CUX2 REGULATES NEUROGENESIS IN THE POSTNATAL MOUSE HIPPOCAMPUS

by

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Abstract

Although once thought to be incapable of regeneration, the adult mammalian brain generates new neurons in two regions: the SVZ of the lateral ventricle and the DG of the hippocampus. While the cell types involved in adult neurogenesis have been broadly characterized, the transcriptional regulation of this process remains poorly understood. Here, we demonstrate that transcription factor Cux2 is important for normal postnatal hippocampal neurogenesis. *Cux2*$^{neo/neo}$ mutant mice generated fewer Dcx-positive neuroblasts, Tbr2-positive transit amplifers, and Calretinin-positive immature neurons, without affecting gliogenesis. Moreover, we show that Cux2 is principally expressed in Type1/Type2a cells. Using cultured embryonic NPCs we show that Cux2 mutants generate fewer neurons. Indeed, Cux2 plays a pro-neuronal role in both the postnatal hippocampus and in cultured embryonic NPCs. Cux2 may thus serve as an important regulator of the neuronal fate and may be a novel marker for neuronally committed Type 1/2a NPCs in the postnatal DG.

List of Abbreviations and Symbols Used

BDNF	Brain-Derived Neurotrophic Factor
CA	Cornu Ammonis
Cux	Cut-like transcription Factor
$Cux2^{neo/neo}$	Cux2 hypomophic mutation
$Cux2^{neo/+}$	Cux2 heterozygote control
Dcx	Doublecortin
DG	Dentate Gyrus
EGF	Epidermal Growth Factor
FACS	Fluorescent Activated Cell Sorting
FGF	Fibroblast Growth Factor
GABA	Gamma Aminobutyric acid
GCL	Granule Cell Layer
GFAP	Glial Fibrillary Acidic Protein
LV	Lateral Ventricle
NeuroD1	Neurogenic Differentiation 1 transcription factor
Ngn2	Neurogenin 2 transcription factor
NPC	Neural Progenitor Cell
NSC	Neural Stem Cell
OB	Olfactory Bulb
PBS	Phosphate Buffered Salline
PBT	Phosphate Buffered Saline with Triton
PFA	Paraformaldehyde
Prox1	Prospero-related homebox gene 1
RMS	Rostral Migratory Stream
SEM	Standard Error of the Mean
SGZ	Subgranular Zone
Sox2	SRY-related homeobox gene
SVZ	Subventricular Zone
Tbr2	T-box transcription Factor 2
VEGF	Vascular Endothelial Growth Factor
Wnt	Wingless transcription factor

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CHAPTER 1: INTRODUCTION

1. Introduction

The mammalian brain develops in the embryo from a simple neuroepithelium composed of neural stem cells (NSCs). NSCs undergo a series of cell fate decisions to give rise to neurons and glia to form the brain (McKay, 1997; Van der Kooy and Weiss, 2000). It was once thought that this process was restricted to development and unlike blood or skin cells, which regenerate continuously, making new neurons in the adult brain was considered an impossible feat (Cajal, 1991). This view was challenged however, when Altman and others showed that certain regions of the adult brain are able to generate new neurons; a process called adult neurogenesis (Altman and Das, 1965; 1967; Kaplan and Hinds, 1977). Despite this evidence, the scientific community remained skeptical until the 1990's when NSCs were discovered in the adult brain (Reynolds and Weiss, 1992; Kilpatrick and Bartlett, 1993; Palmer et al., 1995). NSCs in the adult brain, and not just in the embryo, offered an explanation for the source of adult neurogenesis, which brought support for this biological phenomenon. This was further reinforced by a study that showed that new neurons are generated in the adult human hippocampus (Erikson et al., 1998). However, the regulation of neurogenesis in the adult is poorly understood and its relevance to human health remains unclear.

It has now been well established that at least two areas of the adult mammalian brain generate new neurons throughout life: the subventricular zone (SVZ) of the lateral ventricle in the olfactory system and the dentate gyrus (DG) of the hippocampus (**Figure 1.1**, Altman and Das, 1965; Palmer et al., 1995, 2000). Neurogenesis in the SVZ produces new olfactory interneurons while NSCs in the DG differentiate into new granule cell neurons of the hippocampus. New neurons produced in the subgranular zone (SGZ) of the

hippocampus migrate upwards into the granule cell layer (GCL) and integrate into the circuitry of the hippocampus. In contrast, new neurons born in the SVZ migrate as chains over long distances through a path called the rostral migratory stream (RMS) and differentiate in the olfactory bulb (OB) as interneurons (Palmer and Gage, 2006). Given the importance of the hippocampus in spatial and episodic memory (Rolls, 2000; Dickerson and Eichenbaum, 2010) understanding neurogenesis in this area is of particular interest for human health. While some of the same molecular mechanisms that govern embryonic neurogenesis are also important for adult neurogenesis, this process is not fully understood in the adult brain. We therefore need to identify the network of proteins that instruct NSCs to become new neurons in the adult hippocampus.

1.1. Function and anatomy of the hippocampus

The ventral hippocampus functions in emotion and stress (Henke, 1990), while the dorsal hippocampus is critical for spatial and episodic memory (Moser et al., 1995). The relevance of adult neurogenesis to these functions is not clear and has been disputed based on the small numbers of neurons that are generated relative to the size of the brain (Snyder and Cameron, 2011). Accumulating evidence, however, suggests that levels of adult neurogenesis are likely sufficient to affect human behavior (Snyder and Cameron, 2011). To fully understand the implications of adult neurogenesis in the hippocampus it is important to consider the hippocampal circuitry and the unique characteristics of newly generated granule cell neurons.

The mammalian hippocampus is comprised of four main regions: the DG, the cornu ammonis (CA), the presubiculum and the subiculum. The CA regions are further subdivided into four regions called CA1, CA2, CA3, and CA4 (**Figure 1.2, B**). Together,

the DG and CA regions make up the tripsynaptic hippocampal network. The first synapase is formed by excitatory input from the entorhinal cortex onto the dendrites of granule cell neurons of the DG. The entorhinal cortex is important for memory and navigation as the main interface between the hippocampus and neocortex (Amaral and Lamenex, 2006). In turn, these granule cells extend axons from the DG to form the mossy fiber tract, which contacts the pyramidal neurons of the CA3 region (**Figure 1.2, B**). This connection represents the second synapse of the trisynaptic network. Pyramidal neurons of the CA3 region synapse to the pyramidal neurons of CA1 via the Shaffer collateral pathway to form the third synapse. Closing the network, pyramidal neurons of the CA1 region send axons to the subiculum that, in turn, project back to the entorhinal cortex. Importantly, adult hippocampal neurogenesis only generates new granule cells in the DG, which means that it only directly influences the mossy fiber projections between the DG and the CA3 region (Kempermann, 2006).

While the trisynaptic hippocampal network offers a simplified view of the connections of the hippocampus, it is important to note that the SGZ and DG receive inputs from many different neurotransmitter systems that may influence adult hippocampal neurogenesis. Such inputs include glutamate from the entorhinal cortex, contralateral hippocampus, as well as from hillar mossy cells. Other neurotransmitters include acetylcholine from the basal forebrain and septum, noradrenaline from the locus cereleus, GABA from local interneurons as well as dopamine from the ventral tegmental area. Indeed, dopamine is effective in modulating the activities of newly generated hyperexcitable young neurons in the mouse DG that are important for filtering incoming information (Mu et al., 2011).

Since each individual granule cell contacts roughly ten to fifteen CA3 pyramidal neurons (Ascady et al., 1998), each granule cell can act as a "detonator" through downstream collateral connections (Snyder and Cameron, 2011). Given that the rat DG contains roughly 650,000 new granule cells at 3 months of age (Snyder and Cameron, 2011) and there are 500,000 CA3 pyramidal neurons to receive their input (West et al., 1991), the number of new granule cells appears relatively large. Although it is well established that the number of newly generated neurons decreases with age (Seki and Arai, 1995; Kuhn et al., 1996), the plastic period may be longer in older animals, which may help to compensate for the decreased number (Snyder et al., 2009). Moreover, the mouse DG shows an activation of merely 2-5% of granule cells upon exploration of a new environment (Treves et al., 2008; Chawla et al., 2005). Given that new neurons are up to five times more likely to become activated than old granule cells (Snyder et al., 2009; Ramirez-Amaya, 2006), at least some new neurons must be activated during hippocampus-dependent tasks.

Compared to mature granule cells, newly generated neurons also have several unique characteristics that may indicate a specialized role in hippocampal function. The firing patterns of granule neurons of the DG are critical for encoding experience (Leutgeb et al., 2007). Due to enhanced excitability (Mongiat et al., 2009; Schmidt-Hieber et al., 2000) as well as more varied spike latency (Mongiat et al., 2009) young granule cells have different firing patterns than mature granule cells. Young neurons are also less likely to be inhibited by GABA in the hippocampus since they are depolarized by this neurotransmitter (Pradhan et al., 2007). Further, it has been shown that young granule cells may actually synthesize and release GABA as well as glutamate (Mody et al., 2002; Zhao et al., 2010)

highlighting their different neurotransmitter profile. Indeed, new neurons generated through adult hippocampal neurogenesis may play a distinct role in hippocampal function due to their unique properties.

1.2. The Process of Adult Hippocampal Neurogenesis

Hippocampal neurogenesis is restricted to the SGZ of the DG (Ming and Song, 2011; Sohur et al., 2006) and produces new granule neurons in the granule cell layer (GCL) of the DG. This process begins with a NSC, which is, by definition, both self-renewing and multipotent (McKay, 1997; Van der Kooy and Weiss, 2000). This means that the cell has the ability to replicate itself as well as differentiate into neurons and glia. Upon differentiation, the NSC first gives rise to a more restricted neural progenitor cell (NPC), which is committed to either the neuronal or glial lineage. *In vitro* cells can be tested to determine whether they meet the criteria of a true NSC or have restricted potential (Seaberg and van der Kooy, 2003). *In vivo,* however, it is very challenging to differentiate between an NSC and the more restricted NPC. For this reason, the term *precursor cell* (or NPC) is used as the umbrella term for all cells that may give rise to neurons and glia in the brain (Kempermann, 2006).

DG development in the embryo begins at mid-gestation, in a specialized region of the SVZ of the dentate neuroepithelium. The first dentate granule cells are born and migrate to the presumptive DG site where they become part of a scaffold for forming granule cell layers (Altman and Das, 1965). Throughout the first few postnatal weeks, precursor cells in the hilus proliferate and differentiate into many granule cells to form the DG. After this rapid production of granule cells, remaining precursors settle in the SGZ where they continue to produce neurons at a reduced rate throughout life (Altman and

Bayer, 1990; Pleasure et al., 2000). The final maturation and integration of these new neurons into the adult hippocampal circuitry resembles the precise and sequential process of neonatal hippocampal development (Esposito et al., 2005). However, we do not fully understand the transcriptional regulation of neurogenesis in the adult brain.

