing immature neuronal progenitor cells, which suggests a potential function of NFIA in this cellular subpopulation. Further the high overlap of NFIA with DCX implicated also co-expression of NFIA with Sox11, which was confirmed by immunohistochemical co-stainings for Sox11 and NFIA (Fig 2.15 A). Considering in addition the ChIP-Seq data of a recent publication that revealed Sox11 DNA binding near to the *NFIA* locus (Bergsland et al., 2011), the assumption arose that *NFIA* might be a Sox11 target gene.

Knockout of Sox11 in cKO Sox4/11 monolayer by treatment with HTN-Cre (+ Cre) concomitantly lead to a reduction of *NFIA* mRNA level relative to control (- Cre) (reduced to 0.6 ± 0.0 fold of control level, $p = 0.0372$) (Fig. 2.14 B) as well as relative

to wt monolayer transduced with HTN-Cre (reduced to 0.7 ± 0.0 fold of wt level, p = 0.0005) (Fig. 2.14 B'). Analysis of wt monolayer treated with HTN-Cre resulted in no significant decrease of either *Sox11* or *NFIA* level relative to wt monolayer subjected to only the HTN-Cre solvent (- Cre) (Fig. 2.14 C), which excluded unspecific effects of HTN-Cre on mRNA transcription *per se*.

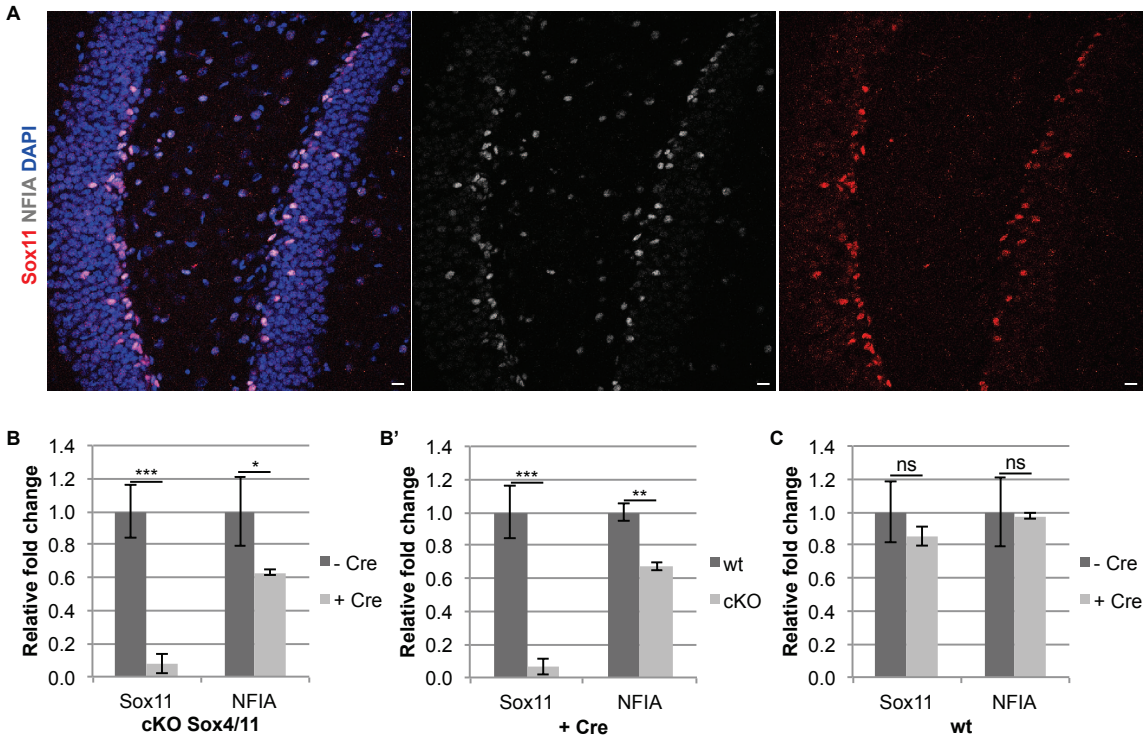


Figure 2.14 Co-expression of NFIA with Sox11 *in vivo* and reduction of *NFIA* level after Sox11 knockout *in vitro*

A Immunohistochemical analysis of the adult DG revealed high overlap of NFIA with Sox11 expression. **B-C** Analysis of *Dll1* mRNA amount in cKO Sox4/11 or wt neural monolayer cultures treated with HTN-Cre (+ Cre) or solvent (- Cre) by qRT-PCR: Quantification of fold change of *Sox11* and *Dll1* mRNA level in HTN-Cre treated relative to control (- Cre) cKO Sox4/11 monolayer depicted drastic reduction of *Sox11* mRNA accompanied by decreased *NFIA* mRNA amount (**B**), which was not detected in wt monolayer (**C**); comparison of cKO Sox4/11 with wt monolayer both exposed to HTN-Cre revealed equivalent reduction of *Sox11* and *NFIA* mRNA level (**B'**). n = 3; scale bar, 10 µm.

A reliable NFIA antibody allowed for analysis of NFIA expression by immunohistochemistry in newborn DG neurons to further confirm a potential regulation of NFIA by Sox11 on protein level *in vivo*. This was done either in the context of conditional Sox11 knockout by stereotactic injection of cKO Sox4/11 mice with a GFP and Cre recombinase-encoding retrovirus (CAG-GFP-IRES-Cre) or after 6-week-lasting Sox11 overexpression in wt mice injected with CAG-Sox11-IRES-GFP (see 2.1.2). In both cases mice injected with a CAG-GFP retrovirus served as control.

Mice treated with CAG-GFP-IRES-Cre (+ Cre) or CAG-GFP (+ GFP) were perfused 5 dpi. After immunohistochemical processing, Sox11 and NFIA protein level were quantified on the basis of staining intensity of individual neurons relative to overall staining intensity whereby a biased quantification due to varying staining efficiencies in different sections and/or animals was eliminated. Intensity indices obtained like this were further normalized to the control group (+ GFP) resulting in a fold amount of Sox11 and NFIA protein level in experimental (+ Cre) normalized to control group. A reduction of Sox11 protein (0.3 ± 0.0 fold, $p = 0.0004$) lead to a concomitant decrease of NFIA protein level (0.6 ± 0.1 fold, $p = 0.0049$) in CAG-GFP-IRES-Cre- relative to CAG-GFP-transduced cells (Fig. 2.15 A-C). Vice versa, retrovirally transduced cells of wt mice that were injected with CAG-Sox11-IRES-GFP (+ Sox11) and perfused 6 wpi exhibited parallel to elevated Sox11 level increased NFIA expression relative to control cells of CAG-GFP-injected mice (+ GFP): Staining intensity of Sox11 was elevated to 1.7 ± 0.2 fold ($p = 0.0057$) and of NFIA to 1.2 ± 0.1 fold ($p = 0.0060$) (Fig. 2.15 D-F).

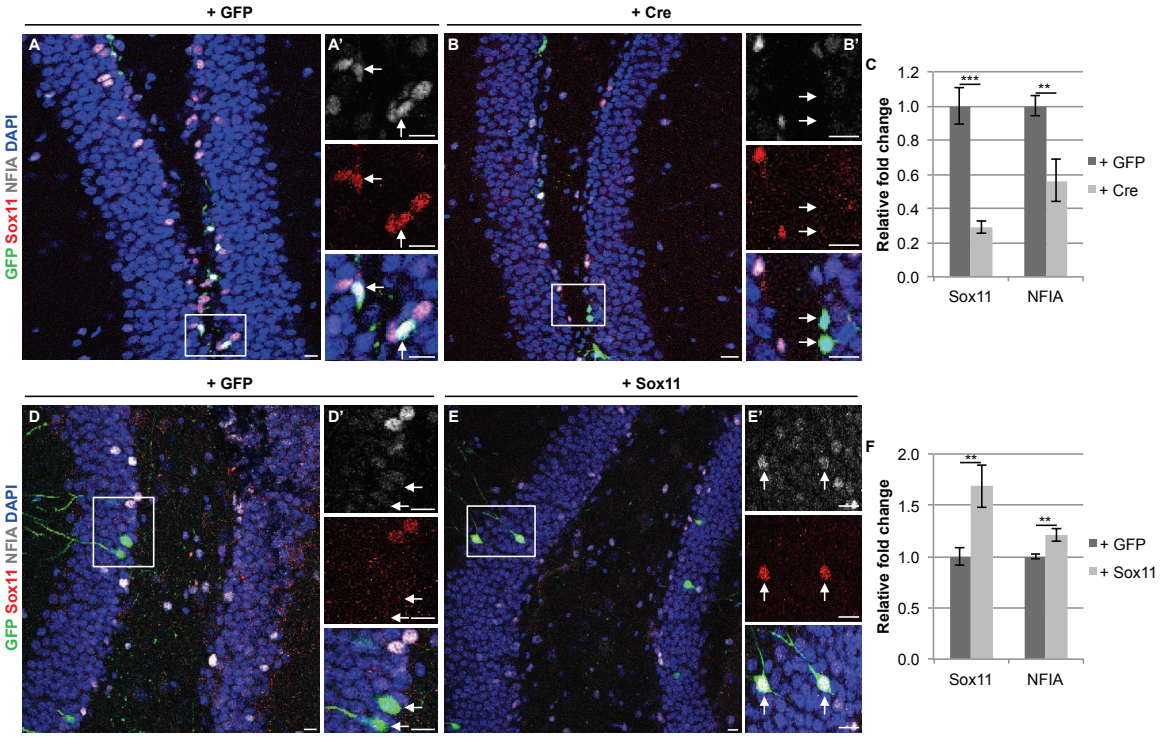


Figure 2.15 Reduction or increase of NFIA protein level following Sox11 knockout or overexpression *in vivo*

A-C Immunohistochemical analysis of Sox11 and NFIA protein level in 5-day-old newborn DG neurons of cKO Sox4/11 mice injected with either CAG-GFP (+ GFP) (**A**) or CAG-GFP-IRES-Cre (+ Cre) (**B**) retrovirus: Images of transduced cells (marked by GFP expression) of control (+ GFP) (**A**) and experimental group (+ Cre) (**B**) shown with respective magnifications (**A'**-**B'**) illustrate efficient knockout of Sox11 concomitant with reduced NFIA expression. **C** Further quantification of Sox11 and NFIA signal intensity confirmed reduction of NFIA along with Sox11 protein level. **D-F** Immunohistochemical analysis of Sox11 and NFIA protein level in 6-week-old newborn DG neurons retrovirally transduced with either CAG-GFP (+ GFP) or CAG-Sox11-IRES-GFP (+ Sox11): Concomitant Sox11 overexpression and increased NFIA expression was only detected in CAG-Sox11-IRES-GFP-transduced cells (**E**) but not in CAG-GFP-transduced cells (**D**) (respective magnifications are shown in **D'-E'**), which was further confirmed by quantification of both Sox11 and NFIA signal intensity (**F**). n = 3; scale bar, 10 µm.

In summary, *in vitro* and *in vivo* data support the notion that *NFIA* represents a Sox11 downstream target gene. But whether it is a direct or indirect target needs to be further clarified. Similarly, additional efforts have to be made to unravel the question whether Sox11 evoked phenotypes might at least partially be implemented by NFIA.

2.2.3 Stathmin 1

Stathmin 1 (STMN1) is a microtubule-regulatory protein that promotes microtubule disassembly by sequestering tubulin. Even though STMN1 is abundantly expressed in the developing mammalian CNS, STMN1 knockout mice do not exhibit a developmental phenotype (Schubart et al., 1996). So far, STMN1 has mainly been studied with respect to cancer biology where it was shown to influence survival of

cancer cells (Nemunaitis, 2012) and migrational properties (Chen et al., 2012; Liu et al., 2013). Effects of STMN1 on cell motility were further identified in mouse embryonic fibroblasts (Shumyatsky et al., 2005). With respect to the CNS, STMN1 was implicated in the migration of newborn neurons in the RMS (Jin et al., 2004) as well as in axonal malformations in aged STMN1 knockout mice (Liedtke et al., 2002) and in neurons of the adult DG (Yamada et al., 2010). Even though Stathmin expression has been discovered in the adult neurogenic niches for a long time (Jin et al., 2004), its function and regulation remains largely unknown. Studies implicating STMN1 in migrational processes together with the migratory phenotype of Sox11 overexpressing neurons revealed in this study and the recently detected binding of Sox11 to DNA in close proximity of the *STMN1* gene (Bergsland et al., 2011), initiated the investigation of *STMN1* as a potential Sox11 target gene.

First of all, the expression pattern of STMN1 in the adult DG was determined by immunohistochemical analysis (Fig. 2.16 A-E). STMN1 expression was restricted to the immature neuronal subpopulation with 88.7 ± 1.0 % and 93.7 ± 2.0 % of STMN1 positive cells being Sox11 and DCX positive, respectively. Only a marginal co-expression with marker proteins of either NSCs, namely Sox2 and GFAP, or mature DG neurons (NeuN and Calbindin) was detected. The finding that STMN1 and Sox11 are highly co-expressed was of particular interest, since it provided the basis for further studies concerning the hypothesis of Sox11 regulating STMN1 expression. First clues, which argued for this hypothesis, came from experiments with either wt or cKO Sox4/11 monolayer treated with HTN-Cre (+ Cre) or only the solvent of HTN-Cre (- Cre) as control. An unspecific effect of HTN-Cre treatment on *Sox11* or *STMN1* transcription could be excluded, since mRNA level of both proteins were unchanged in wt monolayer cells that were subjected to HTN-Cre relative to control wt monolayer (Fig. 2.16 G). In cKO Sox4/11 monolayer amount of *Sox11* mRNA was effectively reduced to 0.1 ± 0.1 fold of control monolayer level ($p = 0.0000$) (Fig. 2.16 F) and to 0.1 ± 0.0 fold of wt monolayer level treated with HTN-Cre ($p = 0.0003$) (Fig. 2.16 F'). Concomitantly, *STMN1* mRNA level decreased to 0.6 ± 0.1 fold ($p = 0.0235$) and 0.4 ± 0.1 fold ($p = 0.0167$), respectively.

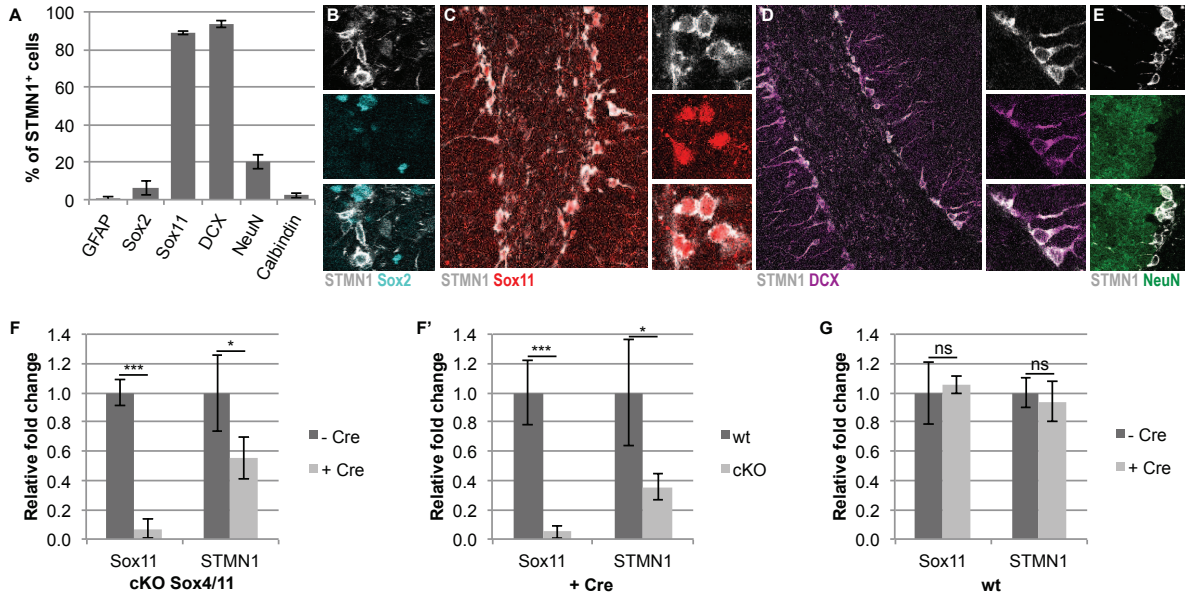


Figure 2.16 Co-expression of STMN1 with Sox11 and DCX *in vivo* and reduction of *STMN1* mRNA amount after Sox11 knockout *in vitro*

A Quantification of immunohistochemical analysis of adult DG with respect to STMN1 expression pattern revealed a strong overlap of STMN1 with Sox11 and DCX expression, but low to no overlap with immature (GFAP, Sox2) or mature (NeuN, Calbindin) marker protein expression as exemplarily illustrated in **B-E**. **F-G** Analysis of *STMN1* mRNA level in cKO Sox4/11 or wt neural monolayer cultures treated with HTN-Cre (+ Cre) or solvent (- Cre) by qRT-PCR: Quantification of fold change of *Sox11* and *STMN1* mRNA level in HTN-Cre treated relative to control (- Cre) cKO Sox4/11 monolayer depicted drastic reduction of *Sox11* mRNA accompanied by decreased *STMN1* mRNA amount (**F**), which was not detected in wt monolayer (**G**); comparison of cKO Sox4/11 with wt monolayer both exposed to HTN-Cre revealed equivalent reduction of *Sox11* and *STMN1* mRNA level (**F'**). n = 3; scale bar, 10 µm.