1.2.1. Cell Types and Their Cellular-marker Profiles in Adult Neurogenesis

Neurogenesis in the hippocampus begins with the division of a Type 1 NPC and ends with the integration of a functional neuron (**Figure 1.2**). This process includes important transitions through several different cell types, which are distinguished by their expression of specific cellular markers (**Figure 1.3**). The first cell type in the cascade is the Type 1 NPC, which is characterized by a radial process that spans the entire thickness of the GCL and expresses Nestin, Glial Fibrillary Acidic Protein (GFAP) and Sox2 (Kempermann et al., 2004; Seri et al., 2004; Seri et al., 2001). These Type 1 cells divide infrequently in an asymmetrical fashion to become another Type 1 cell in addition to a non-radial progenitor called a Type 2a cell (Sohur et al., 2006). Type 2a cells no longer express GFAP, have decreased Nestin staining, and have continued expression of regulatory protein Sox2 (Peveny and Nicolis, 2010). Sox2 is thought to keep hippocampal NSCs in an undifferentiated state and help to maintain their self-renewing ability (Suh et al., 2007).

A third type of progenitor called a transit amplifier (or Type 2b cell) is distinguished by the expression of neurogenic transcription factors Tbr2, NeuroD1, Ngn2 and Mash1 (Hodge et al., 2008; Seki, 2002; **Section 1.4.2.** for more detail). Transit amplifiers divide rapidly before differentiating into neuroblasts (or Type 3 cells) which are identified by the expression of the intermediate filament protein Doublecortin (Dcx) and a

modified form of the cell-surface adhesion molecule called Polysialic Acid-Neural Cell Adhesion Molecule (PSA-NCAM) (Seki and Arai, 1999). These cells migrate from the SGZ upwards to the GCL where they withdraw from the cell cycle and differentiate into neurons (Sohur et al., 2006). Final functional integration requires correct dendritic targeting of new neurons toward the molecular layer of the DG and appropriate axonal projections toward the hippocampal CA3 pyramidal cell layer. At this point, neurons become postimitotic and begin to express calcium binding protein, Calretinin, and mature neuronal protein, NeuN. Prospero-related homebox gene 1 (Prox1) is also expressed in mature adult granule cells and has been used as a specific marker for these DG neurons (Liu et al., 2000; Jessberger et al., 2008).

Type 1 NPCs in the hippocampus are thought to be capable of asymmetrical divisions, resulting in self-renewal and the generation of a non-radial progenitor, which exhibits reduced Nestin and continued Sox2 expression (Bonaguidi et al., 2011; D'Amour and Gage, 2003; Ferri et al., 2004). However, it has not been clearly established whether Type 1 cells in the hippocampus are truly self-renewing multipotent NPCs unlike the multipotent NPs of the SVZ. It has been suggested that they may, instead, display a limited capacity for self-renewal and may have separate NPCs committed to neuronal and glial lineages exclusively (Clarke and van der Kooy, 2011; Encinas et al., 2011; Kempermann, 2011). Indeed, it is unknown when a Type 1 cell first commits to becoming a neuron in the hippocampus and whether subsets of Type1 cells are predestined for one lineage over the other. Identifying a novel marker in Type1 cells to distinguish a subset of neuronally committed NPCs would provide support for this theory.

1.3 The Process of Olfactory System Neurogenesis

The olfactory system contains two neurogenic regions: the olfactory epithelium and the lateral walls of the ventricles (LV). Neurons produced from progenitor cells in the olfactory epithelium become olfactory receptor neurons, while neurons from the SVZ of the LV become interneurons in the OB (Doetsch et al., 1999). Neurogenesis in these regions is important for olfaction in order to discriminate between roughly 10,000 different odors through the action of over ten million olfactory receptor neurons (Gottfried and Wilson, 2011). In early postnatal rodents, roughly tens of thousands of new neurons are born each day in the OB (Lois and Alvarez-Buylla, 1994). However, about half of these newly generated neurons die within days or a few weeks and do not become functionally integrated (Winner et al., 2002). Those that do arrive at the OB disperse radially away from the RMS, differentiate into post-mitotic interneurons, and become functionally integrated in neuronal circuits (Alvarez-Bullya and Garcia-Verdugo, 2002).

As the largest germinal region in the adult mammalian brain (Gates et al., 1995), the SVZ is located along the lateral wall of the LV and houses NPCs which give rise to new neurons. NPCs migrate along the RMS to the OB where they finally become inhibitory neurons which then integrate into the neuronal circuitry of the OB (**Figure 1.1.,** Alvarez-Bullya and Garcia-Verdugo, 2002). Several different types of new interneurons have been characterized but it is unclear whether these different cell types fall into different functional classes (Lledo et al., 2008). The SVZ contains at least 4 different cell types of varying self-renewal ability and neurogenic potential (Sohur et al., 2006; Kempermann, 2006; Miller and Gauthier-Fisher, 2009). The presumptive NPC is a type of glial cell called a Type B cell, which expresses NPC markers GFAP, Sox2, and Nestin. In

the adult brain, these cells are normally quiescent, although, they divide infrequently to produce two daughter cells. This division can be symmetrical to produce two more Type B cells or asymmetrical and give rise to one Type B cell and a proliferating Type C cell destined to become a neuron (Doetsch et al., 2002). Type C cells become neuroblasts, which move to the OB along the RMS where they differentiate into interneurons. While various cell types involved in adult neurogenesis have been described, the genetic regulation underlying the cell type transitions is not fully understood.

1.4. Regulation of Adult Neurogenesis

The rate of adult neurogenesis can be regulated by various factors including voluntary exercise, specific learning paradigms, as well as seizure and ischemia (Van Praag et al., 2000; Parent and Lowestein, 2002). However, we do not fully understand the genetic networks underlying this modulation. The regulation of adult neurogenesis is usually measured as a change in cell numbers but the variance in the number of new neurons within the DG is due to 85% change in "survival"; whereas, changes in proliferation contributes roughly 15% (Kempermann et al., 2006). This suggests that net neurogenesis is largely determined by cell survival and cannot be just attributed to a rapid production of new neurons. Consistent with this idea, cognitive stimulants of adult neurogenesis such as environmental enrichment and learning stimuli appear to primarily affect the survival period of new neurons (Kempermann et al., 1998; Leuner et al., 2004). As in embryonic neurogenesis, more neurons are produced than actually survive long enough to be functionally integrated in the adult DG. Indeed, the neurogenic regions of the adult brain show roughly one hundred times greater apoptotic cell death than that which is observed for the rest of the brain (Biebl et al., 2005). Neurons which do not receive

appropriate synaptic activation (Jessberger and Kempermann, 2003) do not become functionally integrated and eventually die.

NPC regulation in the adult brain involves a fine balance between proliferation and differentiation. The niche environment is a rich source of extrinsic signals which help to mediate this process; however, NPC development is also influenced by the action of intrinsic genetic regulatory networks. How the balance is achieved between intrinsic genetic control and input from the niche environment is unknown and is the focus of intense investigation. We therefore need to identify the network of proteins that regulates the formation of neurons from self-renewing NPCs in the context of the neurogenic niche.

1.4.1. The Neurogenic Niche Environment

The DG and SVZ have a similar structure in that the basement membrane of the vasculature is tightly associated with a mix of precursor cells, astrocytes, endothelial cells and microglia or macrophages. This cellular organization is referred to as the *neurogenic niche* and it keeps multipotent progenitors proliferating and self-renewing (Palmer and Gage, 2000; Kempermann, 2006; Miller and Gauthier-Fisher, 2009). Several components of the niche environment in the adult brain include cell-cell interactions and somatic cell signaling, the vasculature, the extracellular matrix, and basal lamina (Doetsch, 2003). For a region to be truly neurogenic two criteria must be met: NPCs must be present and the local niche environment must be permissive to the development of new neurons. Although NPCs with neurogenic potential can be isolated from many regions of the brain such as the striatum and substantia nigra (Palmer et al., 1995, 1999; Lie et al., 2002) they do not necessarily reside in neurogenic regions. A standard test for determining whether a region is neurogenic is to implant NPCs into the region in question and test whether they are able

to become neurons (Gage et al., 1995; Suhonen et al., 1996; Herrera et al., 1999). If the region is not neurogenic, the implanted cell will become a glial cell or it will die. How both intrinsic and extrinsic signals interact to enable neural progenitors in the SGZ to 'choose' a neuronal fate is not yet clear and thus limits our understanding of the plasticity and regeneration of the brain.

The hippocampal neurogenic niche is restricted to a thin layer of cells at the interface between the hilus and the GCL called the subgranular zone (SGZ) (Sohur et al., 2006, Kemperman, 2006). One key cellular component of this neurogenic niche is local astrocytes, which secrete factors important for neurogenesis (Song et al., 2002). Astrocytes extracted from the neurogenic DG increase precursor cell activity when cultured with NPCs, whereas, astrocytes from non-neurogenic regions such as the cortex do not affect neurogenesis in culture (Song et al., 2002). Interestingly, these hippocampal astrocytes have been shown to increase neurogenic potential of spinal cord NPCs, which do not differentiate into neurons in the spinal cord (Lie et al., 2002). The mechanism of this action may be explained by signaling from the Wingless/int-family (Wnt) of secreted proteins. Several secreted Wnt family members are expressed in hippocampal astrocytes and hippocampal NPCs express receptors for these signaling components (Moon, 2004; Lie et al., 2005). In fact, expression of Wnt3 is sufficient to increase neurogenesis from adult NPCs both *in vivo* and *in vitro* (Lie et al., 2005). More recently it is has been shown that Wnt3a exerts an increase in hippocampal neurogenesis by shortening cell cycle duration of NPCs (Yoshinaga et al., 2010).

In addition to astrocytes, NPCs are closely associated with the vasculature of the stem cell niche (Palmer et al., 2000). Surrounding the lumen of blood vessels, endothelial

cells and pericytes are separated from the brain by a basal lamina and it is thought that angiogenesis and neurogenesis are reciprocally linked (Doetch et al., 2002). In the SVZ, NPCs often make contact with the vasculature at regions lacking pericyte coverage where small circulating molecules in the blood make contact with NPCs (Tavazoie et al., 2008). Newly generated NPCs are able to migrate through this vasculature to sites of injury in order to enhance neurogenesis in response to pathological conditions such as stroke (Massouh and Saghatelyan, 2010). Moreover, vascular endothelial growth factor (VEGF) was found to regulate adult neurogenesis (Jin et al., 2002) providing support for this link. In the SVZ, it was found that circulating small molecules from the blood enter this germinal zone (Tavazoie et al., 2008) providing direct access to signals from the blood. Interestingly, *in vivo* VEGF promoted survival whereas *in vitro* VEGF was found to promote proliferation (Schanzer et al., 2004), highlighting differences between the niche and defined cell culture environment.

NPCs from a variety of embryonic and adult sources in the CNS can be dissected out of the niche environment and cultured to test proliferative potential and differentiation capacity (D'Amour and Gage, 2003; Kilpatrick and Bartlett, 1995; Palmer and Gage, 1995). To test proliferative properties of NPCs the neurosphere method is commonly used (Reynolds and Weiss, 2002) whereby dissected NPCs expand into epithelial balls of cells called neurospheres. To stimulate proliferation, defined neurobasal medium supplemented with Endothelial Growth Factor (EGF) and Fibroblast Growth Factor (FGF2) is used in culture (Reynolds et al., 1992, 1993). EGF is considered to be the strongest mitogen important for proliferation of NPCs and receptors for EGF are expressed on the cell surface of NPCs throughout brain development (Kornblum et al., 2000). FGF2 stimulates

neurogenic potential in cells isolated from various regions including the neocortex and optic nerve (Palmer et al., 1999) and in its absence proliferation decreases (Raballo et al., 2000). When cultured under these conditions dissected NPCs expand quickly into neurospheres. Upon removal of growth factors, the neurospheres stop proliferating and differentiate into brain cells: glia or neurons. Specifically, cells derived from mutant brains can be compared to those from controls to assess the importance of the null gene. Growth rates of loss-of-function NPCs can be evaluated daily over 1-2 weeks by counting the numbers of cells produced following low-density plating. After differentiation, various immunohistochemical markers can be used to label different cell types (i.e. Nestin for progenitors, Dcx for young neurons, GFAP for astrocytes) to determine neuronal differentiation capacity. Since the media supplements are defined, cell culture provides a useful system with which to convey the role of a given gene in neurogenesis.

1.4.2. Transcriptional Regulation of Adult Hippocampal Neurogenesis

The cellular and molecular regulation of adult neurogenesis involves some of the same mechanisms that govern neurogenesis during development (Esposito et al., 2005; Hevner et al., 2006; Hodge et al., 2008). This includes the Notch signaling pathway and the regulators Pax6, Sox2 and Sox9 (Kempermann, 2006; Miller and Gauthier-Fisher, 2009). These proteins are transcription factors expressed in NPCs and are required for their self-renewing ability (Song and Stevens, 2002; Pevny and Nicolis, 2010; Ellis et al., 2004; Graham et al., 2003). Prospero-related homeobox gene 1 (Prox1) is another transcription factor required for differentiation of granule cells in the hippocampus (Karalay et al, 2010). Interestingly, Prox1 appears to be regulated by canonical Wnt

signaling (Karalay et al., 2010) demonstrating an important connection between the local niche astrocytes and an intrinsic target for transcription.

The balance between NPC proliferation and specification of the neuronal fate is dictated by transcription factor cascades in development (Hevner et al., 2006). Sox2 is expressed in undifferentiated cells in this cascade and has recently been characterized as a marker for NPCs in the adult hippocampus (Suh et al., 2007). While Sox2 is thought to predominantly label Type-1 cells within the SGZ, Sox2 also labels some Type-2a cells and can sometimes be coexpressed with Tbr2 (Hodge et al., 2008). As NPCs differentiate into neuronally committed cells, Sox2 expression is down-regulated (Ferri et al., 2004). Sox2 mutant studies have shown that the DG does not develop properly when Sox2 is deleted (Favaro et al., 2009) and conditional Sox2 deletion in the adult hippocampus results in loss of Type-1 progenitors. It has been suggested that Sox2 might help to maintain the NPC pool of proliferating cells in the adult brain through the direct regulation of Sonic hedgehog (Shh) (Favaro et al., 2009). To maintain NPC proliferation, Sox2 represses proneuronal NeuroD1, which must be activated for cells to enter the neuronal differentiation pathway. Other members of the Sox family important for adult hippcampal neurogenesis include Sox3, which is often co-expressed with Dcx (Wang et al., 2006) as well as Sox11, which is also found in DCX-positive cells. Sox11 overexpression in cultured NPCs led to increased neuronal generation (Hashlinger et al., 2009) demonstrating the opposing effect of Sox2.

T-box transcription factors Tbr1 and Tbr2 are both found in the embryonic cerebral cortex and in the adult hippocampus (Hodge and Hevner, 2011). In the adult hippocampus, Tbr2 labels clusters of Type-2/3 cells within the SGZ (Hodge et al., 2008) and recently it

has been demonstrated that Tbr2 is required for normal development of the DG (Arnold et al., 2008). Similar to embryonic development of the cortex, in the adult hippocampus Tbr2 is down-regulated as cells differentiate into post-mitotic neurons while Tbr1 is up-regulated (Hodge et al., 2008). However, the role of Tbr1 has not been characterized in the adult hippocampus.

Differentiation of neurons from NPCs requires activation by "proneural genes" which include the basic helix-loop-helix (bHLH) factors. Various bHLH factors are also involved in maintaining NPC development in the adult hippocampus. Mash1, for example, is found in some Type1 and Type-2a Sox2-positive NPCs in the hippocampus, which, ultimately differentiate into granule neurons (Kim et al., 2008). Interestingly, upon transition from Type-2a to Type-2b Mash1 is downregulated while bHLH-factor Neurogenin-2 (Ngn2) is upregulated (Roybon et al., 2009). Specifically, Neurogenin 2 (Ngn2) directs granule neuroblast production and amplification during hippocampal neurogenesis (Roybon et al., 2009). Also involved in commitment to the neuronal lineage, bHLH factor NeuroD1 is expressed in lineage-determined precursor cells in the adult hippocampus (Kempermann, 2006). However, NeuroD1 is also highly expressed in PSA-NCAM-positive cells within the SGZ, which have already begun terminal differentiation (Seki, 2002). We therefore, need to identify markers which can unambiguously delineate a basal (Type1/2a) NPC that is committed to becoming a neuron.

1.4.2.1. Cut-like (Cux) Factors as Neurogenic Determinants

Homeobox-containing genes encode transcription factors, which are important for the genetic regulation of tissue-specific development. In *Drosophila melanogaster*, the homeodomain-containing protein Cut is required for normal development of the external

sensory organs (Quaggin et al., 1996). In mammals, two homologues of the Drosophila homeobox Cut gene, Cux1 and Cux2, are found (Neufeld et al., 1992; Quaggin et al., 1996). These homologues contain two characteristic DNA binding domains: a *Cut repeat* and a *Homeobox domain* **(Figure 3.4)**. While both Cux1 and Cux2 are expressed in most tissues, Cux2 is more strongly expressed in the CNS and is involved in neuronal specification (Neufeld et al., 1992; Iulianella et al., 2003). Importantly, Cux2 is found in NPCs throughout the developing central nervous system and its loss limits the ability of these progenitors to generate new neurons (Iulianella et al., 2008, 2009; Cubelos et al., 2008). It is currently unclear, however, whether Cux2 plays a role in the formation of new neurons in the postnatal brain.

In embryonic spinal cord neurogenesis, Cux2 was found to regulate cell-cycle progression and development of NPCs (Iulianella et al., 2008). Cux2 expression begins in the ventricular zone of the developing spinal cord at E10, correlating with the birth of the first neurons. Cux2 mutant mice developed smaller spinal cords with decreased neural progenitor development and decreased neuronal differentiation (Iulianella et al., 2008, 2009). Similarly, in Cux2 gain-of-function transgenic mice, the spinal cords were larger and neuroblast formation and neuronal differentiation were enhanced (Iulianella et al., 2008).

Cux2 and Cux1 mark the SVZ and upper layer cortical neurons during development (Nieto et al., 2004; Cubelos et al., 2008). While single mutants have a mild cortical phenotype, *Cux1/Cux2* double mutants display severe losses in upper layer cortical interneurons (Cubelos et al., 2008). It is thought that Cut proteins act as transcriptional regulators that oscillate in activity during the cell cycle (Nepvue, 2001). Indeed, Cux2 has

been shown to bind to the promoters of cell cycle inhibitory proteins such as $p27^{kip1}$ to positively regulate their expression (Iulianella et al., 2008, 2009; Ledford et al., 2002). Interestingly, the proneuronal transcription factor, NeuroD1, is under the genetic regulation of Cux2 in the developing mouse spinal cord (Iulianella et al., 2008) highlighting the importance of Cux2 in promoting neuronal fate during development. Cux2 is also expressed in NPCs in the developing mouse cortex and, similarly, Cux2 mutants display defects in neural differentiation in this region (Nieto et al., 2004; Zimmer et al., 2004).

The adult hippocampus maintains many characteristics typical of embryonic neuronal integration observed in the development of the hippocampus (Esposito et al., 2005). Similar mechanisms required for embryonic neurogenesis are thus likely important for neurogenesis in the adult hippocampus. Given the pro-neurogenic roles of Cux2 in the embryo it is likely that Cux2 also plays a role in neurogenesis in the adult brain. In the present study Cux2 expression in the postnatal hippocampus is characterized and, using $Cux2^{neo/neo}$ mice, the role of Cux2 in postnatal hippocampal neurogenesis is investigated.

Hypothesis

I hypothesize that **Cux2 is important for postnatal neurogenesis in the mouse hippocampus.** Using sagittal sections of the mouse hippocampus and immunohistochemical markers, I predict that $Cux2^{neo/neo}$ mice will generate fewer new neurons compared to their littermate controls.

Figure 1.1. Schematic representation of the two neurogenic regions in the adult mouse brain. Parasagittal view showing the SVZ of the LV where NPCs proliferate and then differentiate along the RMS before becoming postmitotic neurons in the OB. The second neurogenic region shown is the SGZ of the DG within the hippocampus. NPCs proliferate in the SGZ of the DG and then they migrate to the GCL where they differentiate into mature granule neurons (see Figure 2). Schematic adapted from Ma et al., 2009.

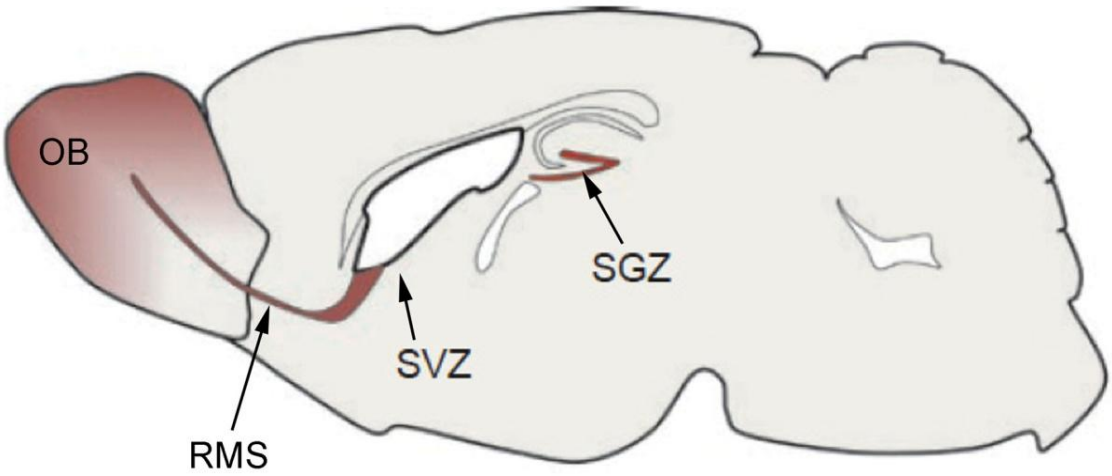

Figure 1.1.