In vitro evidence for a direct regulation of *STMN1* by Sox11 accumulated in view of subsequently performed luciferase assays. A luciferase construct harboring a 1.1 kb sequence upstream of the *STMN1* TSS (p2.9Op18-luc) (Benlhabib and Herrera, 2006; San-Marina et al., 2012), was kindly provided by J. Liu (Toronto). Four variants of this construct were used featuring the full length (FL) sequence or truncated versions Δ 300, Δ 450 and Δ 1000 (Fig. 2.17 A). Two potential Sox11 binding sites were predicted using bioinformatic analysis within the FL construct, one of which is deleted in case of the Δ 300 construct and both in case of the Δ 450 and Δ 1000 construct. Co-transfection of increasing amounts of Sox11 plasmid (20, 50, and 100 ng) with FL luciferase revealed a dose-dependent increase of luciferase activity relative to control, which was already highly significant at the lowest Sox11 concentration of 20 ng (3.6 ± 0.5 fold, p = 0.0013) (Fig. 2.17 B). Thus, the influence of Sox11 on the truncated versions of p2.9Op18-luc was studied at this low yet sufficient plasmid concentration. A reduced luciferase activity relative to the FL

construct was detected for all deletion constructs, but only values of constructs devoid of both predicted Sox11 binding sites (Δ 450 and Δ 1000) reached significance (Δ 450: 0.6 ± 0.0 fold, p = 0.0054; Δ 1000: 0.6 ± 0.1 fold, p = 0.0099) (Fig. 2.17 C). Since the Δ 450 was the one construct lacking both predicted Sox11 binding sites but retaining residual sequences, which are supposedly irrelevant for Sox11 binding, this very construct was analyzed further: Δ 450 luciferase activity was reduced relative to FL at each applied Sox11 plasmid concentration. For instance, Δ 450 luciferase activity changed to 0.3 ± 0.1 fold relative to FL construct (p = 0.0030) at a concentration of 50 ng (Fig. 2.17 D). Furthermore, course of luciferase activation along with increasing Sox11 plasmid concentration was significantly altered between the Δ 450 and the FL versions of p2.9Op18-luc (p = 0.0012) (Fig. 2.17 E).

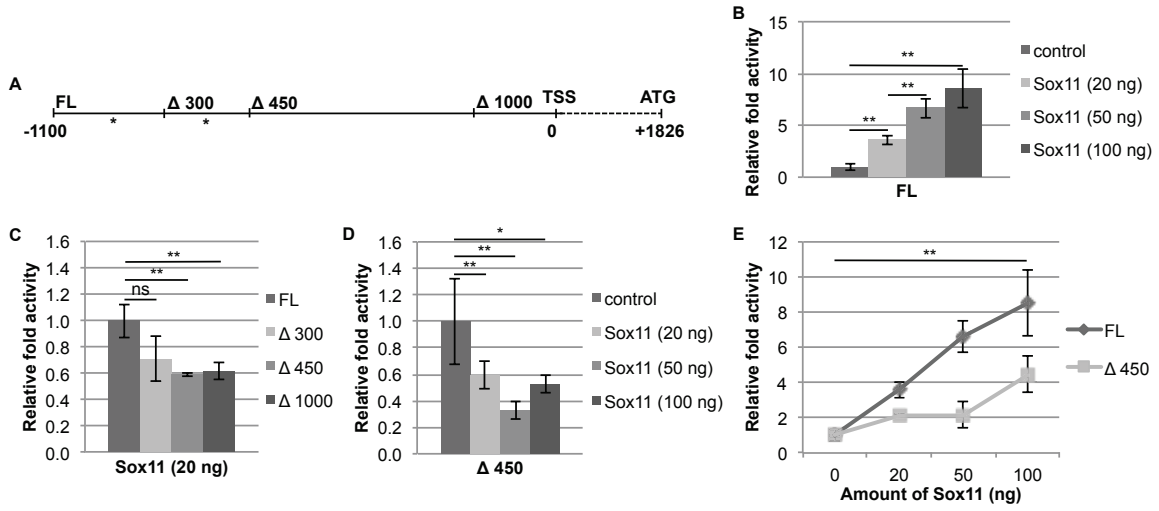


Figure 2.17 *In vitro* activation of *STMN1* promoter by Sox11

A Schematic illustration of the p2.9Op18-luc promoter region featuring a 1100 bp sequence upstream of the *STMN1* transcriptional start site (TSS) up to the start codon ATG (+ 1826 bp), which drives luciferase expression; Full length (FL) and three truncated constructs Δ 300, Δ 450 and Δ 1000 used in this study are schematically depicted; * schematically indicates predicted Sox11 binding sites. **B-E** Evaluation of luciferase assays after co-transfection of various combinations of the four p2.9Op18-luc variants and different amounts of Sox11-encoding plasmid (20, 50, 100 ng) revealed a dose-dependent activation of *STMN1* promoter by Sox11 and the necessity of predicted Sox11 binding sites for activation of *STMN1* promoter: Increasing amounts of Sox11 gradually enhanced luciferase activity relative to control (0 ng Sox11) (**B**); induction of luciferase by Sox11 (20 ng) was significantly less pronounced in case of Δ 450 and Δ 1000 variants relative to FL (**C**); luciferase activity was reduced at all applied Sox11 concentrations in case of the deletion construct Δ 450 relative to FL variant co-transfected with respective Sox11 amounts (control) (**D**); comparing the course of luciferase activation of the FL construct to the Δ 450 variant along with increasing Sox11 amounts depicted reduced luciferase activity in case of the deletion construct Δ 450 (**E**). n = 3.

To complement *in vitro* data clearly indicating a regulative effect of Sox11 on *STMN1* transcription, paradigms of Sox11 overexpression for 21 days or 6 weeks (see 2.1.1 and 2.1.2) were subjected to analysis: *In vivo* STMN1 expression in retrovirally transduced newborn DG neurons identifiable by GFP expression was investigated. Effective Sox11 overexpression was confirmed as described in respective chapters 2.2.1 and 2.2.2. The percentage of GFP positive STMN1 expressing neurons was quantified in both the experimental group, which was injected with CAG-Sox11-IRES-GFP retrovirus (+ Sox11), and the CAG-GFP-injected control group (+ GFP). At 21 dpi, 88.8 ± 4.7 % of Sox11 overexpressing neurons were STMN1 positive in contrast to only 25.2 ± 12.2 % of control neurons ($p = 0.0011$) (Fig. 2.18 A-C). Similarly, 6 wpi the fraction of transduced cells featuring STMN1 expression was higher in the experimental than in the control group: Whereas nearly no STMN1

expression could be detected in control cells (4.8 ± 8.2 %), 49.9 ± 3.0 % of CAG-Sox11-IRES-GFP-transduced cells were STMN1 positive ($p = 0.0009$) (Fig. 2.18 D-F). Additionally, STMN1 expression was analyzed in 5-day-old newborn DG neurons after knockout of Sox11. Stereotactic injection of cKO Sox4/11 mice with a retrovirus encoding for GFP and the Cre recombinase (CAG-GFP-IRES-Cre) efficiently reduced Sox11 expression in CAG-GFP-IRES-Cre-transduced cells (+ Cre) compared to cells of control mice, which were injected with CAG-GFP retrovirus (+ GFP) (see 2.1.2, Fig. 2.15 A - C). Concomitant to loss of Sox11 expression the fraction of STMN1 positive cells within the retrovirally transduced GFP positive cell population was strongly reduced in CAG-GFP-IRES-Cre-injected mice compared to control mice (+ Cre: 39.5 ± 5.5 %, + GFP: 84.7 ± 3.2 %, $p = 0.0001$) (Fig. 2.18 G-I).

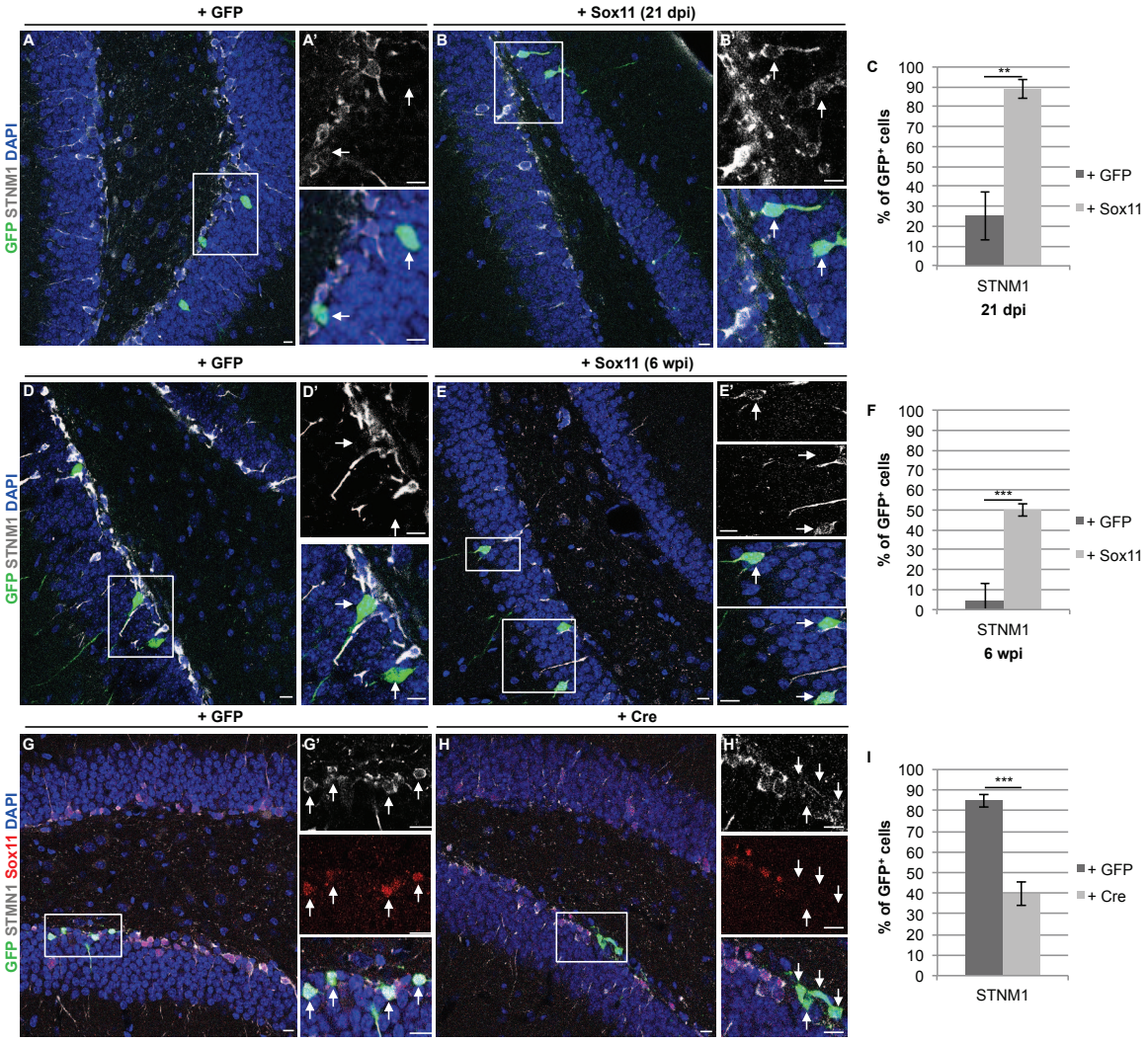


Figure 2.18 Increased or reduced STMN1 expression after Sox11 overexpression or knockout *in vivo*

A-F Immunohistochemical analysis of retrovirally transduced newborn DG neurons (GFP positive) of mice injected with either CAG-GFP (+ GFP) (**A**,**D**) or CAG-Sox11-IRES-GFP (+ Sox11) (**B**,**E**) with respect to STMN1 expression 21 dpi (**A**-**C**) and 6 wpi (**D**-**F**); magnification of boxed areas are shown in **A'**,**B'**,**D'**,**E'**; Quantification of STMN1 positive transduced cells as percentage of all transduced cells showed more STMN1 expressing cells in experimental group (+ Sox11) 21 dpi (**C**) as well as 6 wpi (**F**). **G-I** Immunohistochemical analysis after Sox11 knockout by injection of cKO Sox4/11 mice with CAG-GFP-IRES-Cre retrovirus (+ Cre) (**H**) compared to control mice injected with CAG-GFP (+ GFP) (**G**): Magnified boxes in **G'** and **H'** show STMN1 expression in retrovirally transduced Sox11 positive neurons of control group but not in Sox11 deficient neurons of experimental group (+ Cre) as confirmed by further quantification (**I**). n = 3; scale bar, 10 µm.

In summary, the present study could provide substantial evidence for a direct transcriptional regulation of *STMN1* by Sox11. *In vitro* as well as *in vivo* data clearly point towards this notion: *STMN1*-promoter driven luciferase activity was dose-dependently enhanced by Sox11 depending on the presence of two predicted Sox11 binding sites and STMN1 expression was up- or downregulated parallel to in- or

decreased Sox11 level *in vivo*. Further studies, however, are needed to clarify whether STMN1 mediates Sox11 induced phenotypic changes revealed in this study.