Figure 1.2. Hippocampal neurogenesis. (A) Image depicting the hippocampus (boxed) in close proximity to the lateral ventricle (LV). **(B)** Enlarged schematic of the hippocampus showing the DG and hillus (CA4), CA1, CA2, and CA3 regions. **(C)** Enlarged view of the cells of the DG showing the SGZ where Radial (Type1) NPCs reside. Type 1 NPCs can give rise to non-radial (TypIIa) NPCs, which undergo expansion to become Transit amplifiers and migrate upward to the GCL where they become postmitotic granule cell neurons. Schematic adapted from Kempermann, 1996.

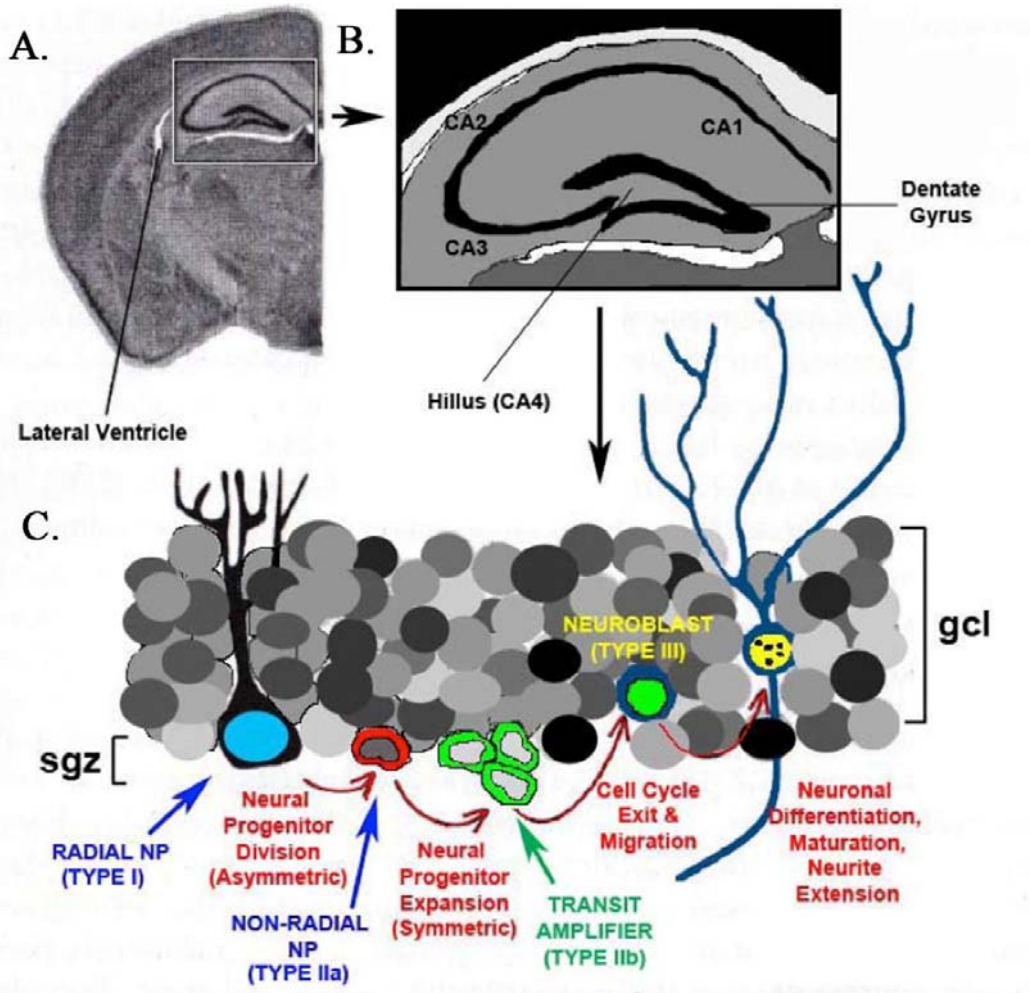
A.
B.
CA2
CA1
CA3
Dentate Gyrus
Hillus (CA4)
Lateral Ventricle
C.
NEUROBLAST (TYPE III)
gcl
sgz
RADIAL NP (TYPE I)
Neural Progenitor Division (Asymmetric)
NON-RADIAL NP (TYPE IIa)
Neural Progenitor Expansion (Symmetric)
TRANSIT AMPLIFIER (TYPE IIb)
Cell Cycle Exit & Migration
Neuronal Differentiation, Maturation, Neurite Extension

Figure 1.2.

Figure 1.3. Cell types involved in hippocampal neurogenesis are distinguished by cellular markers. Type 1 radial glial NPCs are distinguished by their expression of Nestin and GFAP. As Type 1 radial glia transition to non-radial glia they begin to express Sox2 and show decreased expression of Nestin and GFAP to become Type 2a NPCs. Transition to Type 2b NPCs (transit amplifier stage) is characterized by expression of Tbr2 as well as NeuroD1 and Mash1. The following Type 3 stage cells (neuroblasts) express Dcx and PSA-NCAM. Immature neurons begin to express Calretinin followed by NeuN and Calbindin or GABA once they become postmitotic. Schematic adapted from Hodge et al., 2008.

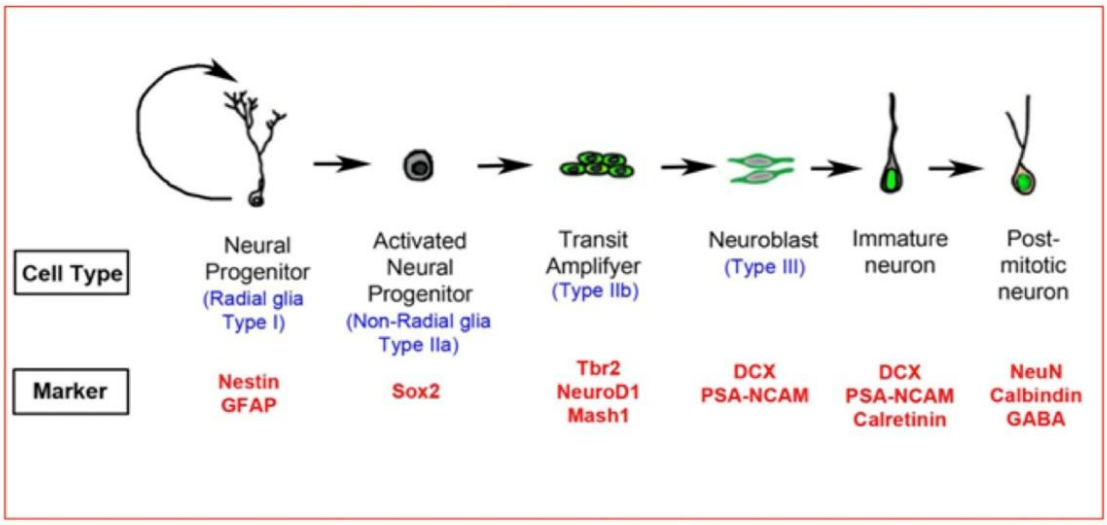
Cell Type
Marker
Neural Progenitor (Radial glia Type I)
Activated Neural Progenitor (Non-Radial glia Type IIa)
Transit Amplifyer (Type IIb)
Neuroblast (Type III)
Immature neuron
Post-mitotic neuron
Nestin GFAP
Sox2
Tbr2 NeuroD1 Mash1
DCX PSA-NCAM
DCX PSA-NCAM Calretinin
NeuN Calbindin GABA

Figure 1.3.

Figure 1.4. Schematic representation of the Cux gene structure. Two members of the Cut family have been identified in mammals called Cux1 and Cux2. The structure consists of a coiled-coil, 3 cut repeats (yellow), and a homeodomain (blue). The cut domains and homeodomain co-operate in DNA binding. Figure obtained from Dr. Iulianella.

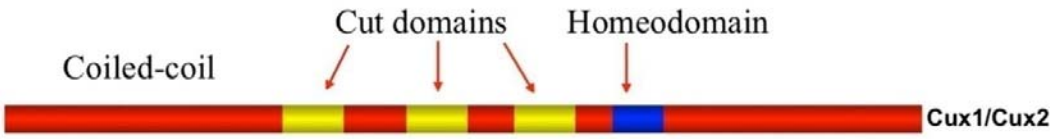

Figure 1.4.

CHAPTER 2: CUX2 REGULATES HIPPOCAMPAL NEUROGENESIS

(MANUSCRIPT)

Cux2 Defines a Subpopulation of Neural Progenitors and Regulates Neurogenesis in the Postnatal Hippocampus

Running Title: Cux2 Regulates Hippocampal Neurogenesis

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2.1. Abstract

Hippocampal neurogenesis occurs in the subgranular zone (SGZ) where it contributes to the generation of granule cell layer (GCL) neurons throughout life. While various cell types involved in postnatal neurogenesis have been described, their underlying genetic regulation is not fully understood. Here we demonstrate that the transcription factor Cux2 is important for hippocampal neurogenesis. Using *Cux2*$^{neo/neo}$ mutant mice we show that *Cux2* deficiency leads to fewer immature neurons, migrating neuroblasts, and transit-amplifiers in the dentate gyrus (DG) of the hippocampus. Although *Cux2* reduction resulted in decreased neurogenesis, it led to increased numbers of Nestin/ Sox2 double-positive neural progenitor cells (NPCs). This cell type is referred to as a Type 1 NPC and is thought to be self-renewing and multipotent. Interestingly, BrdU labeling revealed that the proliferation of Type 1 NPCs was increased in *Cux2* mutants. Consistent with this, we demonstrate that Cux2 is expressed in Type 1/Type2a progenitors, with limited expression in more differentiated cell types of the SGZ. Specifically, Cux2 was restricted to Nestin- and Sox2- positive progenitors and was largely absent from Tbr2-positive transit amplifying cells, Dcx- and PSA-NCAM-positive neuroblasts, and Calretinin-positive immature neurons. Taken together we show that Cux2 is important for normal hippocampal neurogenesis and may be a novel marker for neuronally restricted Type 1/Type2a NPCs.

2.2. Introduction

New neurons are born throughout life in the postnatal hippocampus. Given that hippocampal neurogenesis is important for learning and memory (Dupret et al., 2008; Garthe et al., 2009) the regulation of this process in the postnatal brain is of particular interest for understanding brain function. Progress has been made in the characterization of the cell types involved in hippocampal neurogenesis, but the genetic regulation governing this process is not clear.

Hippocampal neurogenesis is restricted to the SGZ of the DG (Ming and Song, 2011; Sohur et al., 2006). Beginning with the division of a precursor cell and ending with the integration of a functional neuron, neurogenesis involves transitions through various cell types. The Type 1 NPC is characterized by a radial process and the expression of Nestin, Glial Fibrillary Acidic Protein (GFAP) and Sox2 (Kempermann et al., 2004; Seri et al., 2004; Seri et al., 2001). It has not been clearly established whether Type 1 cells are self-renewing multipotent NPCs, or instead display a limited capacity for self-renewal and generate glia or neurons exclusively (Bonaguidi et al., 2011; Clarke and van der Kooy, 2011; Encinas et al., 2011; Kempermann, 2011). Nonetheless, Type I cells differentiate into rapidly dividing transit-amplifying cells characterized by the expression of Tbr2 (Hodge et al., 2008). The amplifiers, in turn, give rise to neuroblasts which express Doublecortin (Dcx) and Polysialic Acid-Neural Cell Adhesion Molecule (PSA-NCAM), and ultimately differentiate into gcl interneurons (Seki and Arai, 1999).

To investigate the genetic regulation of NPC differentiation, we evaluated the role of the Cut-like transcription factor Cux2 in hippocampal neurogenesis. Our previous findings showed that Cux2 is required for neurogenesis in the developing spinal cord (Iulianella et

al., 2008), and others showed that it regulates the formation of cortical interneurons from SVZ progenitors (Cubelos et al., 2008). However, it is unknown whether Cut-like factors regulate neurogenesis in the postnatal brain. Here we show results consistent with decreased neurogenesis upon *Cux2* loss in the postnatal DG. We also show that Cux2 protein is localized to Nestin and Sox2 double-positive progenitors, suggesting that it may be a novel discriminatory marker for NPCs committed to the neuronal lineage.

2.3. Materials and Methods

Animals. Animals used in this study were treated in accordance with the regulations of Dalhousie University Carlton Animal Care facility and guidelines of the Canadian Council on Animal Care. Hypomorphic *Cux2*$^{neo/neo}$ mice were previously generated (Iulianella et al., 2008) using a Cux2 gene trap mouse embryonic stem (ES) cell line. The gene trap insertion was made in the third intron of mouse Cux2 gene on chromosome 5. Agouti mouse progeny were generated which carried the gene trap insertion in the Cux2 locus (Iulianella et al., 2008). Although minor embryo lethality was reported with this mutation, most mutant mice were born viable and fertile and did not appear visibly different from controls upon inspection. Experiments were performed on postnatal day 23 (P23), 3 and 8 months of age.

Histology and Immunohistochemistry. *Cux2*$^{neo/+}$ and *Cux2*$^{neo/neo}$ mice received intraperitoneal injections of the thymidine analog 5-Bromodeoxyuridine (BrdU) (Sigma, St. Louis, MO) at 3mg/Kg, and were sacrificed 30 minutes following injections using the following procedure: Mice were anaesthetized and subsequently perfused with 0.9% saline. Brains were removed and fixed in 4% paraformaldehyde (PFA) (Sigma, St. Louis, MO)

overnight at 4°C, equilibrated in sucrose, and cryoprotected in OCT (Tissue-Tek, Torrence, CA) for parasagittal sectioning. A total of 50 sections per animal at 12µm per section were obtained from *Cux2*$^{neo/+}$ control and *Cux2*$^{neo/neo}$ mutant mice (600 µm total/animal) using a cryostat sectioning device. Hippocampal morphology was assessed by Nissl staining using 0.1% cresyl violet solution.

Immunohistochemistry was performed on parasagittal sections as previously described (Iulianella et al., 2008). Primary antibodies used were anti-Cux2 (Iulianella et al., 2008), anti-CD31/Pecam-1 (BD Biosciences), anti-GFAP (Millipore), anti-Sox2 (Millipore), anti-Nestin (Santa Cruz Biotech), anti-Dcx (Santa Cruz Biotech), anti-PSA-NCAM (Millipore), anti-Calretinin (Dako Cytomation), anti-BrdU (Abcam), anti-Tbr2 (eBioscience), anti-PDGFR (Chemicon), and anti-Ki67 (Dako Cytomation). For BrdU detection, acid nick was performed prior to antibody incubation as described previously (Junek et al., 2010). Appropriate AlexaFluor or DyLight secondary antibodies were obtained from Invitrogen or Jackson ImmunoResearch. Images were captured using a Zeiss Axiovert 200M inverted fluorescence microscope with 20X objectives and confocal sections were imaged using a Zeiss LSM microscope with 40X objectives and processed using Photoshop CS2 (Adobe, San Jose, CA).

Statistics. The effect of *Cux2* loss on postnatal hippocampal neurogenesis was assessed by quantifying neurogenic markers in 20X images of the DG on Photoshop. Counts were performed without knowledge of the genotype to avoid potential counting bias. Counts were completed for 5-7 different control *Cux2*$^{neo/+}$ and *Cux2*$^{neo/neo}$ mutant sections for all stains (Calretinin, Dcx, Tbr2, Nestin, GFAP, and Sox2). Cell proliferation was assessed by quantifying BrdU- and Ki67-positive cells in the DG. For BrdU immunohistochemistry,

acid nick was performed on brain sections prior to resuming regular immunohistochemistry protocol in order to reveal the BrdU antigen. The data was evaluated using a two-tailed t-test with significance taken at $p<0.05$ to compare mutant and control groups.

2.4. Results

Cux2 is expressed in the progenitor cell niche of the SGZ

To investigate the role of Cux2 in the formation of neurons in the postnatal brain, we first characterized the localization of Cux2 protein in parasagittal sections of the mouse hippocampus. Since the neurogenic niche in the SGZ is bound by the vasculature (Palmer et al., 2000), we used a Pecam-1/CD31 antibody to identify blood vessels. Co-immunostaining with Cux2 antibody revealed that Cux2-positive cells associate with the vasculature (arrows; **Figure 2.1A**). Given that NPCs similarly associate with the SGZ vasculature (Palmer et al., 2000), these findings suggested that Cux2 might be expressed in NPC populations.

We next profiled Cux2 levels in the hippocampus at various ages of the maturing mouse brain and found that Cux2 expression was greatest at P23 (**Figure 2.1B**), and decreased with age (**Figure 2.1B-E**). Cux2 expression was significantly lower at 3 months (**Figure 2.1C, E**), and was only minimally expressed by 8 months of age (**Figure 2.1 D, E**). Since the majority of postnatal neurogenesis in the mouse hippocampus occurs within the first few weeks of life (Kuhn et al., 1996), we focused our investigation at P23 when Cux2 levels and hippocampal neurogenesis are maximal.

Reduced postnatal hippocampal neurogenesis in *Cux2*$^{neo/neo}$ mutants

To determine whether Cux2 is required for normal hippocampal neurogenesis we used a hypomorphic *Cux2*$^{neo/neo}$ mutant mouse line (Iulianella et al., 2008). Given that this mutation largely circumvents embryo lethality, it was appropriate for the analysis of Cux2 function in the postnatal brain. Indeed, a hypomorphic analysis has revealed a specific role for Sox2 in the formation of hippocampal interneurons (Cavallaro et al., 2008) demonstrating the utility of using such loss-of function alleles to study postnatal neurogenesis.

Nissl staining showed that the morphology of the DG appeared largely normal in *Cux2*$^{neo/neo}$ mutant mice at P23 (**Figure 2.1 F, G**). Normal hippocampal development was also supported by unchanged numbers of DAPI-positive DG cells in the *Cux2*$^{neo/neo}$ mutants (**Figure 2.1H**). Since the *Cux2*$^{neo/neo}$ hypomorphic mutation did not appear to affect the formation of the hippocampus we were able to investigate the role of this gene during *de novo* postnatal neurogenesis in the SGZ. To this end, we first examined the distribution of Cux2-positive cells relative to newly forming neurons in the DG. Cux2-positive cells were found adjacent to clusters to Calretinin-positive neurons in the SGZ (arrows, **Figure 2.2A, B**). Occasionally, Calretinin-positive neurons expressed Cux2 (arrowhead, **Figure 2.2B, C**), but most immature neurons and their immediate precursors did not express this transcription factor (**Figure 2.2P**). We then compared numbers of Calretinin-positive immature neurons in the DG of *Cux2*$^{neo/neo}$ mice to littermate controls to determine if the loss of *Cux2* attenuated the formation of newborn neurons (**Figure 2.2C, D**). *Cux2*$^{neo/neo}$ mutants had significantly fewer immature neurons ($p=0.034$; **Figure 2.2D, E**), demonstrating that Cux2 is important for normal postnatal hippocampal neurogenesis.

Similarly, Cux2-positive cells were found adjacent to Dcx-positive neuroblasts (arrow, **Figure 2.2F-H**), which were decreased in *Cux2*$^{neo/neo}$ mutants (p=0.03; **Figure 2.2 H-J**). Limited Cux2 expression was observed in Dcx-positive cells, but the majority of Cux2 staining was excluded from neuroblasts in the DG (**Figure 2.2F-H, P**). Importantly, we observed no changes in Cleaved Caspase-3 staining in *Cux2*$^{neo/neo}$ mutant vs. *Cux2*$^{neo/+}$ littermates, indicating that deficits in neurogenesis were not caused by apoptotic elimination (data not shown).

Since Cux2 did not identify neuroblasts, we reasoned that it might instead be expressed in a more undifferentiated progenitor cell type. To investigate this possibility we compared the distribution of Cux2 staining with the transit-amplifier marker Tbr2. Most Cux2-positive cells did not co-express Tbr2 (**Figure 2.2K-M, P**), although Cux2-positive cells were occasionally found adjacent to clusters of Tbr2-positive cells (arrow, **Figure 2.2K-M**). Similar to Calretinin and Dcx, the numbers of Tbr2-positive transit amplifiers were also significantly reduced in *Cux2*$^{neo/neo}$ mice (p=0.01; **Figure 2.2N, O**). Taken together, we have shown Cux2 promotes neurogenesis in the SGZ, but is not principally expressed in transit amplifiers or differentiating neurons.

We then investigated the possibility that *Cux2* loss might affect gliogenesis by quantifying the numbers of Platelet-Derived Growth Factor Receptor α (PDGFRα)-positive oligodendrocytes (Nishiyama et al., 1996) and GFAP-positive astrocytes (Wei et al., 2002). We found that numbers of PDGFRα-positive oligodendrocytes were unchanged in *Cux2*$^{neo/neo}$ mice (data not shown), and that PDGFRα did not co-localize with Cux2 (**Figure 2.2P**). Similarly, we found that numbers of GFAP-positive cells were unchanged in *Cux2*$^{neo/neo}$ mutants (p=0.87; **Figure 2.3 M-O**), although GFAP also labels glial-like

NPCs (see below). Taken together these results indicated that *Cux2* loss did not affect glial cell numbers, and suggested that Cux2 function was specific to the neuronal lineage.