2.3 Sox11 expression following aberrant neuronal activity

A previous study reported that electroconvulsive shock (ECS) treatment, which induces aberrant neuronal network activity, increased expression of *Sox11* mRNA in the DG of adult rats (Sun et al., 2005). However, open questions remained whether this finding can be also observed in adult mice, whether not only Sox11 mRNA but also protein level was similarly affected, and which cells of the adult DG show Sox11 expression. Here, the effect of aberrant neuronal network activity on Sox11 expression was examined on mRNA as well as on protein level in the DG of adult mice. cDNA transcribed from RNA of microdissected DG of either ECS treated (ECS) or sham treated (sham) mice, which served as control, was analyzed by qRT-PCR. Three points in time, namely 30 min, 3 h, and 6 h after ECS treatment were analyzed, for each of which the relative fold change of target gene level over control was calculated based on $2^{-\Delta\Delta CT}$ data. CT values of *Srp14* (signal recognition particle 14) were used for internal normalization. Analyzed target genes were *Sox11* and the immediate-early gene (IEG) *Arc (*activity-regulated cytoskeleton-associated protein) that served as positive control for ECS induced neuronal activity (Lyford et al., 1995). *Arc* mRNA level peaked shortly after ECS (30 min: 43.4 ± 9.4 fold) and gradually declined thereafter (3 h: 29.8 ± 2.3 fold, 6 h: 7.2 ± 1.3 fold) (Fig. 2.19 D). *Sox11* mRNA level were unchanged 30 min after ECS (0.56 ± 0.1 fold), peaked at 3 h (7.7 ± 3.9 fold), and remained elevated at 6 h (6 h: 6.8 ± 1.2 fold) (Fig. 2.19 E). Thus, *Sox11* mRNA upregulation in context of aberrant electrophysiological activity occurs also in the DG of adult mice. A similar finding was obtained in the adult DG after pilocarpin-induced status epilepticus (6 h-28 d) in collaboration with Prof. Albert Becker and Dr. Julika Pitsch, Bonn. *Sox11* mRNA level peaked 6 h after induction of status epilepticus and returned to baseline level at 72 h and 28 d (data not shown). To clarify whether the change of *Sox11* mRNA expression was translated onto protein level mice were perfused 3, 6 or 24 h after ECS or sham treatment and analyzed immunohistochemically. The effectiveness of ECS treatment was once more verified *in vivo* by the detected increase in number of DG neurons expressing another IEG gene, *c-fos* (Fig. 2.19 A-C). Preliminary visual analysis of Sox11 expression gave in fact the impression that Sox11 is, at least 6 h after ECS

treatment, expressed in the GCL of ECS but not sham treated mice throughout the DG (Fig. 2.19 F-K). This suggested that Sox11 was also upregulated in mature DG neurons. To quantitatively describe this observation, staining intensity of Sox11 in the GCL of ECS treated mice was determined as fold change over sham treated mice providing a measure for expressed protein amount. Quantification of the expression of the TF NeuroD, which exhibits under physiological circumstances a DG expression pattern very similar to Sox11, was conducted in parallel with Sox11. Therefore, NeuroD served as negative control for potential staining artifacts, such as unspecific staining of secondary antibodies. NeuroD staining intensity and thus protein level was not significantly affected by ECS at any point in time (3 h: 1.1 ± 0.1 fold, 6 h: 0.8 ± 0.1 fold, 24 h: 1.0 ± 0.4 fold) (Fig. 2.19 M). In contrast, Sox11 immunoreactivity changed after ECS: While no significant alteration was detected 3 h after ECS (1.0 ± 0.1 fold), Sox11 immunoreactivity was significantly increased to 1.4 ± 0.1 fold ($p = 0.0148$) at 6 h (Fig. 2.19 L) and gradually decreased again afterwards (24 h: 1.3 ± 0.2 fold). The quantitative course of Sox11 and NeuroD protein expression within the GCL was also comprehensible by visual analysis (Fig. Fig. 2.19 F-K). To ensure this finding and to once more exclude that staining artifacts were responsible for the detected Sox11 signal in the GCL, Sox11 staining intensity in the DG was additionally normalized to the CA1 region prior to calculation of the fold change over sham at 6 h after ECS. Similar results were obtained, namely a significant upregulation of Sox11 protein level to 1.4 ± 0.0 fold ($p = 0.0019$) (Fig. 2.19 N-O) in the DG of ECS compared to sham treated mice.

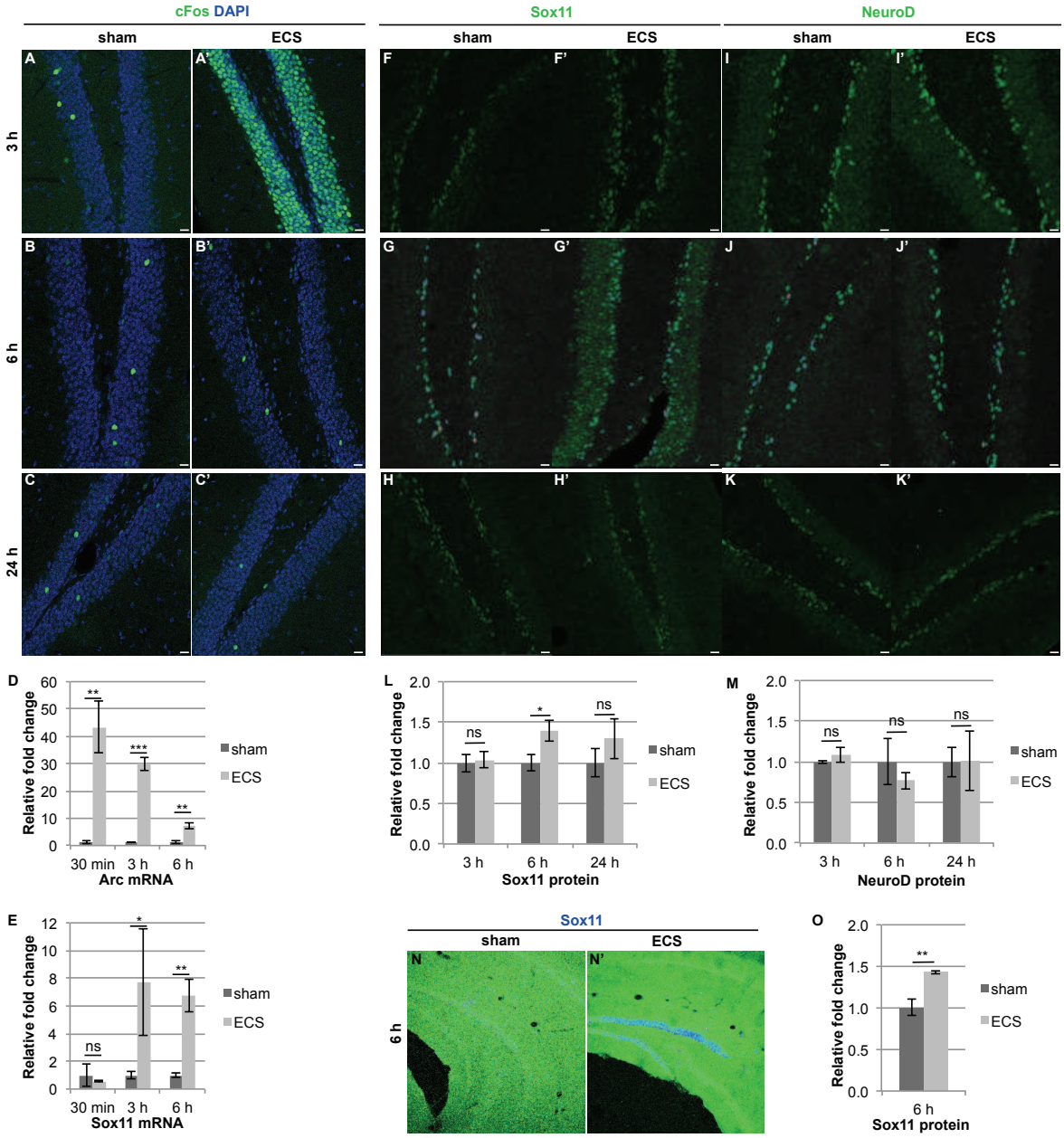


Figure 2.19 Increased Sox11 expression on mRNA and protein level after electroconvulsive shock treatment

A-C Immunohistochemical analysis of c-fos protein expression at distinct points in time (3, 6, and 24 h) after sham (**A-C**) or ECS treatment (**A'-C'**) revealed high c-fos expression in the entire DG 3 h after ECS treatment. **D-E** Analysis of *Arc* (**D**) and *Sox11* (**E**) expression 30 min, 3 h, and 6 h after sham or ECS treatment by qRT-PCR: Normalized to *Srp14*, mRNA level of *Arc* were significantly increased after ECS relative to sham treatment; *Sox11* mRNA level was unchanged 30 min after ECS but increased thereafter significantly. **F-K** Immunohistochemical analysis of Sox11 (**F-H**) and NeuroD (**I-K**) protein expression at distinct points in time (3, 6, and 24 h) after sham (**A-C**) or ECS treatment (**A'-C'**). **L-M** Quantitative analysis of signal intensities of Sox11 and NeuroD in GCL depicted as fold change after ECS relative to sham treatment revealed Sox11 upregulation on protein level 6 h after ECS. **N** Immunohistochemical analysis of Sox11 protein expression 6 h after sham (**N**) or ECS (**N'**) treatment in DG and CA1 region. **O** Similar quantification as in **L-M** detected an increased Sox11 protein expression in DG of ECS compared to sham treated mice. n = 3; scale bar, 10 µm.

In summary, increased Sox11 expression was detected both on mRNA level in microdissected DG tissue and on protein level in brain sections of mice subjected to ECS treatment. Moreover, it was found that Sox11 expression is upregulated in an animal model of mTLE. Thus, this study provides evidence for an upregulation of Sox11 protein in mature DG neurons after aberrant electrophysiological activity suggesting that Sox11 might have a functional role in this population under non-physiological conditions.

3 Discussion

Newborn DG neurons of the adult HC are considered to be crucial for plasticity of the hippocampal network and for hippocampal function (Song et al., 2012a). The functional relevance of adult hippocampal neurogenesis in physiology and pathophysiology is underlined by the findings that a) loss of adult hippocampal neurogenesis is associated with hippocampal learning and memory deficits and increased anxiety behavior, b) age-associated cognitive deficits correlate with a decrease in hippocampal neurogenesis, c) adult neurogenesis is perturbed in neurodegenerative diseases, and d) the ameliorative effect of certain classes of antidepressants requires intact adult hippocampal neurogenesis (Zhao et al., 2008). Thus, comprehensive understanding of how adult hippocampal neurogenesis is regulated is of particular basic and clinical interest. This will permit, in the long term, modifications of adult hippocampal neurogenesis in a way that positively influence hippocampal function under physiological and especially pathological conditions. Furthermore, increasing the knowledge about the mechanisms controlling adult neurogenesis is expected to contribute to the development of cell-based therapies for replacement of lost neurons in neurodegenerative diseases (Lie et al., 2004).

Sox proteins are implicated in many developmental processes including the development of the nervous system (Lefebvre et al., 2007; Sarkar and Hochedlinger, 2013) and it was recently found that members of the Sox TF family coordinate sequential gene expression during neuronal development (Bergsland et al., 2011). Hence, analyzing the specific relevance of certain Sox proteins for neurogenic processes can contribute to further our understanding of the complex regulatory mechanisms controlling adult hippocampal neurogenesis. This represented the central aim of the present study. Sox11 was investigated with respect to its impact on neuronal maturation and integration during adult hippocampal neurogenesis as well as to possible Sox11 downstream targets. The emphasis was placed on Sox11 since Sox11 was shown to be restrictively expressed in immature neurons of the adult hippocampus (Haslinger et al., 2009) and, most interestingly, to be crucial for neuronal differentiation (Mu et al., 2012).

3.1 Sox11 - a bivalent regulator of adult hippocampal neurogenesis

Mu and colleagues demonstrated the requirement of Sox4 and Sox11 for neuronal differentiation (Mu et al., 2012): *In vitro*, both proteins were shown to be able to induce neuronal differentiation as well as to promote neuronal reprogramming of astrocytes. Furthermore, after *in vivo* conditional knockout of both proteins in newborn granule neurons of the adult DG, Sox4/11 deficient cells failed, in contrast to control cells, to initiate or maintain neuronal differentiation, which was indicated by the absence of both neuron-specific marker expression and a neuron-like morphology.

Data of this previous study investigating the *in vivo* effect of conditional Sox4/11 ablation are complemented by the results of the present study. Various phenotypical changes were discovered after Sox11 misexpression in newborn granule neurons of the adult DG. These included delayed neuronal maturation and integration as well as mispositioning of newborn neurons, while the latter one could even be detected in mature adult-born neurons.

Taken together, results from both studies identify Sox11 as a bivalent regulator of adult hippocampal neurogenesis: At early developmental stages, on the one hand, the presence of Sox11 protein is crucial to allow for proper neuronal fate commitment. On the other hand, during the further course of neuronal development the downregulation and persistent absence of Sox11 protein is required for appropriate maturation and integration of newborn DG neurons as well as for the integrity of mature adult-born DG neurons.

First indications for disturbed neuronal maturation after failed Sox11 downregulation were observed in case of Sox11 overexpression at 21 d after neuronal birth (see 2.1.1). Persistent expression of immature marker proteins (Fig. 2.1) and existence of more than one basal dendrite (Fig. 2.2) as well as reduction of morphological complexity (Fig. 2.3) and impaired spine development (Fig. 2.4) represented clear signs of delayed progression of neuronal development. Several studies revealed the importance of adult hippocampal neurogenesis in general or, more specifically, of cohorts of newborn DG neurons for hippocampal plasticity and ultimately hippocampal function. Disruption of adult hippocampal neurogenesis either unspecifically by applying antimitotic drugs or X-irradiation (Clelland et al., 2009; Gilbert et al., 2001; Kim and Lee, 2011; Rola et al., 2004; Saxe et al., 2006; Shors et

al., 2001; Snyder et al., 2005; Winocur et al., 2006), or specifically by manipulation of newborn cell populations making use of different inducible genetic approaches, such as the Cre-ERT2 system (Deng et al., 2009; Dupret et al., 2008; Guo et al., 2011; Imayoshi et al., 2008; Kheirbek et al., 2012b; Nakashiba et al., 2012; Sahay et al., 2011; Saxe et al., 2006; Tronel et al., 2012), resulted in impaired hippocampal function. The appropriate development of the functionally relevant adult-born DG neurons was in the present study shown to be heavily dependent on Sox11. Thus, precise regulation of Sox11 expression appears to be crucial to ensure functional integrity of the adult HC.

This notion is further underlined by the finding that misexpression of Sox11 delayed electrophysiological development of newborn DG neurons (see 2.1.1 and 2.1.2). Proper electrophysiological innervation of newly generated cells by GABA and glutamate was reported to be important for proliferation of neuronal progenitors and survival of newborn neurons (Bruel-Jungerman et al., 2006; Ge et al., 2007a; Song et al., 2012b; Tozuka et al., 2005). In addition, disturbance of electrophysiological properties and/or integration of newborn neurons was shown to impair hippocampal neurogenesis and function (Bergami and Berninger, 2012; Kheirbek et al., 2012b; Tashiro et al., 2006a). Thus, impaired integration (Fig. 2.4) and electrophysiological development (Fig. 2.8) of newborn neurons that were the result of failed Sox11 downregulation might possibly result in lost or at least delayed fulfillment of their functional contribution to the hippocampal network. The importance of precise timing maturation and integration of new DG neurons is illustrated by the finding of Farioli-Vecchioli and colleagues that misexpression of Tis21, which lead to accelerated maturation of new DG neurons, resulted in severely impaired hippocampal function (Farioli-Vecchioli et al., 2008). Moreover, electrophysiologically impaired newborn neurons might not be able to successfully compete for pre-existing synaptic contacts within the adult hippocampal network, which was further suggested to be a limiting factor for neuronal survival (Tashiro et al., 2006a). Hence, failed integration due to lost competition and perhaps even subsequent neuronal death might as well contribute to lost functional fulfillment of newborn DG neurons.

Hence, the present study provides conclusive evidence for a crucial role of Sox11 within adult hippocampal neurogenesis and, moreover, for an ultimate implication of Sox11 in hippocampal function.

Data of this study revealed a delayed maturation and integration of newborn granule neurons in the adult DG as a consequence of failed Sox11 downregulation. But further effects of Sox11 on DG neurons were detected after overexpressing Sox11 for 6 w in newborn or in mature adult-born neurons (see 2.1.2 and 2.1.3): Neurons were mispositioned with their somata residing more frequently in the outer part of the GCL in closer proximity to the MCL (Fig. 2.9 and Fig. 2.12). Besides, newborn neurons featured altered branching characteristics including more primary dendrites and dendrites that branched closer to the soma (Fig. 2.7). The latter phenomenon might be either evoked directly by Sox11 or a secondary effect of Sox11-induced mispositioning, since mislocated neurons have a reduced distance between their soma and the GCL-MCL border from where dendritic branching can occur.

A recent study reported that presynaptic strength in the hippocampal network depended on dendritic position of the postsynaptic cell with the strongest terminals closest to the soma, even though this phenomenon was less pronounced for DG compared to pyramidal neurons (de Jong et al., 2012). Furthermore, it was shown that the greater the distance between the dendritic synapse and the granule cell soma the more pronounced the voltage attenuation is (Krueppel et al., 2011). Both studies suggest that synaptic characteristics depend on the relative location of the soma to the dendrite, more precisely the synapse. Sox11 overexpressing newborn DG neurons were shown to reside closer to the MCL resulting in an altered distance between their somata and synapses formed with projections from the EC. Moreover, Scholl analysis of these neurons revealed altered branching characteristics such as more primary dendrites, more dendrites closer to the soma, and less dendrites at greater distance to the soma. Considering both of these observations and the experimental evidence for a correlation between synaptic strength and synaptic distance to the soma, an effect of Sox11-dependent positioning on relative synaptic strength may be assumed. Moreover, projections from the EC terminate in distinct regions of the MCL, e.g. those of the perforant pathway mainly in the outer two thirds. But whether a Sox11-induced shift of DG neurons towards the MCL might result in termination of afferent projections from the EC on inappropriate regions of the dendritic tree remains to be analyzed. Likewise, it will be interesting to prove the hypothesis that a shift in relative cell and synapse location induced by Sox11 has functional consequences for DG neurons.

3.2 Regulation of Sox11 expression

Sox11 downregulation is crucial for timing and completion of neuronal maturation in the adult DG. This raises the intriguing question, how Sox11 expression itself is mechanistically regulated. Based on previous studies, different regulatory mechanisms are conceivable. A study of the developing chicken spinal cord demonstrated that Sox11 operated downstream of neurogenins (Ngn) (Bergsland et al., 2006). This might also apply for the DG, since Ngn2 expression was detected in early intermediate progenitors of the adult DG (Ozen et al., 2007) and thus marginally preceded Sox11 expression. Moreover, unpublished data from a cooperation with Dr. G. Masserdotti and Prof. B. Berninger revealed increased *SoxC* mRNA level upon Ngn2-mediated astrocytic reprogramming. However, Ngn2 expression was reported to be limited to early stages of neuronal development in the adult DG, namely to Type II cells at the beginning of the transiently amplifying progenitor phase (Roybon et al., 2009). Thus, Ngn2 might be upstream of Sox11 but certainly not involved in Sox11 maintenance, since it is absent from the DCX-positive Type III cell population. The same might prove true for Mash1 that was, on the one hand, shown to influence SoxC expression in the developing brain (Castro et al., 2011), but, on the other hand, was only detected in early Type II cells of the adult DG (Roybon et al., 2009).

Besides straightforward regulation of Sox11 transcription by TFs, a contribution of epigenetic modifications to the regulation of Sox11 expression should be considered. Data from several studies suggest that activating or repressive histone modifications represent one possibility to influence Sox11 transcription: During retinal development declining levels of activating histone modifications were associated with decreased Sox4 and Sox11 expression (Usui et al., 2013a). An association of activating and silencing histone modifications with Sox11 expression and repression, respectively, was further described in a study on lymphoid neoplasms (Vegliante et al., 2011). Moreover, Swiss and colleagues described that, during oligodendrocyte differentiation, Sox11 expression was negatively regulated by histone deacetylase (HDAC)-catalyzed removal of activating acetyl groups from histones (Swiss et al., 2011).

Next to histone modifications, DNA methylation and demethylation represents a further epigenetic mechanism to influence gene transcription in a negative of positive manner, respectively. In Sox11 positive lymphoid neoplasms Sox11 expression was revealed to be associated with unmethylated DNA, whereas a tendency to DNA

hypermethylation was detected in Sox11 deficient lymphoid neoplasms (Vegliante et al., 2011). In cooperation with Prof. H. Song, Baltimore, dynamic methylation of the *Sox11* locus in DG neurons was observed in response to neuronal activity and was correlated with Sox11 mRNA and protein expression (data not shown). Thus, *Sox11* transcription might indeed depend on the DNA methylation status of its promoter region.

Regulation of protein expression is not restricted to the control of transcription but includes further modulations on the posttranscriptional level, e.g. micro-RNA mediated reduction of transcribed mRNA level. A very recent study on eye development identified Sox11 as a new target of miR-204 (Shaham et al., 2013). With respect to NSCs and neurogenesis, several studies brought forth the concept that microRNAs are crucial to balance stem cell maintenance and fate determination (Shi et al., 2010). Together with the finding that *Sox11* mRNA is present already in not yet differentiated cells (Beckervordersandforth et al., 2010), this suggests that Sox11 expression might indeed be posttranscriptionally regulated by microRNA interference.

Finally, posttranslational modifications of Sox11 protein, such as phosphorylation, ubiquitination, and neddylation, represent another possibility to control cellular Sox11 protein level. So far, modification of Sox protein activity, but not expression level, by phosphorylation was only reported in one *in vitro* study on the SoxE group member Sox9, which exhibited increased DNA binding efficiency after phosphorylation (Huang et al., 2000).

3.3 Sox11 - Presumable mechanisms of action

Similar to regulation of Sox11 itself, only sparse knowledge exists on how Sox11 mediates its effects, i.e. how it mechanistically controls gene expression.

Due to the capability of Sox proteins in general to bend DNA upon binding to the minor groove (Ferrari et al., 1992), Sox11 might regulate gene expression by increasing accessibility of and changing the distance to DNA for other DNA binding proteins. However, experimental evidence confirming this assumption for Sox11 has not yet been provided.

Direct interaction with and/or recruitment of other DNA binding proteins including TFs, factors of the transcriptional machinery, and epigenetic modulatory proteins was demonstrated for various members of the Sox TF family (Lefebvre et al., 2007; Sarkar and Hochedlinger, 2013): SoxE group members were shown to interact with

various types of TFs (Wissmuller et al., 2006); Chen and colleagues reported transcriptional activation upon recruitment of the transcriptional co-activator p300 by Sox2 (Chen et al., 2008); and experimental evidence was provided that interaction of Sox2 with the polycomb complex resulted in gene silencing (Boyer et al., 2006). The importance of functional interaction of Sox proteins with other TFs was even more conclusively demonstrated for Sox2 that lost its ability to induce pluripotency following mutation of amino acids responsible for Oct4 binding (Jauch et al., 2011). TFs of the POU family were shown to be able to increase SoxC target gene activation (Kuhlbrodt et al., 1998; Wiebe et al., 2003). With respect to Sox11, a synergistic effect with class II POU factors on Nestin expression in neural primordial cells was reported (Tanaka et al., 2004). Thus, a functional interaction of POU TFs with Sox11 might represent a downstream mechanism of target gene regulation. Most interestingly, the regulatory region of *DCX*, which was identified as a Sox11 target gene in our laboratory (Mu et al., 2012), features putative POU binding sites (Karl 2005) in close proximity to Sox11 binding sites. Furthermore, *in vitro* data of the present study suggest a synergistic effect of Sox11 together with Mash1 since Mash1-induced *Dll1* promoter activity was further augmented by Sox11 (Fig. 2.13). Analysis of a possible interaction of Sox11 with POU TFs and Mash1 as well as with unknown interaction partners, e.g., by an unbiased proteomic approach, represents an interesting topic for future studies.

Finally, Sox11 might exert its effects by modulation of other signaling pathways. This hypothesis is encouraged by the observation that the second relevant SoxC group member Sox4 positively modulated the canonical Wnt signaling pathway (Lee et al., 2011; Sinner et al., 2007). To increase the knowledge about crosstalk of Sox11 with other signaling pathways and proteins represents a major challenge for future experiments. Initial hints might be accomplished by comparison of phenotypical changes induced by either Sox11 overexpression or Sox11 knockout with the phenotypic outcome after alteration of signaling pathways and/or mutation of proteins implicated in neurogenic processes. A candidate pathway for interaction is the Reelin pathway. Reelin has been associated with many developmental and adult neurogenic processes including neuronal migration, morphology, and synaptic plasticity (Herz and Chen, 2006; Pujadas et al., 2010; Tissir and Goffinet, 2003). With respect to the HC, Reelin was implicated in granule cell migration, since in the neonatal and adult DG aberrant migration of granule cell progenitors was detected after loss of Reelin

expression (Gong et al., 2007). This phenomenon was further described for adult-born hippocampal neurons after inactivation of the Reelin pathway (Teixeira et al., 2012). In consideration of the fact that one major phenotypical outcome of Sox11 overexpression in the adult DG was disturbed migration of newborn and mature adult-born granule neurons, determining a possible interaction of Sox11 with the Reelin pathway constitutes an interesting topic for future research. Similarly, a crosstalk of Sox11 with DISC1 should be considered: DISC1 knockdown lead to mispositioning of DG neurons and aberrant branching characteristics (Duan et al., 2007; Zhou et al., 2013) resembling the phenotype induced by Sox11 overexpression.

Even though the specific mode of action of Sox11 in adult hippocampal neurogenesis could not be revealed within the present study, the identification of several potential Sox11 transcriptional targets might provide first hints on downstream effects initiated by Sox11.

3.4 Potential transcriptional targets of Sox11

Only few Sox11 target genes are known, so far. Sox proteins can bind DNA at sequences that only partially match the consensus sequence. Thus, potential imperfect Sox binding sites are abundant and can be found throughout the genome at high frequency (Lefebvre et al., 2007) hampering the identification of Sox target genes. Three potential Sox11 target genes were identified in this study. This might on the one hand help to at least partially explain phenotypic changes induced by Sox11 and on the other hand implicate Sox11 in additional processes of neuronal development.

In vitro results from Sox4/11 conditional knockout experiments and luciferase assays (Fig. 2.13) provided the indication that the Notch ligand *Dll1* might be one potential Sox11 downstream target (see 2.2.1). The plausibility of this finding is increased by a study on prostate cancer cells that demonstrated a direct regulation of *Dll1* transcription by Sox4, which, as a member of the SoxC group, potentially acts in redundancy with Sox11 and targets similar loci as Sox11 (Scharer et al., 2009). Moreover, luciferase assays of this study revealed an increased *Dll1* promoter activation in the presence of Sox11 together with Mash1. Interestingly, Mash1 was reported to be involved in the regulation of *Dll1* (Casarosa et al., 1999; Ito et al., 2000; Nelson and Reh, 2008) (Fig. 2.13). Hence, Sox11 and Mash1 might

synergistically influence *Dll1* transcription at early stages of neuronal development (i.e. in Type II cells), at which Sox11 and Mash1 are co-expressed, but not in Mash1 deficient Type III cells.

Notch ligands such as Dll1 are expressed by differentiated cells and can exert a negative feedback on neighboring progenitor cells. By activation of Notch signaling via this so-called lateral inhibition Notch ligands can modulate the genetic program of adjacent cells and regulate for instance proliferation of progenitor cells. Several studies strongly implicated Notch signaling in various aspects of adult neurogenesis including regulation of stem cell maintenance and neuronal fate decision (Ables et al., 2011; Imayoshi and Kageyama, 2011). Also Dll1 was, a one Notch ligand, associated with adult neurogenic processes: The zebrafish ortholog of mammalian Dll1, DeltaA, was shown to potentially control Notch activity levels and thereby the balance between quiescence and recruitment of NSCs in the adult zebrafish brain (Chapouton et al., 2010). Furthermore, Dll1 was reported to control neurogenesis in the mammalian spinal cord (Ramos et al., 2010) and it was suggested that Dll1-activated Notch signaling negatively influences neuronal differentiation by Hes1-mediated *Mash1* repression (Kiparissides et al., 2011). Even though the role of Dll1 in the adult DG has not been studied so far, the importance of Notch signaling specifically for adult hippocampal neurogenesis was demonstrated: Regulation of cell fate and dendrite morphology in the postnatal DG as well as maintenance of adult hippocampal NSCs are dependent on Notch signaling (Breunig et al., 2007; Ehm et al., 2010). Activation of the Notch pathway relies on the binding of Notch to its ligands. Thus, Sox11 might via regulation of *Dll1* expression contribute to the control of proper dendrite morphology and to the maintenance of the balance between NSC proliferation and quiescence. Indeed, the Sox11 overexpression phenotype with shorter apical dendrites (Fig. 2.3) and altered dendritic branching characteristics (Fig. 2.7) resembles the reduced branching complexity in Notch1 cKO mice (Breunig et al., 2007). Potential effects of Sox11 overexpression on NSC maintenance could not be determined with the present strategy. Since only a cohort of DG neurons born at the time of retroviral injection was affected by Sox11 overexpression, cells featuring Dll1 expression might have been too few to exert a detectable effect on the NSC pool. Thus, to finally address this issue, it would be necessary to overexpress Sox11 in larger cohorts of newborn neurons.

Further studies have to confirm the hypothesis that Sox11 regulates Dll1 expression and thereby modulates adult hippocampal neurogenesis. A correlation of Sox11 and Dll1 expression has to be analyzed *in vivo* and the role of Dll1 in adult hippocampal neurogenesis has to be identified.

NFIA, which belongs to the nuclear factor one (NFI) family of TFs, was identified as another potential target gene of Sox11. *In vitro* conditional knockout of Sox4/11 (Fig. 2.14), analysis of *in vivo* NFIA expression pattern (Fig. 2.14), and determination of NFIA protein level after either knockout of Sox4/11 or Sox11 overexpression (Fig. 2.15) (see 2.2.2) suggested that *NFIA* transcription is regulated by Sox11. Further indication for this hypothesis is provided by a study in mouse and chick spinal chord revealing that NFIA was under the control of another TF of the Sox family, namely Sox9 (SoxE group) (Kang et al., 2012). Moreover, ChIP-Seq analysis detected Sox11 DNA binding near to the *NFIA* locus (Bergsland et al., 2011). Interestingly, in cortical embryonic neural precursor cells NFIA expression was reported to be activated by Notch signaling (Namihira et al., 2009). Such an regulation is unlikely in the adult DG, since NFIA is expressed immature granule neurons (Schwarz, 2013)(this study), which are Notch signaling deficient. Thus, NFIA might be differentially regulated during embryonic development and adult neurogenesis.

Recently, the expression pattern of NFIA was reported for both adult neurogenic niches: NFIA expression was detected in progenitor cells, migrating neuroblasts, and astrocytes in the adult SVZ, RMS, and OB (Plachez et al., 2012). Its expression in the adult DG was restricted to the SGZ, more specifically to the DCX expressing immature neuronal cell population (Schwarz, 2013). Moreover, co-expression with Sox11 was observed in the present study (Fig. 2.14). During embryogenesis NFI proteins regulate multiple aspects of neural development (Mason et al., 2009), such as progenitor self-renewal (Piper et al., 2010), fate determination (Namihira et al., 2009), neuronal differentiation as well as migration (Kilpatrick et al., 2012), and gliogenesis (Deneen et al., 2006; Kang et al., 2012). But, so far, no studies have been conducted with respect to their function in adult neurogenesis. It is worth mentioning that the expression of NFIA in neuronally committed cells of the adult DG is in stark contrast to the positive regulation of gliogenesis and astrocytic fate commitment during embryonic development by NFIA (Deneen et al., 2006; Kang et

al., 2012; Namihira et al., 2009). Thus, NFIA may fulfill a different role in the context of adult hippocampal neurogenesis. Few studies identified NFIA functions that might be of significance for adult hippocampal neurogenesis. Wang and colleagues provided experimental evidence that in cerebellar granule neurons (CGNs) NFI(A) regulates the expression of a certain GABA A receptor (GABAR) subunit (Wang et al., 2004a). Taking into account the importance of GABA signaling for proper neuronal maturation (Ge et al., 2007a; Song et al., 2012b; Tozuka et al., 2005) together with the delayed neuronal maturation induced by Sox11 overexpression, this finding hints at the possibility that Sox11 might regulate GABAR expression via NFIA. Moreover, it was discovered that migration and dendrite formation of postmitotic CGNs was severely disrupted by repression of NFI function (Wang et al., 2007). This finding implicates NFI(A) in the regulation of cellular migration and dendritic outgrowth, both of which were as well affected by Sox11 overexpression. Thus, not only electrophysiological properties might be influenced by NFIA expression but also migration and dendritic development of newborn DG neurons. Since Sox11 was within this study implicated in the very same processes, the assumption emerges that NFIA might indeed act downstream of Sox11 partly mediating its function.
NFIA represents an interesting candidate for further experimental approaches, for instance with the aim to copy Sox11-dependent phenotypes. Unfortunately, first attempts to carry out such experiments, namely NFIA overexpression or competitive inhibition of NFIA by dnNFIA by stereotactic injection of respective retroviruses, were not successful.