Increased numbers of hippocampal neural progenitors in *Cux2*$^{neo/neo}$ mutants

Given that Cux2 was not expressed in differentiating hippocampal neurons, we investigated whether Cux2 is expressed in Type 1/Type 2a NPs by staining for Nestin, Sox2, and GFAP. We found that high Cux2 levels co-localized with Nestin (arrows, **Figures 2.3A-C, 2.2P**), and that numbers of Nestin-positive radial glial progenitors were significantly increased in *Cux2*$^{neo/neo}$ mice (p=0.002; **Figure 2.3D, E**). Similarly, Cux2 extensively co-localized with Sox2 in SGZ (**Figures 2.3F-H, 2.2 P**), and Sox2-positive cells were significantly increased in *Cux2*$^{neo/neo}$ mutants (p=0.019; **Figure 2.3I, J**).

Given that more differentiated cell types within the SGZ can also express Nestin (Filippov et al., 2003), we wanted to confirm that Cux2 is principally localized in a Type 1/Type 2a NP characterized by the co-expression of both Nestin and Sox2. To this end, we triple-labeled control hippocampi with Cux2, Nestin, and Sox2 antibodies and found that 75% of Cux2 expressing cells co-localized to Sox2- and Nestin-double positive progenitors (arrowhead, **Figure 2.3P-R**). Our findings are therefore consistent with a role for Cux2 in identifying Type 1/Type 2a NPs destined for a neuronal fate in the hippocampus. Further, we also showed that the loss of *Cux2* led to decreased SGZ neurogenesis accompanied by an increase in Type 1/Type2a NPs.

To determine why *Cux2*$^{neo/neo}$ mice have more Nestin- and Sox2- positive NPCs, we examined proliferation following an acute pulse of BrdU, which detects NPCs in the SGZ (Palmer et al., 1997). We found that the numbers of BrdU-positive cells were significantly increased in *Cux2*$^{neo/neo}$ mice after a 30-minute pulse (p=0.03; **Figure 2.4A-C**). To confirm

the cell-type identity of BrdU-positive cells, we co-stained for Nestin, Sox2, and Dcx. As expected, BrdU predominantly labeled progenitors expressing Nestin (**Figure 2.4D-F**) and Sox2 (**Figure 2.4G-I**), and not neurons expressing Dcx (**Figure 2.4J-L**). The increased proliferation of SGZ progenitors in *Cux2*$^{neo/neo}$ mutants was confirmed by staining with the proliferative marker Ki67 ($p= 0.001$; **Figure 2.4M-O**). Taken together, these findings demonstrate that *Cux2* loss leads to increased division (or self-renewal) of SGZ progenitors.

Figure 2.1. Cux2 is expressed in the SGZ and co-localizes with the vasculature.

A*,** Niche vasculature immunostained with Pecam/CD31 (red) in and Cux2 (green) in P23 parasagittal mouse hippocampal sections (arrows). ***B-D*,** Cux2 staining (white) in the SGZ at P23 **(*B*)**, 3 **(*C*)**, and 8 **(*D*)** months of age. **E,** Bar chart quantifying the significant decrease in Cux2 expression with age. Nissl staining reveals normal neuronal development of the DG in *Cux2*$^{neo/neo}$ mice **(G)** compared to *Cux2*$^{neo/+}$ littermate controls **(F)**. ***H, Numbers of DAPI-positive cells were unchanged in the *Cux2*$^{neo/neo}$ hippocampus compared to littermate controls (p=0.89). Images were counterstained with DAPI**.** Abbreviations: DG, dentate gyrus; h, hilus; SGZ, subgranular zone.

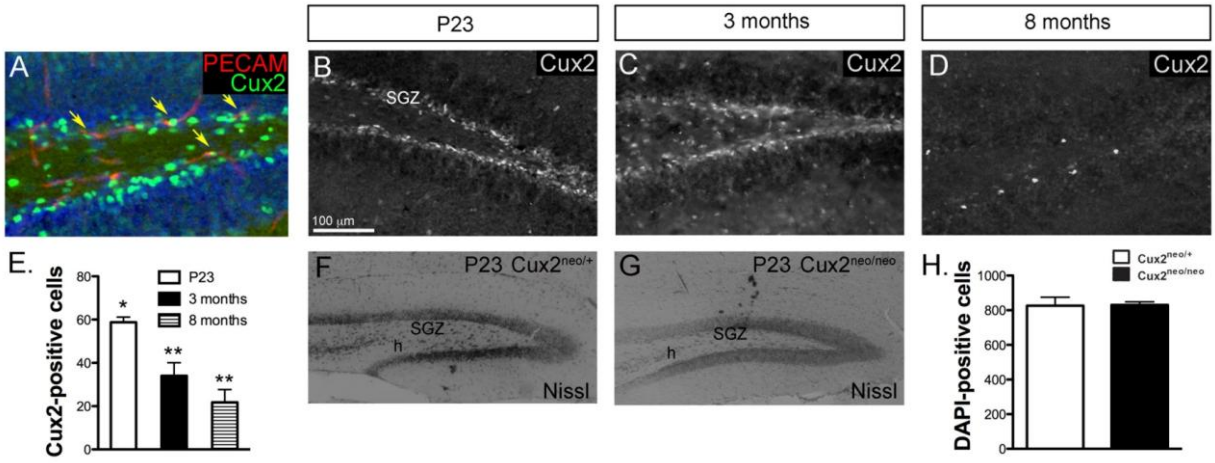
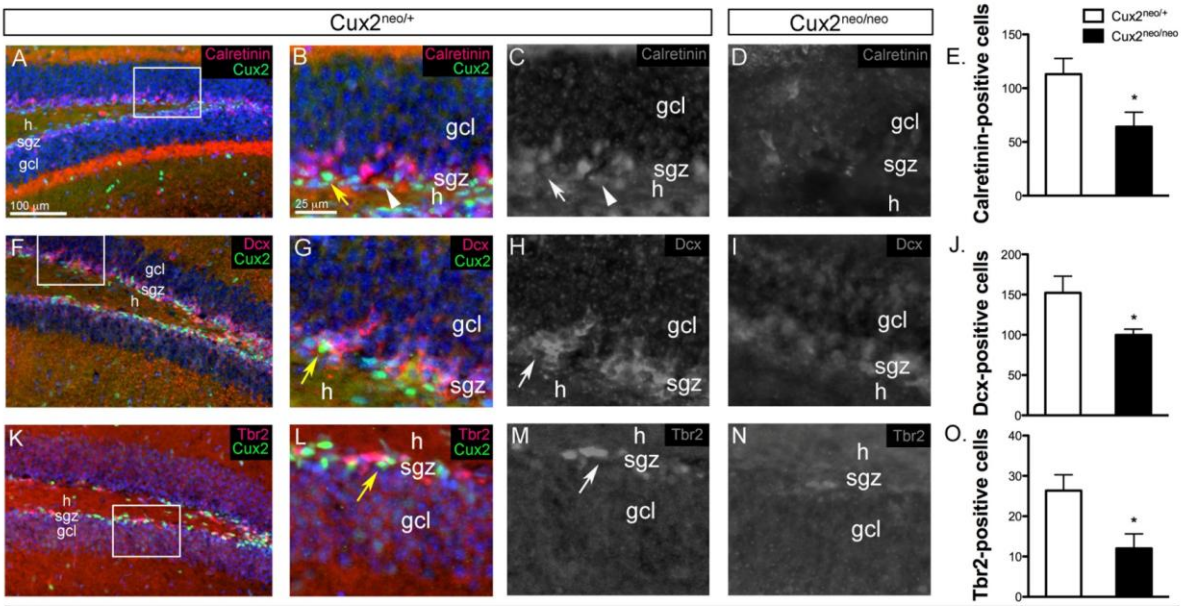

P23
3 months
8 months
A
PECAM
Cux2
B
Cux2
SGZ
100 μm
C
Cux2
D
Cux2
E.
Cux2-positive cells
P23
3 months
8 months
F
P23 Cux2neo/+
SGZ
h
Nissl
G
P23 Cux2neo/neo
SGZ
h
Nissl
H.
DAPI-positive cells
Cux2neo/+
Cux2neo/neo

Figure 2.1.

Figure 2.2. Reduced postnatal SGZ neurogenesis in *Cux2*$^{neo/neo}$ mutant mice. ***A-C***, Cux2-positive cells (***A,B***, green, arrow) adjacent to Calretinin-positive immature neurons (***A,B***, red). ***B, C,*** Cux2 staining was occasionally detected in Calretinin-positive cells (white; arrowhead). ***D,*** Reduced Calretinin staining in *Cux2*$^{neo/neo}$ mutants (p=0.028; ***E***). ***F-H,*** Cux2-positive cells (***G***, green) in the SGZ adjacent to clusters of Dcx-positive neuroblasts (***F, G***, arrow). ***H, I,*** Reduced Dcx staining in the *Cux2*$^{neo/neo}$ DG (p=0.033; ***I***). ***K,L,*** Cux2-positive cells (green) adjacent to clusters of Tbr2-positive transit amplifiers in the SGZ (***L*** red, arrow; ***M*** white). ***N,*** Reduced Tbr2-positive transit amplifiers (white) in *Cux2*$^{neo/neo}$ mutants (p=0.01; ***O***). ***P***, Co-localization with Cux2 was highest for Sox2 (64.2%). Limited or no co-localization with Tbr2, Dcx, PSA-NCAM, Calretinin and PDGFRα. Images counterstained with DAPI. Abbreviations as in Fig. 1.



P.	Sox2	[1]Tbr2	[1]Dcx	[1]PSA-NCAM	[1]Calretinin	PDGFRα
%Co-localization with Cux2	64.2 +/- 4.4	4.5 +/- 0.5	11.3 +/- 1.2	4.4 +/- 0.82	6.2 +/- 1.3	0

[1]Weak Cux2 expression in co-localized cells

Figure 2.2.