Most conclusive experimental evidence for a Sox11-dependent transcriptional regulation was acquired for *STMN1,* which encodes for a microtubule-regulatory protein promoting microtubule disassembly: *In vitro* conditional knockout of Sox4/11 resulted in a concomitant decrease of *STMN1* mRNA level (Fig. 2.16); luciferase assays demonstrated that *STMN1*-promoter driven luciferase activity was dose-dependently enhanced by Sox11, which most importantly additionally depended on the presence of two predicted Sox11 binding sites (Fig. 2.17); and *in vivo* STMN1 expression, which nearly completely overlapped with Sox11 expression (Fig. 2.17), was up- or downregulated in parallel to in- or decreased Sox11 level, respectively (Fig. 2.18) (see 2.2.3). ChIP-Seq data that demonstrated Sox11 DNA binding near to the *STMN1* locus (Bergsland et al., 2011) further strengthen the hypothesis of a

potential direct transcriptional regulation of *STMN1* by Sox11. Luciferase assays of this study identified a synergistic effect of Sox11 together with Mash1 on *Dll1* promoter activity (Fig. 2.17). Interestingly, Mash1 was also implicated in the transcriptional regulation of *STMN1* (Yamada et al., 2010). Hence, a similar effect might be involved in the induction of STMN1 expression in early neuronal progenitor cells (Type II cells), which co-express Sox11 and Mash1. However, this synergy certainly is not responsible for maintained STMN1 expression in Type III cells, which are Mash1 deficient.

The STMN1 expression pattern was previously determined in the adult neurogenic niches (Jin et al., 2004), and was in this study further confirmed to be restricted to the immature neuronally committed subpopulation of newborn granule neurons in the adult DG (Fig. 2.16). STMN1 was discovered to regulate migrational processes and cell motility not only of non-neuronal cell types such as cancer cells and mouse embryonic fibroblast (Chen et al., 2012; Liu et al., 2013), but even more interestingly also of newborn neurons in the RMS (Jin et al., 2004). The pronounced migrational phenotype after Sox11 overexpression, which resulted in mispositioned adult-born DG neurons (Fig. 2.9 and Fig. 2.12), suggests that the effect of Sox11 on granule cell migration might be mediated by STMN1 and *STMN1* indeed represents a direct Sox11 downstream target.

Further experiments are required to confirm that Sox11 regulates STMN1 expression and thereby modulates adult hippocampal neurogenesis. For instance, a retroviral experimental approach overexpressing STMN1 in newborn DG neurons could reveal, whether the Sox11-dependent phenotype can be copied by STMN1.

Considering the sparse knowledge about putative downstream targets of Sox11, studies like the present one aiming to identify new Sox11-regulated genes are particularly important. Results can facilitate a better understanding of the downstream events triggered by Sox11.

3.5 Sox11's potential involvement in neurological disorders

Epilepsy represents one of the most common neurological disorders with roughly 50 million people being affected worldwide (Brodie et al., 1997). Medial temporal lobe epilepsy (mTLE) is presumably the most common refractory form of epilepsy (Wiebe, 2000). In animal models mTLE is mimicked by seizures induced either electrically or by application of a chemoconvulsant (Parent and Kron, 2012). With several studies

associating mTLE with adult neurogenesis, abnormal adult hippocampal neurogenesis emerged as a prominent hallmark of mTLE (Hattiangady and Shetty, 2008; Kuruba et al., 2009; Parent and Kron, 2012). Observed alterations of neurogenesis in the context of mTLE include augmented DG cell proliferation (Gray and Sundstrom, 1998; Jessberger et al., 2005; Jiruska et al., 2013; Parent et al., 1997), migration of newborn DG neurons into ectopic regions such as the hilus and the MCL of the DG, as well as mossy fiber sprouting (Jessberger et al., 2007b; Overstreet-Wadiche et al., 2006). The two latter findings point to disturbed migration and impaired morphological development of newborn neurons if exposed to epilepsy-mimicking conditions. Such alterations of adult hippocampal neurogenesis, which can partly also be detected in human TLE tissue (Parent, 2007), might contribute to the pathology and chronic manifestation of mTLE. Moreover, comorbidities associated with epilepsy such as anxiety, cognitive impairment, and depression (Jacobs et al., 2009) are phenotypes associated with impaired adult hippocampal neurogenesis. Thus, modulation of the adult hippocampal neurogenic process represents an intriguing option for therapeutic interventions. Jessberger and colleagues showed that normalizing neurogenesis in a mTLE mouse model by treatment with the antiepileptic drug valproic acid protected mice from seizure-induced cognitive impairment in a hippocampus-dependent learning task (Jessberger et al., 2007a). To achieve the aim of effective therapeutic treatment of epilepsy, knowledge about molecular events and molecules involved in both mTLE and adult hippocampal neurogenesis is required. This might, for instance, enable the identification of molecular targets involved in epileptic pathology. Increased *Sox11* mRNA level in the adult DG were detected after pilocarpin-induced seizures in a collaboration with Prof. Albert Becker and Dr. Julika Pitsch, Bonn (data not shown). Aberrant neuronal network activity evoked by ECS treatment of adult rats similarly resulted in increased *Sox11* mRNA expression (Sun et al., 2005). Moreover, data from a collaboration with Prof. H. Song, Baltimore, suggested epigenetic changes of the Sox11 locus after ECS treatment (data not shown). In the present study these observations were complemented revealing that after ECS treatment Sox11 expression was increased on RNA and, importantly, also on protein level in the mouse adult DG (Fig. 2.19) (see 2.3). Moreover, increased Sox11 expression was shown to negatively influence adult hippocampal neurogenesis. This included alterations such as more basal dendrites and cellular mislocation (Fig. 2.2, Fig. 2.9, and Fig. 2.12) that resemble pathological

DG neuron phenotypes detected in animal models of mTLE (Parent and Kron, 2012). Thus, Sox11 might indeed be a mediator of pathological alterations in the adult epileptic DG and therefore represent a therapeutic target.
Moreover, not only newborn DG neurons were reported to be mislocated after epilepsy, but also a reaction of fully differentiated DG neurons in terms of somatic translocation was described in a chemoconvulsant model of mTLE (Murphy and Danzer, 2011). This becomes particularly interesting, since Sox11 misexpression induced cellular mispositioning not only in newborn neurons but also in fully matured adult-born neurons, as revealed in this study by adeno-associated viral overexpression of Sox11 (Fig. 2.12). This experimental paradigm elucidated the influence of Sox11 on adult-born DG neurons, since only these neurons were marked by retroviral injections into the adult DG prior to adeno-associated viral transduction. An intriguing question for future research is whether Sox11 can also affect postnatally generated DG neurons. To address this issue, labeling of granule neurons during postnatal development of the DG has to be achieved prior to adeno-associated viral Sox11 overexpression in the adult DG.

Depression is another common neurological disorder, which is supposed to be the second most prevalent cause of illness-induced disability by 2020 (Tanti and Belzung, 2010). Thus, elucidating the molecular mechanisms of depression and antidepressant treatment is a major challenge for future research. Intriguingly, an association of Sox11 with depression was revealed: Antidepressant treatment of mice with paroxetine was shown to be accompanied by an increase of *Sox11* mRNA level (Sillaber et al., 2008). The requirement of intact adult hippocampal neurogenesis for effective antidepressant treatment was revealed in several studies (Sahay and Hen, 2007). For instance, it was reported that impairment of adult hippocampal neurogenesis by X-irradiation blocked the ameliorative effect of antidepressants (Jiang et al., 2005; Santarelli et al., 2003). So far, the precise correlation of adult hippocampal neurogenesis and effective antidepressant treatment as well as whether altered adult hippocampal neurogenesis might even be an etiological factor for depression is still a subject of debate (Eisch and Petrik, 2012; Sahay and Hen, 2007). Taking into account that a) effectiveness of certain antidepressants most probably depends on an intact neurogenic process in the adult

DG, b) Sox11 was shown to be critically involved in this very process (Mu et al., 2012)(this study), and c) Sox11 expression is affected by antidepressant treatment (Sillaber et al., 2008), an implication of Sox11 in depression and its treatment can be assumed. But whether Sox11 upregulation after antidepressant treatment is beneficial for the ameliorative effect of antidepressants has to be critically analyzed, since a pathological effect of Sox11 misexpression on DG neurons was discovered in the present study. Conditional knockout of Sox4/11 in the adult DG for example by stereotactic injections of cKO Sox4/11 mice with a Cre-recombinase-encoding AAV (AAV-Cre) in context of antidepressant treatment might provide more clarity on whether effective antidepressant treatment depends on Sox11: Analyzing the effect of antidepressant treatment by behavioral analysis of AAV-Cre-injected mice compared to mice injected with a control AAV would allow for primary conclusions on Sox11's role for the amelioration of depressive symptoms.

Finally, a recent study revealed that Sox11 was induced by administration of morphine in the nucleus accumbens and that its knockdown modified the reward response (Koo et al., 2012). To mechanistically explain this finding and to understand how plasticity of neurons outside of the DG might be influenced by Sox11 represents an interesting topic for future studies. The study of Koo and colleagues discovered a potential extra-hippocampal influence of Sox11 on addiction. This might expand the importance of Sox11 beyond the adult hippocampal niche and beyond neurological disorders associated with this restricted brain region.

4 Materials and Methods

4.1 Materials

4.1.1 Chemicals and reagents

1 kb DNA ladder	New England Biolabs
100 bp DNA ladder	Fermentas
Acrylamide/Bis Solution (40 %)	Biorad
Ampicillin	Sigma-Aldrich
Anexate (0.1 mg/ml)	Hexal
Antibiotic-Antimycotic (100 x)	Gibco
Antisedan (5 mg/ml)	Pfizer
APS	Sigma-Aldrich
B27 (50 x)	Gibco
Bovine serum albumin (2 mg/ml)	Pierce
Bromophenol blue	Sigma-Aldrich
Chloroform	Roth
DAPI dilactate	Sigma-Aldrich
DMEM, High Glucose, Pyruvate (# 41966)	GIBCO/Invitrogen
DMEM/F-12, GlutaMAX™ (# 31331)	GIBCO/Invitrogen
DNA agarose	Biozym
Domitor (1 mg/ml)	Pfizer
Dormicum (5 mg/ml)	Roche
ECL Western Blotting Detection Reagents	Amersham
EDTA	Sigma-Aldrich
EGF	PeproTech
EtBr (1 mg/ml)	Roth
EtOH (100 %)	Merck
FBS (ultra low endotoxin)	PAA
Fentanyl (0.1 mg/ml)	Janssen-Cilag
FGF	PeproTech
Geneticin	GIBCO/Invitrogen
Glycerin/Glycerol (≥ 99,5 %)	Roth
HCl (32 %)	Merck
HEPES (1 M)	GIBCO/Invitrogen

HTN-Cre	provided by the laboratory
Isopropanol (100 %)	Merck
KCl	Merck
Laminin	Invitrogen
Lipofectamine 2000	Invitrogen
Loading dye (6 x)	BioLabs
MeOH (100 %)	Merck
Milk powder	BioChemica
NaAc	Merck
NaCl	Merck
NaOH tablets	Merck
Normal Donkey Serum	Chemicon
Page Ruler Plus Prestained Protein Ladder	Fermentas
PBS (1 x)	Gibco
PFA	Roth
PMSF	BioChemica
Poly-D-Lysine	Sigma
Power SYBR Green PCR Master Mix	Applied Biosystems
Protease inhibitor cocktail tablets (complete Mini)	Roche
Puromycin	Sigma-Aldrich
Random Primer Mix (60 µM)	NEB
SDS	Roth
Sunflower seed oil	Sigma
Tamoxifen	Sigma
Temgesic (0.3 mg/ml)	Essex Pharma
Tris-Base	Riedel-de Haen
Triton X-100	Roth
Trizol Reagent	Invitrogen
Trypsin-EDTA (0.5 %)	GIBCO/Invitrogen
β-Mercaptoethanol	Sigma-Aldrich

4.1.2 Buffers, solutions, and media

	Component	**Final c**	**Amount**	
10 x APS	APS	10 %(v/v)	1	g
	H_2O		10	ml
Awake	Temgesic	3.4 %(v/v)	0.17	ml
	Antisedan	5 %(v/v)	0.25	ml
	Anexate	50 %(v/v)	2.5	ml
	NaCl		2.08	ml
0.1 M Borate buffer (pH 8.5)	Boric acid	100 mM	3.09	g
	H_2O	*fill up to*	*500*	*ml*
2 M $CaCl_2$	$CaCl_2$	2 M	111	g
	H_2O	*fill up to*	*500*	*ml*
0.01 M Citrate buffer (pH 6.0)	Citric acid	10 mM	0.29	g
	H_2O	*fill up to*	*100*	*ml*
Cryoprotectant solution	Glycerol ≥ 99,5 %	25 %v/v)	250	ml
	Ethylene glycol	25 %v/v)	250	ml
	PO_4 buffer (0.1 M)	50 %(v/v)	500	ml
2 x HBS (pH 7.05, sterile)	NaCl	16 %(w/v)	8.0	g
	KCl	0.74 %(w/v)	0.37	g
	Na_2HPO_4	0.40 %(w/v)	0.20	g
	Glucose	2 %(w/v)	1.0	g
	HEPES	10 %(w/v)	5.0	g
	H_2O	*fill up to*	*500*	*ml*
HCl	HCl (32 %)	2 M	2.49	ml
	H_2O		9.51	ml

HEK293T cell medium	DMEM (# 41966)		500	ml
	Antibiotic-Antimycotic (100 x)	1 %(v/v)	5	ml
	FBS	10 %(v/v)	56	ml
HTN-Cre solvent	Glycerol (≥ 99,5 %)	50 %(v/v)	1	ml
	NaCl (5 M	0.5 M	200	µl
	HEPES (0.5 M	20 mM	80	µl
	H_2O		720	µl
4 x Laemmli	Tris	250 mM)	3	g
	SDS	4 % (v/v)	4	g
	Glycerol	40 % (v/v)	40	ml
	β-Mercaptoethanol	20 % (v/v)	20	ml
	(Bromophenol blue)			
	H_2O	*fill up to*	*100*	*ml*
Monolayer medium	DMEM/F-12 (# 31331)		500	ml
	Antibiotic-Antimycotic (100 x)	1 %(v/v)	5	ml
	B27 (50 x)	2 %(v/v)	10	ml
	HEPES (1 M)	1 %(v/v)	5	ml
	(FGF	freshly added)		
	(EGF	freshly added)		
3 M NaAc pH 5.0)	NaAc	3 M	12.3	g
	H_2O	*fill up to*	*50*	*ml*
10 x PAGE running buffer	Tris	250 mM	60.4	g
	Glycine	1.92 M	288	g
	SDS	1 %(w/v)	20	g
	H_2O	*fill up to*	*2*	*l*

10 x PAGE transfer buffer	Tris	250 mM	6.4	g
	Glycine	1.92 M	28.8	g
	MeOH	20 %(v/v)	400	ml
	H_2O	*fill up to*	*2*	*l*
PFA (pH 7.4)	PFA	4 %	40	g
	NaOH	tablets	1-2	
	PO_4 buffer (0.1 M)	*fill up to*	*500*	*ml*
0.2 M Phosphate buffer	NaH_2PO_4	0.55 %(w/v)	16.6	g
	Na_2HPO_4	2.19 %(w/v)	65.7	g
	H_2O	*fill up to*	*3*	*l*
RIPA	Tris (1 M)	1 mM	1	ml
	NaCl (5 M)	0.15 mM	3	ml
	Triton X-100 (25 %)	1 %(v/v)	4	ml
	DOC (10 %)	0.5 % (v/v)	5	ml
	SDS (10 %)	0.1 %(v/v)	1	ml
	EDTA (0.5 M)	1 mM	200	µl
	H_2O		86	ml
	(Protease inhibitor tablets	freshly added)		
	(PMSF (0.1 M)	freshly added)		
Sleep	Fentanyl	5 %(v/v)	0.25	ml
	Dormicum	10 %(v/v)	0.5	ml
	Domitor	5 %(v/v)	0.25	ml
	NaCl		4.0	ml
30 % Sucrose	Sucrose	30 %(w/v)	150	g
	PO_4 buffer (0.1 M)	*fill up to*	*500*	*ml*

50 x TAE	Tris	2 M	242	g
	Glacial acetic acid	1 M	57.1	ml
	EDTA (0.5 M)	0.05 M	100	ml
	H_2O	*fill up to*	*1*	*l*
10 x TBS (pH 7)	NaCl	1.37 M	80	g
	KCl	26 M	2	g
	Tris	250 mM	250	ml
	H_2O		750	ml
TBS++	Normal donkey serum	3 %(v/v)	1.5	ml
	Triton X-100 (10 %)	0.25 %(v/v)	1.25	ml
	TBS (1 x)	*fill up to*	*50*	*ml*
1 x TBST	Tween-20	0.1 %(v/v)	1	ml
	TBS (1 x)		1	l

4.1.3 Enzymes and enzyme buffers

5 PRIME Master Mix (2.5 x)	5 PRIME
Antarctic phosphatase buffer	New England Biolabs
Antarctic phosphatase (5 U/µl)	New England Biolabs
Buffer NEB4	New England Biolabs
iProof buffer HF(5)	Biorad
iProof polymerase (2 U/µl)	Biorad
PmeI	New England Biolabs
SfiI	New England Biolabs
T4 ligase (400 U/µl)	New England Biolabs
T4 ligase buffer with ATP (10 x)	New England Biolabs
RiboLock RNAse I	Fermentas
RevertAid Premium Reverse Transcriptase	Fermentas
RT Buffer (10 x)	Fermentas
DNAseQ	Promega
DNAse Buffer (10 x)	Promega

Taq DNA polymerase	5 PRIME
BSA (100 x)	New England Biolabs

4.1.4 Antibodies

Primary antibodies

Antigen	**Species**	**Dilution (IHC)**	**Company**
BrdU	rat	1:250	AbD Serotec
Calbindin	ms	1:1,000	Swant
Calrethinin	rb	1:1,000	Swant
DCX	gt	1:250	Santa Cruz Biotechnologies
DCX	rb	1:500	Abcam
GFP	ch	1:500	Aveslab
GFP	rb	1:1000 (WB)	Aveslab
Ki67	rb	1:500	Dako
NeuN	ms	1:50	Chemicon
NeuroD	gt	1:200	Santa Cruz Biotechnologies
Prox1	rb	1:500	Chemicon
Sox11	gt	1:500	Santa Cruz Biotechnologies
Sox11	rb	1:500	Millipore
β-gal	ch	1:500	ProSci Incorporated
β-gal	rb	1:2,000	Biogenesis
β-actin	ms	1:10,000 (WB)	Abcam
Stathmin	rb	1:5,000	Abcam
PSA-NCAM	ms	1:400	Millipore
NFIA	rb	1:1,000	Active Motif
Dll1	sh	various 1:1,000 (WB)	R&D Systems
Sox2	rb	1:250	Chemicon
Sox2	gt	1:500	Santa Cruz Biotechnologies
c-fos	rb	1:500	Santa Cruz Biotechnologies
His	rb	1:200 (WB)	Santa Cruz Biotechnologies
RFP	rat	1:50	(Rottach et al., 2008)

Secondary antibodies

All secondary antibodies were produced in donkey.

Fluorophore	Species	Dilution (IHC)	Company
633CF	gt	1:1,000	Biotium
633CF	ms	1:1,000	Biotium
633CF	rb	1:1,000	Biotium
Alexa488	gt	1:250	Jackson ImmunoResearch
Alexa647	sh	various	Jackson ImmunoResearch
Bio	sh	1:400	Jackson ImmunoResearch
Cy3	gt	1:250	Jackson ImmunoResearch
Cy3	ms	1:250	Jackson ImmunoResearch
Cy3	rb	1:250	Jackson ImmunoResearch
Cy3	sh	1:250	Jackson ImmunoResearch
Cy3	strep	1:500	Jackson ImmunoResearch
Cy5	gt	1:250	Jackson ImmunoResearch
Cy5	ms	1:250	Jackson ImmunoResearch
Cy5	rb	1:250	Jackson ImmunoResearch
Fitc	ch	1:250	Jackson ImmunoResearch
HRP	ms	1:10,000 (WB)	Jackson ImmunoResearch
HRP	rb	1:10,000 (WB)	Jackson ImmunoResearch
HRP	sh	1:10,000 (WB)	Jackson ImmunoResearch

4.1.5 Primers

All primers were purchased from Metabion (desalted and lyophilized).

qRT-PCR

Primer	Sequence (5'-3')	Efficacy (%)
Arc fwd	ACTCATTTGGCTGGCATAGG	99
Arc rev	AGATGGTATGGGCAGACAGC	99
DCX fwd	TGCTCAAGCCAGAGAGAACA	101
DCX rev	CTGCTTTCCATCAAGGGTGT	101
Dll1 fwd	CCCATCCGATTCCCCTTCG	88
Dll1 fwd	GGTTTTCTGTTGCGAGGTCATC	88
NFIA fwd	TGGCATACTTTGTACATGCAGC	93

Primer	Sequence (5'-3')	Efficacy (%)
NFIA rev	ACCTGATGTGACAAAGCTGTCC	93
Sox11 fwd	CCCTGTCGCTGGTGGATAAG	96
Sox11 rev	GGTCGGAGAAGTTCGCCTC	96
Srp14 fwd	CAGCGTGTTCATCACCCTCAA	92
Srp14 rev	GGCTCTCAACAGACACTTGTTTT	92
Stathmin fwd	AAAAGAATCTGTCCCCGATTTCC	102
Stathmin rev	TGTTAGCCTCCATTTTGTGGG	102

Overlap extension PCR

Primer	Sequence (5'-3')
ERT2 F fwd	GTGTTCACGTATCTCGAGCCATCTGCTGGAGA
Sox11 F rev	AGATGGCTCGAGATACGTGAACACCAGGTCGGA
Sox11-ERT2 F fwd	GAGGCCGCCTGGGCCATGGTGCAGCAGGCCGAG
Sox11-ERT2 F rev	GAGTTTAAACTCAAGCTGTGGCAGGGAAAC

4.1.6 Plasmids

pCAG-GFP	provided by the laboratory
pCAG-RFP	provided by the laboratory
pCAG-IRES-mtdsRed	provided by the laboratory
pCAG-GFP-IRES-Cre	provided by the laboratory
pCAG-CreERT2-IRES-GFP	provided by the laboratory
pCAG-Sox11-IRES-GFP	provided by the laboratory
pCAG-Mash1-IRES-RFP	provided by the laboratory
pCAG-NFIA-IRES-GFP	provided by the laboratory
pCAG-dnNFIA-IRES-GFP	provided by the laboratory
p2.9Op18-luc FL / Δ 300 / Δ 450 / Δ 1000	provided by J. Liu (Toronto) (Benlhabib and Herrera, 2006) (San-Marina et al., 2012)
Dll1-4.3p::luc	provided by Ph D. J. Breunig (Los Angeles)
phEF-Renilla	provided by the laboratory
pKSSP	provided by the laboratory
pKSPS	provided by the laboratory

4.1.7 Organisms

E. Coli cells (TOP10 and DH5α)	provided by the laboratory
HEK 293T cells	provided by the laboratory
Mouse adult neural stem cells (C57BL/6 and cKO Sox4/11)	provided by the laboratory
C57BL/6 mouse strain	Charles River Harlan provided by the HMGU
cKO Sox4/11 mouse strain	provided by PD Dr. E. Sock (Universität Erlangen) (Penzo-Mendez et al., 2007) (Bhattaram et al., 2010)

4.1.8 Kits

BCA Protein Assay Kit	Pierce
NucleoSpin Plasmid	Macherey-Nagel
NucleoBond Xtra Midi EF	Macherey-Nagel
NucleoSpin Extract II	Macherey-Nagel
QIAquick PCR Purification Kit	Qiagen
QIAquick Gel Extraction Kit	Qiagen
PureYield Plasmid Midiprep System	Promega
Dual-Luciferase Reporter Assay System	Promega
QuikChange II Site-Directed Mutagenesis Kit	Stratagene

4.1.9 Other material

Syringe Filter Units (22 µm)	Millipore
24 well plates	Falcon
Syringes (5 ml and 10 ml)	Braun
Multidish (6, 24, and 96 well)	Nunc
96-well polystyrene plates	Nunc
MicroAmp 96-well plates	Applied Biosystems
Aqua-Poly/Mount	Polysciences
BioTrace PVDF	Pall

Cell culture dish (100 x 20 mm)	Falcon
Cell scraper	Sarstedt
Cellstar pipettes	Greiner bio-one
Counting chamber (0.0025 mm^2)	Neubauer
Cover slips	Menzel-Glaser
Cryoblock mounting medium	Medite Medizintechnik GmbH
Glas capillaries	World precision instruments
Cannula needles	Braun
Microliter syringe	Hamilton
PCR 12-tube strips	Roth
PVDF membrane	Roche
Reaction tube 15 ml	Falcon
Reaction tube 50 ml	Falcon
Round bottom tube 14ml	Falcon
Safe lock tube 1.5 ml	Eppendorf
Safe lock tube 2.0 ml	Eppendorf
Superfrost Plus microscope slides	Menzel-Glaser
Surgical disposable scalpels	Braun
Tube 1.5 ml	Eppendorf
Tube 2.0 ml	Eppendorf
Whatman paper	GE Healthcare

4.1.10 Equipment

Hardware

Centrifuge 5415 D	Eppendorf
Centrifuge 5417 R	Eppendorf
Centro LB960 luminometer	Berthold Technologies
Digital Lab Standard Stereotaxic Instrument	Stoelting
DMI6000 B fluorescence microscope	Leica Biosystems
Fluorescence- and chemiluminescence system	Peqlab
FluoView1000 confocal fluorescence microscope	Olympus
Gelsystem Mini	Peqlab
HeraCell incubator	Thermo Scientific (Kendro)
HeraCell Tissue Culture hood	Thermo Scientific (Kendro)

Leica SP5 confocal microscope	Leica
Mini Protean Tetra Cell	Bio-Rad
ND-1000 spectrophotometer (Nanodrop)	Peqlab
PC-10 Puller	Narishige
PerfectBlue Twin-gel wide-format-system	Peqlab
Powersupply	Bio-Rad
Rotamax 150	Heidolph
Semidry blotting chamber	Bio-Rad
Sliding microtome Leica SM2000 R	Leica Biosystems
Sorvall Evolution High Speed Centrifuge	Thermo Science
Stereotactic chamber	Stoelting
Thermomixer comfort	Eppendorf
PCR cycler	Eppendorf
StepOne Real-time PCR System	Applied Biosystems

Software

FluoView 1.7	Olympus, Hamburg, Germany
Fusion	Vilber Lourmat, Eberhardzell, Germany
Imaris 7.5	Bitplane Scientific Software, Zurich, Switzerland
Leica Application Suite AF	Leica Microsystems, Wetzlar, Germany
NetPrimer	PREMIER Biosoft (http://www.premierbiosoft.com/netprimer/)
Primer3 (v. 0.4.0)	http://frodo.wi.mit.edu/
StepOne Real-Time PCR	Applied Biosystems, life technologies, CA, USA
Vector NTI	Invitrogen, Karlsruhe, Germany

4.2 Methods

4.2.1 Plasmid production

Heat shock transformation

100 µl competent *E. coli* cells were gently thawed on ice before DNA (1 µl-10 µl) was added. After incubation on ice for 15 min, cells were heat-shocked at 42 °C for 45 sec using a thermomixer and immediately placed back on ice for another 15 min. 1 ml LB medium was added and cells were incubated under agitation (350 rpm) at 37 °C for 1 h. After short centrifugation at 13,000 rpm supernatant was discarded except for 100 µl with which cells were resuspended and plated on agar plates containing 100 µg/µl ampicillin.
In case of critical transformation (e.g. after ligation) competent cells were transferred to 14 ml round-bottom Falcon tube prior to adding DNA. Heat shock was performed in a pre-warmed water bath. Centrifugation was omitted and whole cell suspension was plated on pre-warmed agar plates.
Plates were incubated at 37 °C over night (o/n).

Cultivation of bacteria and plasmid isolation

One colony of transformed E. coli was picked from the agar plate and transferred into 3 ml LB-medium containing 100 µg/ml ampicillin. Cells were cultured at 350 rpm and 37 °C for a minimum of 6 h or o/n.
Depending on the required amount of plasmid DNA two different procedures were followed. For purification of small amounts o/n cultures were subjected to NucleoSpin Plasmid kit according to manufacturer's protocol. If higher amount of DNA was desired, approximately 100 µl (depending on cell culture density) of cell suspension was transferred to 200 ml LB-medium containing 100 µg/ml ampicillin, as previously, and again incubated at 37 °C o/n. O/n cultures were processed with NucleoBond Xtra Midi EF kit or PureYield Plasmid Midiprep System kit following manufacturer's protocol. In the latter case the volume of resuspension and cell lysis solution was adjusted to 12 ml instead of 6 ml and of neutralization solution to 15 ml instead of 10 ml to improve recovery. Furthermore the centrifugation step after neutralization was extended to 30 min.
Plasmid DNA concentration and quality was measured using a Nanodrop spectrophotometer.

EtOH precipitation

If required (e.g. for subsequent virus production), DNA was further purified by EtOH precipitation. 1/10 volume 3 M NaAc (pH 5.0) and 2.5 volume 100 % EtOH were added to DNA and incubated for 1 h at -20 °C. After a 30 min centrifugation step at 14,000 rpm and 4 °C, DNA pellet was washed by covering with 70 % EtOH and repeating centrifugation. Finally pellet was air-dried, resuspended in 50 µl MilliQ, and DNA concentration was determined as described above.

Cloning

The DNA of interest to be cloned (insert) was either obtained by restriction digest of plasmid containing the insert with suitable restriction endonucleases (REs), amplified by PCR using specific primers, or obtained by overlap extension PCR (Horton et al., 1989). Restriction digest of plasmid providing the backbone (backbone) and the insert was pipetted and incubated as required by the protocol (NEB) choosing appropriate REs, buffers, incubation temperature, and duration.

To prevent backbone self-ligation and re-circularization an additional de-phosphorylation step was performed subsequent to restriction digest. The backbone was incubated with antarctic phosphatase (5 U/µl) at 37 °C for 30 min. 3 U of phosphatase were used for 1 µg of DNA.

Digested DNA fragments were subjected to agarose gel electrophoresis and extracted using NucleoSpin Extract II kit according to manufacturer's protocol.

For ligation amount of insert (i) was calculated relative to amount of backbone (b) and with respect to length of fragments (l), while multiplying factor (mf) varied between 3 and 10.

$$m_i = \frac{m_b \times l_i}{l_b} \times mf$$

Ligation was performed at a temperature ranging between 16 °C to 4 °C o/n or for up to three days. To exclude re-ligation of the backbone a control ligation without insert was performed. Ligation product was transformed as described above (s. Heat shock transformation). Plasmid was purified with NucleoSpin Plasmid kit. Subsequent restriction digest or colony-PCR using Taq-polymerase and 5 PRIME Master Mix according to manufacturer's protocol was performed to identify positive clones.

4.2.2 HEK 293T cells

Culturing

HEK 293T cells were cultured in DMEM (# 41966) supplemented with 1 % Antibiotic-Antimycotic and 10 % FBS. Cells were grown on uncoated cell culture dishes (10 cm) at 5 % CO_2 and 37 °C and passaged by trypsination when confluency of app. 70 % was reached. Cells were rinsed once with 1 x PBS and detached by adding 2 ml Trypsin-EDTA. 5 ml medium was added to neutralize trypsin, cell suspension was centrifuged at 0.3 rcf for 2 min, supernatant was discarded, and cells resuspended in 1 ml medium. Depending on desired dilution appropriate amount of cell suspension was transferred to a new cell culture dish with 8 ml fresh medium.