Figure 2.3. Increased numbers of hippocampal progenitors in *Cux2*$^{neo/neo}$ mutants. ***A,B,*** Cux2 (green) co-localized with Nestin-positive (red) radial glial NPCs in control *Cux2*$^{neo/+}$ SGZ (arrows). ***D, E,*** Increased Nestin-positive radial glia (white) in *Cux2*$^{neo/neo}$ mutants (p=0.002; ***E***). ***F,G,*** Cux2 (green) co-localized with Sox2-positive NPCs (red; arrows). Inset in ***(G)*** of unmerged Cux2 staining in Sox2-positive cells (***G,*** red***,*** arrows). ***I, J,*** Increased Sox2-positive SGZ progenitors (white) in *Cux2*$^{neo/neo}$ mutants relative to *Cux2*$^{neo/+}$ littermate controls (p=0.019; ***J***). ***K-N,*** Cux2 co-localization with GFAP. **O,** Numbers of GFAP-positive cells were unchanged in *Cux2*$^{neo/neo}$ mutants (***M***, white) relative to *Cux2*$^{neo/+}$ controls (***N***, white; p=0.76). ***P, Q,*** Confocal microscopy of a Cux2-positive SGZ cell (blue) co-expressing both Nestin (green) and Sox2 (red) (arrowhead). Arrow identifies a Nestin and Sox2 double-positive cell that did not express Cux2. ***R,*** Chart summarizing the majority of Cux2-expressing cells (75%) co-expressed both Nestin and Sox2. Images counterstained with DAPI. Abbreviations as in Figs. 2.1 and 2.2.

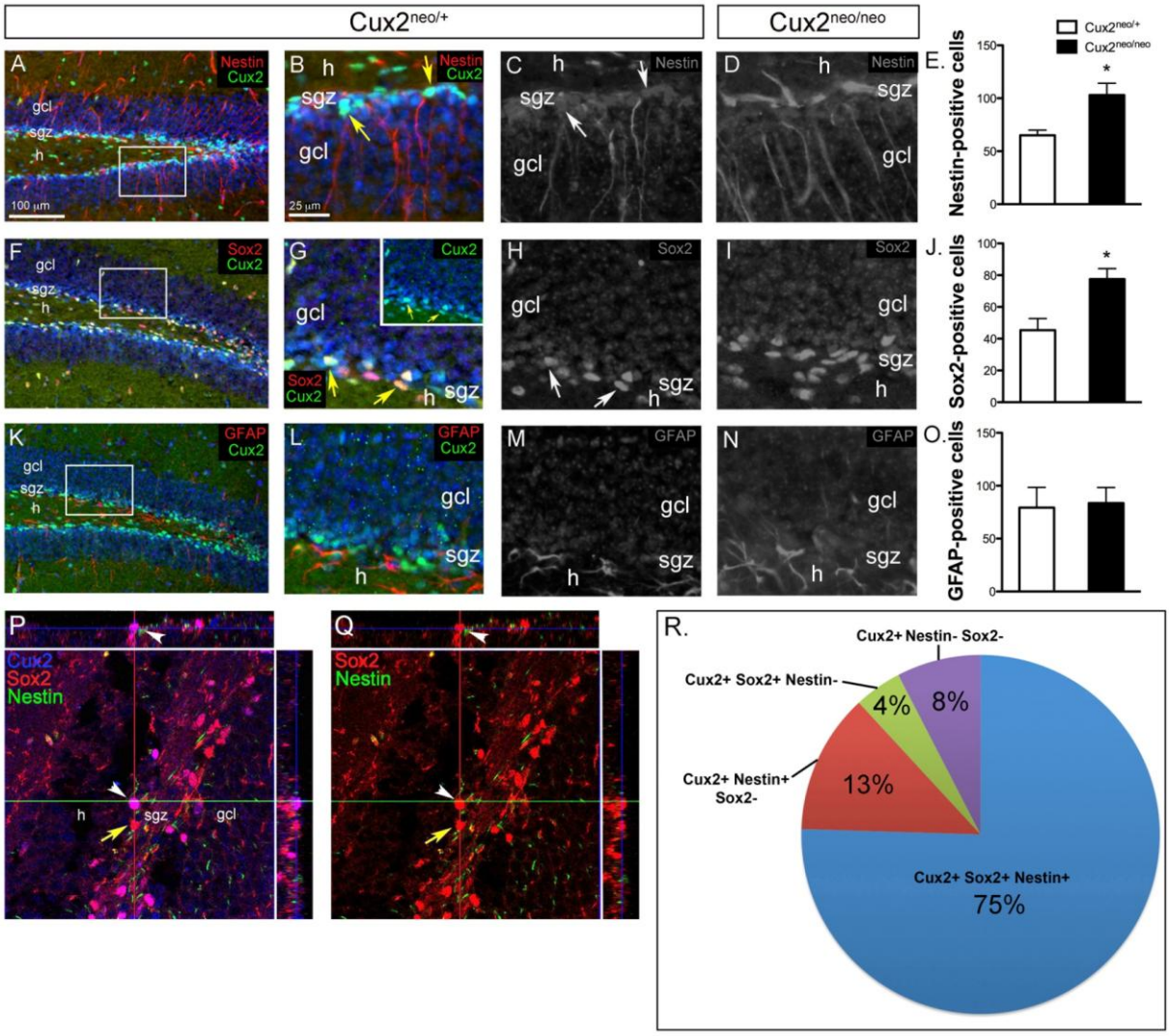

Cux2neo/+
Cux2neo/neo
A
Nestin
Cux2
gcl
sgz
h
100 μm
B
25 μm
C
Nestin
D
E.
Cux2neo/+
Cux2neo/neo
Nestin-positive cells
F
Sox2
Cux2
G
H
Sox2
I
J.
Sox2-positive cells
K
GFAP
L
M
GFAP
N
O.
GFAP-positive cells
P
Cux2
Sox2
Nestin
Q
R.
Cux2+ Nestin- Sox2-
Cux2+ Sox2+ Nestin-
4%
8%
Cux2+ Nestin+ Sox2-
13%
Cux2+ Sox2+ Nestin+
75%

Figure 2.3.

Figure 2.4. Increased proliferation of hippocampal progenitors in *Cux2*$^{neo/neo}$ mutants. ***A,B,*** Anti-BrdU immunohistochemistry (red) following an acute BrdU pulse revealed increased proliferation of SGZ cells in *Cux2*$^{neo/neo}$ mutants relative to *Cux2*$^{neo/+}$ littermates (p=0.030; ***C***). BrdU-positive cells (***D***, red, arrow; ***F***, white) co-localized with Nestin- (***D***, green, arrow; ***E***, white) and Sox2-positive (***G***, green, arrow; ***H***, white) SGZ progenitors . The acute BrdU pulse (***J,*** red, arrow; ***L***, white*)* did not label Dcx-positive neuroblasts (***J***, green, arrow; ***K***, white). ***M-O,*** Increased Ki67 immunostaining in *Cux2*$^{neo/neo}$ mutants relative to *Cux2*$^{neo/+}$ littermates (p=0.001; ***O***). Images counterstained with DAPI. Abbreviations as in Figs. 2.1 and 2.2.

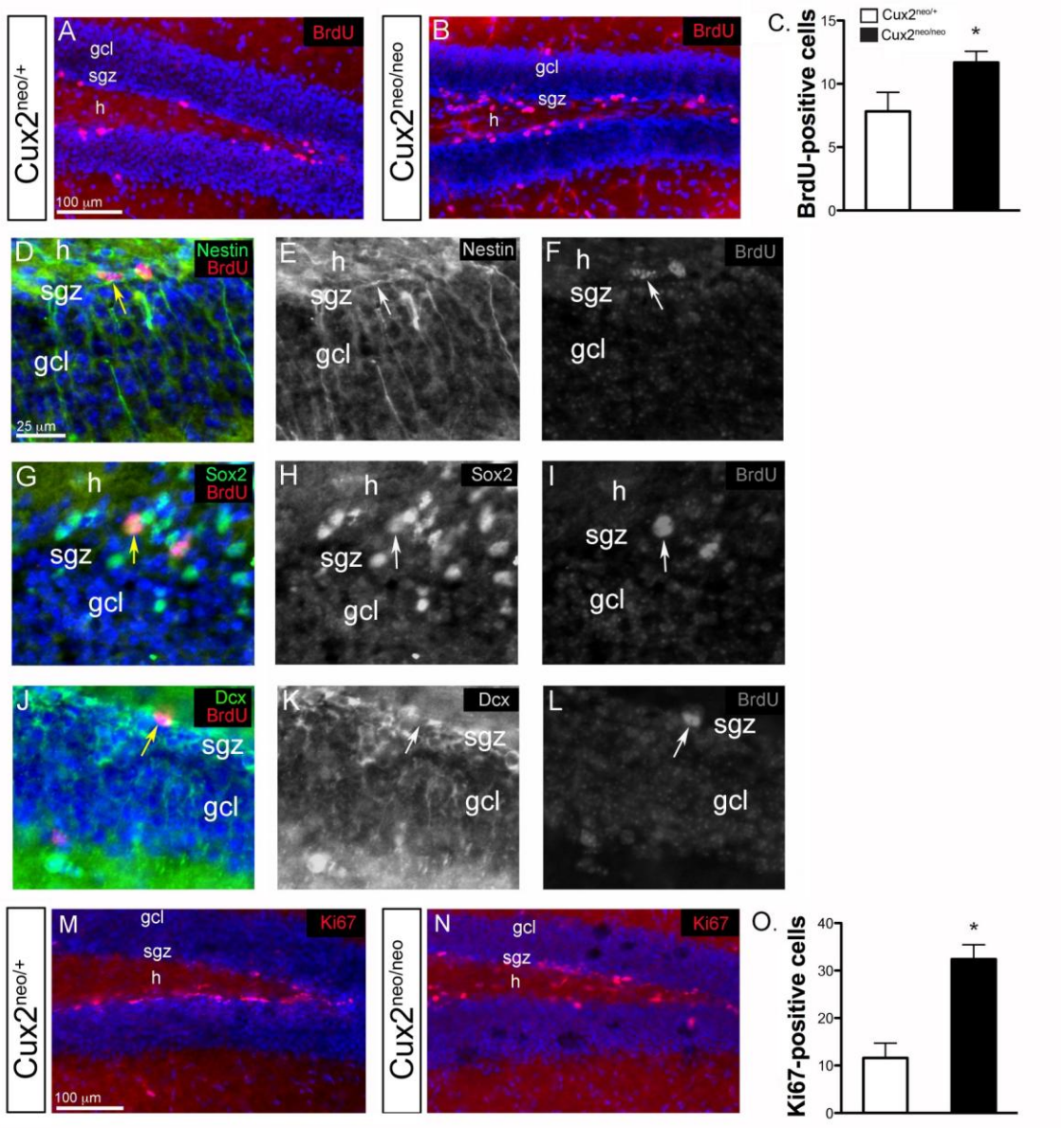

Cux2neo/+
A
gcl
sgz
h
BrdU
100 μm
Cux2neo/neo
B
BrdU
C.
Cux2neo/+
Cux2neo/neo
BrdU-positive cells
D h
Nestin
BrdU
sgz
gcl
25 μm
E h
Nestin
F h
BrdU
G h
Sox2
BrdU
H h
Sox2
I h
BrdU
J
Dcx
BrdU
K
Dcx
L
BrdU
M
Ki67
N
Ki67
O.
Ki67-positive cells

Figure 2.4.