$CaCl_2$ mediated transfection

One day before transient transfection cells were trypsinized as described above (s. Culturing). 10 µl of the cell suspension was diluted with 90 µl medium (dilution factor 10) and 15 µl of the dilution were transferred to a counting chamber. Four quadrants were counted and cell concentration (c) was determined:

$$c(\frac{cells}{ml}) = \frac{cell\ count}{4} \times dilution\ factor \times 10^4$$

Knowing the number of cells needed for transfection (n_c) the adequate volume of the cell suspension (V) was calculated:

$$V(ml) = \frac{n_c}{c}$$

The cell suspension was filled up with medium to the overall volume needed for transfection. For later luciferase assay cells were incubated in 24 well plates o/n (approximately 30,000 cells in 1 ml medium per well).

For transfection 2 x HBS and 2 M $CaCl_2$ were pre-warmed under the hood at room temperature (RT) for 30 min. DNA to be transfected was composed of different plasmids: Firefly luciferase-reporter construct (Table 4.1), renilla luciferase (phEF-Renilla) as internal control, plasmid encoding for protein-of-interest, and corresponding empty plasmid to equalize DNA amount. For each approach total amount of DNA was constantly 500 ng and total volume of DNA solution remained 43.8 µl. A Master Mix (MM) of luciferase-reporter (150 ng) and renilla (5 ng) was prepared. For each experimental condition triplicates were prepared individually. Starting with the suitable volume of MilliQ to adjust the end volume of 43.8 µl, 2 µl of

MM was added, followed by plasmid with gene for protein-of-interest and empty plasmid.

DNA was mixed properly with 6.2 µl 2 M $CaCl_2$. Subsequently, 2 x HBS was added and mixed thoroughly generating air bubbles. The transfection reaction was further incubated at RT for 30 min and added dropwise to the cells. After o/n incubation medium was replaced in the early morning to prevent acidification. By estimation of the percentage of cells expressing green fluorescent protein (GFP) or red fluorescent protein (RFP) (depending on fluorophore expressed by transfected plasmids) transfection efficiency was assessed 24 h later.

Lipofectamine mediated transfection

HEK293T cells were prepared on 24 well plates as in case of $CaCl_2$ mediated transfection. DNA solution consisting of luciferase-reporter, renilla, plasmid encoding for protein-of-interest, and empty plasmid was also pipetted as described for $CaCl_2$ mediated transfection but the end volume added up to 50 µl. Per well 2 µl Lipofectamine 2000 in 50 µl medium without antibiotics was needed. According to number of wells a MM of both components was prepared and incubated at RT for 5 min. 50 µl MM was mixed thoroughly with 50 µl DNA solution, shortly centrifuged, and incubated at RT for 20 min before being added to cells. Further procedure is again in accordance with $CaCl_2$ mediated transfection.

Luciferase assay

Dual-Luciferase Reporter Assay System kit was used to perform luciferase assays. 5 x Lysis buffer was diluted with MilliQ and firefly luciferase buffer as well as renilla luciferase buffer was prepared according to manufacturer's protocol. 48 h after transfection medium was aspired and cells were lysated in 200 µl 1 x Lysis buffer at 250 rpm for 10 min. Cell lysates were resuspended several times before 15 µl were transferred to a 96-well polystyrene plate. Measurement was performed using a Centro LB960 luminometer.

For analysis of luciferase assays the values of the ratio between firefly and renilla activity were used to calculate the fold induction of firefly luciferase activity. Values were divided by the so-called basal value, i.e. value for cells transfected only with the luciferase-reporter, the internal control, and the empty plasmid but without plasmid encoding for protein-of-interest. For this basal value a 1-fold increase was determined.

Table 4.1 Firefly luciferase-reporter constructs for luciferase assays.

Name	Region upstream of TSS (kb)
p2.9Op18-luc FL	-1.10 (+ 1.90 upstream of ATG)
p2.9Op18-luc Δ 300	-0.80 (+ 1.60 upstream of ATG)
p2.9Op18-luc Δ 450	-0.65 (+ 1.45 upstream of ATG)
p2.9Op18-luc Δ 1000	-0.10 (+ 0.90 upstream of ATG)
Dll1-4.3p::luc	-4.3

4.2.3 Mouse adult neural stem cells

Culturing

Mouse adult neural stem cells were cultured in DMEM/F-12 (# 31331) supplemented with 1 % Antibiotic-Antimycotic, 1 % B27, 2 % HEPES, and EGF and FGF (20 ng/µl), which were supplemented every second day. Cells were grown as adherent cultures on Poly-D-Lysine/Laminin coated cell culture dishes (10 cm) at 5 % CO_2 and 37 °C and passaged by trypsination when confluency of app. 70 % was reached. Except for different amount of Trypsin-EDTA (1 ml) and of medium for neutralization (8 ml), trypsination was performed as described above (s. 4.2.2).

Viral transduction

Prior to viral transduction cells were passaged as described above. Cell density was determined using a counting chamber and 1-2 x 10^6 cells per cell culture dish were seeded. Cells were incubated for 4 h. Retroviruses were gently thawed on ice. 1-2 µl of virus was added to each dish, which was gently shaken to ensure equal viral distribution. By estimation of the percentage of cells expressing GFP or RFP (depending on fluorophore encoded by transduced virus) transduction efficiency was assessed 24 h later. One day later RNA was isolated from cells (s. 4.2.7).

HTN-Cre transduction

As an alternative to viral transduction protein transduction of the HTN-Cre protein was performed with the aim to improve recombination of conditional knockout (cKO) Sox4/11 monolayer cells. The HTN-Cre protein is a fusion protein consisting of a His-tag (H), a protein transduction domain (T), a nuclear localization sequence (N) and

the Cre recombinase (Cre). This composition enables purification of the protein (by H), endocytosis into the cytoplasm (by T), and nuclear import (by N).

Optimal HTN-Cre concentration, at which recombination was sufficient and side effects or toxicity were minimal, was determined by titration. Monolayer cells, either wild type (wt) or cKO Sox4/11, were treated with 0 µM, 0.5 µM, 1.0 µM, or 2.0 µM HTN-Cre, while best results were obtained at a concentration of 0.5 µM under proliferative conditions (differentiation by withdrawal of GFs resulted in considerable loss of cells with time).

One day prior to transduction cells were passaged as described above. Cell density was determined using a counting chamber and 2 x 10^6 cells were seeded per cell culture dish. Cells were incubated o/n. On the next day fresh medium plus GFs (6 ml per dish) was prepared. HTN-Cre (stored at -20 °C) was gently thawed on ice. As control solvent only (-Cre) or HTN-Cre (+Cre) at a final concentration of 0.5 µM was added to pre-warmed medium. Old medium was aspirated and medium +/- Cre was added to monolayer cells. Incubation time varied between 16 h and 20 h depending on viability of cells. Medium with or without Cre was removed, monolayer cells were washed once with 1 x PBS, and fresh pre-warmed medium plus GFs was added. Monolayer cells were kept under proliferating condition for one or two more days (depending on cell density) prior to RNA isolation.

4.2.4 Viral approaches

Virus production

Single-cell genetic manipulation in an intact brain environment was achieved by stereotactic injections of a retrovirus and/or adeno-associated virus (AAV).

Production of replication incompetent retrovirus was performed in HEK293T cells as described previously (Tashiro et al., 2006b). The cytomegalovirus (CMV) promoter, which is based on the Moloney murine leukemia virus (MoMLV), drives transcription of the modified viral genome, while structural and enzymatic proteins (gag/pol) and envelope proteins (vsv-g) were separately encoded on two plasmids, CMV-gag/pol and CMV-vsv-g. The third plasmid to be transfected was the retroviral vector that contained the cDNA of interest (xx), CAG-xx (Table 4.2). Transfection was performed according to the Lipofectamine 2000 protocol. Virus was harvested four times (2, 4, 6, and 8 d after transfection) and concentrated by two rounds of ultra-centrifugation. Katrin Wassmer performed retrovirus production.

Viral titers were determined by transduction of HEK293T with a serial dilution of the retrovirus. Fluorescent cells were counted under the DMI6000 B fluorescence microscope and viral titer was calculated as colony-forming units (cfu) per ml. Viral titers ranged between 1 x 10^8 cfu/ml and 1 × 10^9 cfu/ml, while retrovirus was injected at a titer of 1-2 x 10^8 cfu/ml.

AAVs (serotype 9) containing different cDNA of interest (xx) (AAV-xx, Table 4.2) were not produced in our laboratory but were kindly provided by B. K. Kaspar, Ohio (Kaspar et al., 2002; Xiao et al., 1998). Viral titers ranged between 1 x 10^{10} cfu/ml and 1 × 10^{11} cfu/ml. A titer of 1 x $10^{10\text{-}11}$ cfu/ml was used for AAV-injections.

Table 4.2 Retro- (CAG) and adeno-associated (AAV) viruses used in this study.
Denomination of viruses according to the cDNA of interest together with the fluorophore (GFP, RFP, or mtdsRed) encoded by the virus. Each virus is further assigned to the paradigm for which it was used.

Virus	**Paradigm**
CAG-IRES-GFP	Control virus for CAG-Cre, 5 dpi Control virus for Sox11, 21 dpi and 6 wpi
CAG-IRES-RFP	Control virus for Sox11, 21 dpi and 6 wpi Birth dating for AAV-Sox11 vs. AAV-GFP
CAG-GFP-IRES-Cre	Conditional knockout of Sox4/11, 5 dpi
CAG-Sox11-IRES-GFP	Overexpression of Sox11, 21 dpi and 6 wpi
CAG-NFIA-IRES-GFP	Overexpression of NFIA, 23 dpi
CAG-dnNFIA-IRES-GFP	Expression of dnNFIA, 10 dpi
CAG-CreERT2-IRES-GFP	Tamoxifen-mediated knockout of Sox4/11, 11 and 14 dpi
CAG-IRES-mtdsRed	Control virus for Sox11, 6 wpi
CAG-Mash1-IRES-RFP	Overexpression of Mash1, *in vitro*
AAV-GFP	Control virus for AAV-Cre/GFP and AAV-Sox11
AAV-Cre/GFP	Conditional knockout of Sox4/11
AAV-Sox11	Overexpression of Sox11

Stereotactic injections

All experiments were carried out in accordance with the European Communities Council Directive (86/609/EEC). The Government of Upper Bavaria approved the stereotactic viral injections into the brain of adult mice. For all experiments 7 w to 12 w old mice (either C57BL/6 or cKO Sox4/11) were used and grouped housed in standard cages with *ad libitum* access to food and water under a 12 h light/dark cycle. One week prior to injection until one week after injection cages were equipped with running wheels to increase the number of transduced cells for later analysis.
Anesthetics (Sleep and Awake) were prepared as described in 4.1.2. Mice were anesthetized by intraperitoneal (ip) injection of 300-400 µl Sleep (depending on weight). As soon as mice were deeply narcotized they were fixed in a stereotactic chamber and the scalp was opened with a scalpel at a length of app. 0.5-1 cm. Coordinates for injections into the HC were set relative to bregma (medial/lateral = ±1.6; anterior/posterior = -1.9) and holes for injections into both hemispheres were drilled into the skull. 0.9 µl virus, which was diluted with 1 x PBS to appropriate concentration, was injected into the hilus of each hemisphere (coordinates dorsal/ventral from *dura mater* = -1.9) at a speed of 250 nl/min using a Digital Lab Standard Stereotaxic Instrument. Coordinates for injection of the SVZ/RMS were medial/lateral = ± 0.8, anterior/posterior = +3.3 and dorsal/ventral = -2.9. Transferring mice onto a heating plate normalized body temperature, scalp was sutured, and 400 µl Awake was injected ip to antagonize Sleep and wake up mice.
According to experimental paradigm mice were perfused at distinct points in time post injection (pi). In case of adeno-associated viral overexpression of Sox11 in mature DG neurons retroviral injection of CAG-RFP was followed by a second injection of the AAV as described above.

Tamoxifen treatment

To induce recombination in cKO Sox4/11 mice, ip injections of Tamoxifen were required after retroviral injection of CAG-CreERT2-IRES-GFP (7 dpi). Tamoxifen was dissolved in 100 % EtOH (9 parts) and sunflower seed oil (1 part) shaking at 4 °C to reach a concentration of 10 mg/ml. Solution was stored at -20 °C during daily use for a maximum of two weeks. Each mouse was injected with 100 µl of this solution (i.e. 1 mg Tamoxifen) twice a day in the morning and in the evening for five consecutive days to ensure effective recombination (Mori et al., 2006).

BrdU treatment

For proliferation analysis after AAV-Cre injections, 100 mg/kg BrdU, which was dissolved in NaCl at a concentration of 10 mg/ml and sterile-filtered, was administered to mice according to their weight by ip injection. Mice were injected on two consecutive days in the morning, evening, and morning again. Perfusion was performed on the evening of the second day.

4.2.5 Electroconvulsive shock

Tissue of mice that received electroconvulsive shock (ECS) treatment was kindly provided by the laboratory Prof. H. Song, Baltimore. ECS was delivered via bilateral ear clip electrodes for 0.5 sec at 55 mA and 100 Hz. Sham mice, which served as control, were handled identically but received no shock (Warner-Schmidt et al., 2008). Mice were perfused 3, 6, or 24 h after ECS and immunohistochemically analyzed for protein expression (s. 4.2.6), or DG of sacrificed mice (cervical dislocation 30 min, 3 h or 6 h after ECS) was microdissected for further RNA isolation and analysis (s. 4.2.7).

4.2.6 Immunohistochemical analysis

Perfusion

Animals were deeply anesthetized with CO_2. Transcardial perfusion was performed at a speed of 10 ml/min applying first 1 x PBS followed by 4 % PFA (pH 7.4) each for 5 min. Brains were removed and post-fixed in 4 % PFA at 4 °C o/n. Subsequently, brains were transferred into 30 % sucrose in which they were stored at 4 °C until further use. Perfusion was performed with the help of Fabian Gruhn.

Tissue processing

Brains were cut coronally for analysis of the HC or sagitally for analysis of the SVZ/RMS/OB using a sliding microtome. Thickness of sections varied according to further application, 40 µm or 50 µm for immunohistochemical analysis of protein expression or 100 µm for morphological analysis. Sections were stored in 96-well plates at -20 °C, each well filled with 200 µl cryoprotectant solution.

Free-floating staining procedure

At least three appropriate sections containing the region of interest were selected for each mouse at intervals of four to six wells and transferred to 12 well plates with

nets. Sections were rinsed 3 times for 15 min in 1 x TBS followed by a blocking step with TBS++ at RT for 1 h to 2 h. Primary antibody solution (maximum of three different antibodies from different species) was prepared in TBS++ according to known optimal dilution (s. 4.1.4) Sections were incubated with primary antibodies in 1.5 ml Eppendorf tubes under rotation at 4 °C for 2 d. Sections were washed again three times (as described above) and subsequently blocked with TBS++ at RT for 30 min. Meanwhile secondary antibodies (s. 4.1.4) were diluted in TBS++. To prevent bleaching of fluorophore linked to secondary antibodies following steps were performed in the dark. Sections were incubated in secondary antibody solution in 1.5 ml Eppendorf tubes under agitation at RT for 2 h or at 4 °C o/n. After one washing step with 1 x TBS for 15 min and incubation with DAPI (1:10,000 in 1 x TBS) for 15 min, sections were washed two more times before being mounted on Superfrost Plus microscope slides with cover slips using Aqua-Poly/Mount. Slides were dried at RT o/n and stored at 4 °C until further use.

For BrdU staining, sections had to be pre-incubated in 2 M HCl at 37 °C for 30 min to make DNA accessible for the antibody. Sections were incubated in 0.1 M borate buffer twice for 10 min to regain former pH. After 6 washing steps in 1 x TBS à 15 min, sections were further processed according to standard staining protocol (s. above).

If signal amplification was desired, sections were incubated with a biotinylated (bio) secondary antibody o/n followed by incubation with a streptavidin (strep) -conjugated fluorophore for 4 h or up to 1 d.

Imaging

After immunohistochemical treatment cells were imaged using a Leica SP5 confocal microscope at a resolution of 1024x1024. Scanning parameters as objective, zoom, and step size varied depending on which analysis was to be conducted.

For subsequent analysis of protein expression and somatic positioning a 40 times objective (immersion oil) was used without zoom and the step size ranged between 1.0 µm and 1.5 µm. In case of further morphological analysis, only cells located in the middle of the upper blade of the DG were scanned to exclude effects of cellular localization on morphological properties. A 40 times objective (immersion oil) or 63 times objective (glycerol) was used without zoom and the step size was 0.4 µm. For later spine analysis dendrites of cells, which were chosen the same way as for

morphological analysis, were scanned in the middle of the MCL using a 63 times objective (immersion oil) with 5 times zoom and a step size of 0.2 µm.

Analysis

Depending on type of analysis different software and different number of retrovirally transduced cells per animal were used.

Leica Application Suite AF was used to analyze protein expression, somatic positioning, number of basal processes, length of dendritic extension towards the hippocampal fissure, and number and type of spines. Protein expression was analyzed either absolutely whether or not protein was expressed or quantitatively by measuring signal intensity relative to overall staining intensity. 50-100 cells were analyzed per animal. Somatic positioning was measured as the distance of the soma to the SGZ-hilus border relative to total GCL thickness visualized by DAPI staining. This resulted in a so-called positioning index with values ranging between 0 and 1, which was determined for approximately 40 cells. Cellular processes directed towards the hilus were counted as basal processes. For this quantification approximately 40 cells were analyzed. Length of dendritic extension was determined by measuring the distance between the soma and the endpoint of the longest dendrite relative to the distance of the some to the dendritic fissure visualized by DAPI. The values of this analysis, which included approximately 40 cells, are given as percentage of potential dendritic extension. Number of spines of 5-8 dendrites per animal was counted manually and diameter of spine heads was measured, while spines with a diameter bigger than 0.6 µm were defined as mushroom spines.

For analysis of morphological parameters as dendritic length, number of dendrite branching points, and dendrite branching characteristics Imaris 7.5 software was used. Cells (1-4 cells per animal, at least 12 cells per group) that expressed the retrovirally encoded fluorophore in sufficient amount to reliably detect dendrites were reconstructed by semi-automatically tracing the dendritic tree. This procedure provided data on dendritic length and branching points as well as for Scholl analysis.

4.2.7 Analysis on RNA level

RNA isolation

RNA was isolated either from micro-dissected DG or from mouse adult neural monolayer cells. Tissue (50-100 ng) was homogenized in 2 ml safe lock tubes by resuspension in 1 ml trizol using a cannula needle. In the latter case, medium of cells

was aspirated and 1 ml trizol per dish was added. Cells were scratched of cell culture dish and cell suspension was transferred to a 2 ml safe lock tube. If later continuation of RNA isolation was desired, tubes were frozen at -80 °C.

0.2 ml chloroform per 1 ml trizol was added to tubes. Solution was mixed well by inverting tubes several times (app. 15 sec), incubated at RT for 3 min, and centrifuged at 12,000 rcf and 4 °C for 15 min. Upper aqueous phase, which contains RNA, was transferred into new 1.5 ml safe lock tube. To precipitate RNA 0.5 ml (per 1 ml trizol) was added. After incubation at RT for 10 min and centrifugation at 12,000 rcf and 4 °C for 30 min, supernatant was carefully removed. Pellet was briefly vortexed in 1 ml 70 % EtOH (prepared with RNAse free water) and centrifuged once more at 12,000 rcf and 4 °C for 10 min. Supernatant was removed and air-dried pellet was dissolved in 10-50 µl RNAse free water, while shaking in a thermomixer at 450 rpm and 55 °C for 10 min. RNA concentration and quality was measured using a Nanodrop spectrophotometer. RNA was stored at -80 °C or directly used for cDNA synthesis.

cDNA synthesis

RNA amounts varied between 500 ng and 3 µg limited by the lowest RNA concentration of samples to be analyzed. For each sample the same RNA amount was used. Preceding cDNA synthesis, RNA was subjected to DNAseQ treatment according to manufacturer's protocol (Promega). All steps of cDNA synthesis were pipetted on ice and for incubation a PCR cycler was used. Each sample was divided in half, while one half to which no reverse transcriptase (RT) was added served as a negative control (-RT). 2 µl Random Primer Mix, which includes dNTPs (1 mM), and 7.5 µl RNAse free water were added to RNA. After incubation at 65 °C for 5 min, 4 µl RT Buffer, 0.5 µl RiboLock RNAse, and 1 µl RevertAid premium RT (not in case of -RT) were added. Reaction was incubated at 25 °C for 10 min, followed by 60 °C for 30 min and finally terminated at 85 °C for 5 min. cDNA was stored at -20 °C.

qRT-PCR

StepOne Real-Time PCR software was used for quantitative real time PCR (qRT - PCR) experiments, which were run on a StepOne Real-time PCR System.

NetPrimer and Primer3 software was used for design of primers (4.1.5). If not published or yet tested in the laboratory, primer efficiency was calculated on the basis of C_T values measured at different cDNA concentrations.

Primer stocks (100 µM) were diluted 1:10. cDNA dilution varied between 1:2 and 1:20 depending on effectiveness of cDNA synthesis. The reaction volume per sample was 20 µl composed of MilliQ (8.2 µl), fwd and rev primer (each 0.4 µl), Power SYBR Green PCR Master Mix (10 µl), and cDNA template (1 µl). For each primer a MM was prepared except for cDNA template and distributed into MicroAmp 96-well plates (19 µl per well). cDNA was added, while triplicates were pipetted for each sample. C_T values were exported from StepOne Real-Time PCR software and evaluated using Excel.

4.2.8 Analysis on protein level

Protein isolation

Proteins were isolated from monolayer cultures that were scraped of cell culture dishes in 10 ml of ice-cold 1 x PBS, centrifuged in 15 ml falcons at 0.3 rcf and 4 °C for 5 min and washed twice more by resuspending cell pellet in 1 ml of ice-cold 1 x PBS and centrifugation. Protease inhibitor cocktail tablets were dissolved in MilliQ (1 tablet / 1.5 ml) and 150 µl of Protease inhibitor cocktail was freshly added together with 10 µl PMSF (0.1 M) to 845 µl of ice-cold RIPA buffer, while 300 µl were needed for each cell culture dish. Pellet was resuspended in RIPA buffer and left under agitation at 4 °C for 1 h. After centrifugation at 16.000 rcf and 4 °C for 20 min supernatant representing the total cell extract was transferred into a 1.5 ml Eppendorf tube. Protein concentration was determined using the BCA Protein Assay kit according to manufacturer's instructions or by measurement at a Nanodrop spectrophotometer. Protein solution was stored at -80 °C until further use.

SDS polyacrylamide gel electrophoresis

To separate and analyze proteins SDS polyacrylamide gel electrophoresis (SDS-PAGE) was performed. Gels composed of stacking and resolving gel (Table 4.3) were prepared in a MINI protean system or PerfectBlue Twin-gel wide-format-system.

Proteins were denaturized in 4 x Laemmli buffer at 95 °C for 5 min before being loaded on the gel together with 10 µl Page Ruler Plus Prestained Protein Ladder using a microliter syringe. Once proteins reached the separating gel (90 V for app. 10 min), proteins were separated at 120 V in 1 x PAGE running buffer until desired separation was reached.

Table 4.3 Stacking and resolving gel for SDS-PAGE.
Different acrylamide concentrations in the resolving gel (ranging from 7.5 % to 15 %) were chosen depending on size of proteins to be separated.

Stacking gel	**5 %**
Upper Tris (0.5 M, 0.4 % SDS, pH 6.8) (µl)	625
H_2O (ml)	1.56
40 % Acrylamide (µl)	312
TEMED (µl)	3
10 % APS (µl)	15

Separating Gel	**7.5 %**	**10 %**	**12 %**	**15 %**
Lower Tris (1.5 M, 0.4 % SDS, pH 8.8) (ml)	1.25			
H_2O (ml)	2.81	2.50	2.25	1.88
40 % Acrylamid (ml)	0.94	1.25	1.50	1.88
TEMED (µl)	5			
10 % APS (µl)	20			

Western Blot

Proteins separated by SDS-PAGE were further analyzed by Western Blot (WB) using a semidry blotting chamber. Whatman papers were soaked in 1 x PAGE transfer buffer (diluted with MilliQ) prior to use, while PVDF membrane had to be activated in 100 % MeOH for 1 min. Components were stacked in the blotting chamber according to following scheme.

Bottom 3 Whatman paper - gel - PVDF membrane - 3 Whatman paper **Top**

Transfer settings varied between 12-20 V and 30-60 min according to molecular weight (MW) of proteins (the higher the MW the lower the voltage and longer the duration). After blocking membrane in 5 % milk powder dissolved in 1 x TBST (5 % milk) at RT for 1 h, it was incubated in 50 ml falcon tubes with primary antibodies (s.4.1.4) diluted in 5 % milk at 4 °C o/n. The membrane was washed three times in 1 x TBST for 10 min and then incubated with secondary antibodies (s. 4.1.4) diluted

in 1 x TBST at RT for 60 min. After six washing steps à 5 min, the membrane was covered with approximately 1 ml ECL solution, which was freshly prepared by mixing ECL Western Blotting Detection Reagents 1 and 2 (1:1), and incubated for at least 1 min. Signals were visualized by camera detection using a fluorescence- and chemiluminescence system in combination with the Fusion software.

4.2.9 Statistics

Excel was used for statistical analysis. Significances were determined with homo- or heteroscedastic student t-test or two-factor ANOVA with replication. Error bars represent standard deviation (SD) if not indicated otherwise and results are represented as mean ± SD. Number of biological replicates (n) is individually specified for each analysis. Significance is depicted by * for $p < 0.05$, ** for $p < 0.001$, and *** for $p < 0.0001$.

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6 Appendix

Curriculum Vitae

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Ausbildung

10/2009 - aktuell	**Technische Universität München** Doktor der Naturwissenschaften Dissertation: „Sox11 als Regulator der neuronalen Reifung & Integration neugeborener Neuronen im adulten Hippocampus“
10/2007 - 08/2009	**Technische Universität München** M. Sc. in Molekularer Biotechnologie (Note 1,1) Masterarbeit: „Die Rolle des beta catenin-Tcf/LEF Signalwegs in der adulten hippocampalen Neurogenese“
10/2004 - 09/2007	**Technische Universität München** B. Sc. in Biochemie (Note 1,4) Bachelorarbeit: „Die Synthese multimerer RGD-Peptide für biologische Anwendungen“
06/2003	**Gymnasium Kirchheim b. München** Abitur (Note 1,2)

Berufserfahrung

10/2008-12/2008	**The Boston Consulting Group, München** ***Visiting Associate*** *Aufgaben*: Analyse des internationalen pharmazeutischen Marktes, Dokumentation, Präsentationsvorbereitung
07/2008-09/2008	**National University of Singapore, Singapur** ***Praktikantin*** *Aufgaben*: Zellkultur, Fluoreszenzmikroskopie *Projekt*: Untersuchung des Proteintransports (Sec13 und Sec24) in T. burcei

03/2008-04/2008 **Technische Universität München, München**
Praktikantin
Aufgaben: Zellkultur, molekularbiologische Methoden

03/2006-04/2006 **Technische Universität München, München**
Praktikantin
Aufgaben: Peptidsynthese, Flüssigchromatographie, Kernresonanzspektroskopie, Massenspektrometrie
Projekt: Diastereoselektive Synthese von Phosphinodipeptidisosteren

10/2003-12/2003 **Micromet, München**
Studentische Hilfskraft
Aufgaben: Proteinanalytik, Assistenz Proteinchromatographie

Stipendien und Auszeichnungen

05/2010-05/2013 **Universität Bayern e. V.**
Graduiertenstipendium nach dem Bayerischen Eliteförderungsgesetz

01/2004-05/2013 **e-fellows.net**
Online-Stipendium

11/2012 **ForNeuroCell II Symposium, München, Deutschland**
Preis für bestes Poster („Role of SoxC transcription factors in adult hippocampal neurogenesis“)

03/2009 **German/Polish Life Science Meeting, Krakau, Polen**
Preis für beste Präsentation („Wnt/beta-catenin as regulators in the development of neural stem cells“)

07/2008 **Deutscher Akademischer Austauschdienst**
Reisekostenzuschuss

Publikationen

Mu, L., Berti, L., Masserdotti, G., Covic, M., Michaelidis, T.M., Doberauer, K., Merz, K., Rehfeld, F., Haslinger, A., Wegner, M.*, et al.* (2012). SoxC transcription factors are required for neuronal differentiation in adult hippocampal neurogenesis. The Journal of Neuroscience *32*, 3067-3080.

Karalay, O., Doberauer, K., Vadodaria, K.C., Knobloch, M., Berti, L., Miquelajauregui, A., Schwark, M., Jagasia, R., Taketo, M.M., Tarabykin, V.*, et al.* (2011). Prospero-related homeobox 1 gene (Prox1) is regulated by canonical Wnt signaling and has a stage-specific role in adult hippocampal neurogenesis. Proceedings of the National Academy of Sciences of the United States of America *108*, 5807-5812.

Fortbildung

12/2012	**Helmholtz Zentrum München, München, Deutschland** Seminar „Systematische Karriereplanung und -entwicklung“
11/2012	**ForNeuroCell II Symposium, München, Deutschland**
07/2012	**Helmholtz Zentrum München, München, Deutschland** Seminar „Good manufacturing practice - Basis-Schulung“
04/2011	**ForNeuroCell Symposium, München, Deutschland**
10/2010	**4th International Symposium "Stem Cells, Development and Regulation", Amsterdam, Netherlands**
03/2009	**German/Polish Life Science Meeting, Krakau, Polen**
12/2007	**Roche, Penzberg, Deutschland** Workshop „Professionell Präsentieren und Moderieren“
11/2007	**McKinsey, Kitzbühel, Österreich** Workshop

Zusatzqualifikationen und Interessen

Sprachen	Englisch: Verhandlungssicher Französisch: Grundkenntnisse
Computerkenntnisse	Microsoft Office (Word, Excel, PowerPoint)
Interessen	Yoga, Wandern, Kochen, Backen, Volleyball, Mountainbiken, Theater, Lesen
Soziales Engagement	Patenschaft Indien, Institute for Indian Mother and Child (11/2005 - 08/2012)
Reisen	Namibia (08/2009 - 09/2009), Skandinavien (08/2007 - 09/2007), Costa Rica (09/2006 - 10/2006), Transsibirische Eisenbahn (03/2004 - 07/2004)

Eidesstaatliche Erklärung

Ich erkläre an Eides statt, dass ich die bei der Fakultät Wissenschaftszentrum Weihenstephan für Ernährung, Landnutzung und Umwelt der Technischen Universität München zur Promotionsprüfung vorgelegte Arbeit mit dem Titel:

Sox11 as a regulator of neuronal maturation & integration of newborn neurons in the adult hippocampus

Am Lehrstuhl für Entwicklungsgenetik unter der Anleitung und Betreuung durch

Univ.-Prof. Dr. W. Wurst

ohne sonstige Hilfe erstellt und bei der Abfassung nur die gemäß § 6 Abs. 6 und 7 Satz 2 angegebenen Hilfsmittel benutzt habe.

Ich habe keine Organisation eingeschaltet, die gegen Entgelt Betreuerinnen und Betreuer für die Anfertigung von Dissertationen sucht, oder die mir obliegende Pflichten hinsichtlich der Prüfungsleistungen für mich ganz oder teilweise erledigt.

Ich habe diese Dissertation in dieser oder ähnlicher Form in keinem anderen Prüfungsverfahren als Prüfungsleistung vorgelegt.

Ich habe den angestrebten Doktorgrad **noch nicht** erworben und bin **nicht** in einem früheren Promotionsverfahren für den angestrebten Doktorgrad endgültig gescheitert.

Die öffentlich zugängliche Promotionsordnung der TUM ist mir bekannt, insbesondere habe ich die Bedeutung von § 28 (Nichtigkeit der Promotion) und § 29 (Entzug des Doktorgrades) zur Kenntnis genommen. Ich bin mir der Konsequenzen einer falschen Eidesstattlichen Erklärung bewusst.

München, den ....