2.5. Discussion

The regulation of postnatal neurogenesis is central to our understanding of the regenerative ability of the brain. Much attention has been focused on the elucidation of the mechanisms governing the birth of neurons in two regions of the adult brain: the SVZ of the lateral ventricle and the SGZ of the hippocampus. Although progress has been made, our knowledge of the neurogenic factors remains rudimentary. Here we report that the transcription factor Cux2 is expressed in Nestin and Sox2 double-positive NPs and is important for hippocampal neurogenesis.

In particular, we show that *Cux2* deficiency led to fewer immature neurons (Calretinin+ cells), migrating neuroblasts (Dcx+ cells), and transit-amplifiers (Tbr2+ cells) without affecting gliogenesis. Interestingly, we also observed an increase in Type 1/Type 2a Nestin- and Sox2- positive progenitors in the $Cux2^{neo/neo}$ mutant SGZ, indicating that Cux2 normally acts to restrict self-renewal and promote neuronal differentiation. Consistent with this, *Cux2* loss led to increased progenitor proliferation as revealed by BrdU immunstaining following an acute pulse. One interpretation of these findings is that Cux2 acts within Type 1/Type 2a hippocampal progenitors to drive their neuronal differentiation. Upon *Cux2* loss, NPCs continue to self-renew and build up in numbers because they cannot differentiate efficiently.

Previous work has shown that *Cux2* is expressed in progenitors of the embryonic spinal cord and fetal cortical SVZ (Cubelos et al., 2008; Iulianella et al., 2008). Since Cux2 regulates both the proliferation and differentiation of NPCs during development, it is possible that the decreases in postnatal hippocampal neurogenesis we observed in $Cux2^{neo/neo}$ mutants reflect changes that occurred during development. However, we noted

that the *Cux2* mutant hippocampus appeared to have normal neuronal development as revealed by Nissl staining and DAPI cell counts (Fig. 1). The Cux2 mutation is hypomorphic and since it largely circumvents embryonic lethality it is suitable for postnatal analysis. Furthermore, we restricted our analysis to markers of *de novo* neurogenesis, such as the immature neuronal marker, Calretinin (Brandt et al., 2003). Our findings are therefore consistent with a role for Cux2 in regulating postnatal hippocampal neurogenesis, although embryonic effects cannot be entirely ruled out.

Other regulatory proteins, such as the basic helix-loop-helix (bHLH) transcription factors Neurogenin 2 (Ngn2) and NeuroD1, have been shown to regulate hippocampal neurogenesis (Roybon et al., 2009). However, NeuroD1 and Ngn2 are principally expressed in proliferating Tbr2-positive transit-amplifying NPCs (Ozen et al., 2007; Roybon et al., 2009), whereas Cux2 mainly localized to Type 1/Type 2a NPCs and not in Tbr2-positive cells (Fig. 2P). This suggests that Cux2 may function upstream of the bHLH proneurogenic factors in the postnatal brain. Indeed, we previously showed that Cux2 acts upstream of NeuroD1 to activate a neurogenic program in spinal cord progenitors (Iulianella et al., 2008). Consistent with this, both *NeuroD1* (Roybon et al., 2009) and *Cux2* (current study) loss led to deficits in neuronal production in the postnatal SGZ. Cux2 may thus act on Type 1/Type 2a NPs to promote their commitment to the neuronal fate.

There has been controversy as to whether the postnatal hippocampus contains multipotent self-renewing progenitors or lineage-restricted non-self-renewing progenitors (Bonaguidi et al., 2011; Clarke and van der Kooy, 2011; Encinas et al., 2011; Kempermann, 2011). Some evidence suggests the hippocampal progenitors contain separate glial and neuronal lineages and have limited self-renewal (Bull and Bartlett, 2005;

Clarke and van der Kooy, 2011; Encinas et al., 2011). On the other hand, *in vivo* clonal analyses suggest that these NPCs are indeed multipotent and are capable of self-renewal in the postnatal DG (Bonaguidi et al., 2011; Suh et al., 2007). Part of the issue may relate to the limited availability of markers for undifferentiated but neuronally committed progenitors. Such a marker may help to identify when a Type 1 hippocampal progenitor first commits to neuronal differentiation. We now provide evidence that Cux2 is a key regulator of this cell fate decision and may serve as novel discriminatory marker for neuronally committed NPCs. Future work using cell fate analyses of Cux2-positive NPs may help determine whether the hippocampus contains multipotent or lineage-restricted progenitors.

2.6. Acknowledgements

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2.7. References

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CHAPTER 3: CELL CULTURE ANALYSES OF CUX2 FUNCTION

3.1 Introduction

NPCs within the adult hippocampus and SVZ are part of specialized niches which support both their self-renewal and differentiation. These niche environments include cell-cell interactions and cell signaling as well as association with the vasculature and endothelial cells (Doestch et al., 2002). Findings presented in **Chapter 2** demonstrated that transcription factor Cux2 is required for normal hippocampal neurogenesis within the endogenous niche. However, the intrinsic specification of adult NPCs and the extent to which they depend on their niche environment is not well understood. Given the complexity of this interaction, the role of Cux2 in neurogenesis was investigated independent of the complex niche environment utilizing a cell culture system.

It was hypothesized that NPCs derived from the *Cux2*$^{neo/neo}$ mutant postnatal hippocampus would exhibit reduced numbers of neurons in culture. Since Cux2 is known to drive neurogenesis in both the developing cortex and spinal cord (Cubelos et al.,2008; Iulianella et al., 2008) it may serve as a 'master' regulator of the neuronal fate independent of the niche environment. Indeed, certain transcription factors act in an intrinsic manner independent of outside influences. For example, mice deficient in the transcription factor *Sox2*, show reduced proliferation of NPCs in the SVZ. When these NPCs were cultured *in vitro* outside of the SVZ niche environment, the effect of *Sox2* loss was recapitulated (Cavallaro et al., 2008). However, previous studies have shown that adult hippocampal-derived NPCs may be primarily gliogenic in culture (Bull and Bartlett, 2005; Seaberg and van der Kooy, 2002; Clarke and van der Kooy, 2011), unlike those from the SVZ. Indeed, when the association of niche endothelial cells and SGZ precursors is disrupted neurogenic potential is lost and precursors become glia (Monje et al., 2002). With this in mind, the

relatively young postnatal P7 hippocampal-derived neurospheres were used to evaluate their neurogenic potential in culture. The goal of this experiment was to (i) determine whether P7 hippocampal-derived NPCs are neurogenic in culture and if yes, (ii) examine any possible differences in differentiation in *Cux2*$^{neo/neo}$ mutant versus control cultures.

After differentiation for 7 days, cells were immunostained for neurogenic, gliogenic, and oligodendrocytic markers to evaluate numbers of cell types in both control and mutant cultures. However, P7 hippocampal NPCs were found to be primarily gliogenic in culture and so they could not be used to investigate the cell-autonomous role of Cux2 in neuronal differentiation. This limitation required the usage of an alternative source of NPCs for culture neurogenesis. Embryonic NPCs derived from the E14.5 dorsal telencephalon are multipotent in culture (Kelly et al., 2009) and so they were used to test the hypothesis that Cux2 is required for normal neurogenesis outside of the brain in culture conditions.

3.2 Methods

3.2.1 Animals

All animals utilized in this study were treated in accordance with the regulations of Dalhousie University Carlton Animal Care facility and guidelines of the Canadian Council on Animal Care. Hypomorphic *Cux2*$^{neo/neo}$ mice were previously generated (Iulianella et al., 2008) and although minor embryo lethality was reported with this mutation, most mutant mice were born viable and fertile and appeared grossly normal.

3.2.2. P7 Hippocampus and E14.5 Dorsal Telencephalon Dissections

Neurosphere Proliferation Complete Medium (Stem Cell Technology) was prepared according to the manufacturer's instruction. The final concentration of rhEGF (Stem Cell Technology) was 20 ng/mL and rhFGF2 (R & D Systems) of 10ug/mL. In preparation for NSC dissections from both P7 hippocampus and E14.5 dorsal telencepthalon, T25 flasks were prepared with 8 mL of preheated Neurosphere Proliferation Complete Medium. Crude dissections to remove intact brains were performed on the bench-top and brains were placed singularly into sterile Petri dishes of autoclaved PBS containing pen-strep (1uL/mL) and glucose (2mg/mL). Hippocampal or dorsal telencephalon regions were dissected from intact brains in a sterile bench using a dissecting microscope. All fine dissections were performed using sterile technique with brains immersed in PBS containing pen-strep and glucose (**Figure 1 and 2).**

Dissected targets (either P7 hippocampus or E14.5 dorsal telencephalon) were placed in 15-mL falcon tubes containing 2 mL of Neurosphere Complete Proliferation Medium supplemented with antibiotics. Cells were mechanically triturated in a sterile tissue culture hood using fire-polished Pasteur pipettes. After trituration cells were spun @1000 rpm for 3 min in a centrifuge, supernatant was removed, and cells were resuspended in pre-warmed Neurosphere Proliferation Complete Medium. Resuspended cells were cultured in suspension in T-25 flasks at 37°C in 5% CO_2 for 7 days.

3.2.3. Experimental Procedure

3.2.3.1. Proliferation

For proliferation assays, complete proliferation media was refreshed every 2 days as follows: the growing neurospheres were transferred to sterile 15-mL Falcon tubes and spun at 400 rpm for 3 min followed by the removal of supernatant. Cell pellets were gently resuspended in 5 mL of media and placed into a new pre-warmed T-25 flask and incubated at 37ºC. Cells were allowed to proliferate for 7 days at which time neurospheres were harvested for quantitative analysis. Neurospheres were counted using a hemacytometer. After the first round of Proliferation for 7 days, cells were triturated and either plated under differentiation conditions at a density of 500,000 cells/mL or re-plated for a second round of proliferation at 100, 000 cells/mL as described above.

3.2.3.2. Differentiation

To differentiate neurospheres, proliferating neurospheres were triturated, counted and resuspended in Neurosphere Differentiation Medium (Stem Cell Technology) according to manufacturer's instruction. Briefly, neurospheres were collected in 15-mL falcon tubes and spun at 800 rpm for 3 minutes. Old proliferation medium was removed and cells were re-suspended in Neurosphere Differentiation Complete Medium. Cells were counted using a hemacytometer and plated at equal densities (500,000 cells/mL) on matrigel-coated coverslips for adherance. Media was changed every 2 days by aspirating half of the old media and infusing with new media. After 7 days of differentiation, cells were processed for immunocytochemistry as follows to determine cellular identity and allow quantification of numbers of neurons.

Immunocytochemistry

Once the cells were cultured in differentiation conditions for 7 days, the medium was aspirated, cells were fixed with 4% PFA for 20 minutes at room temperature, and processed for immunocytochemistry. Briefly, cells were washed twice in PBS for 5 min/wash followed by permeabilization in 0.1% PBT for 10 min and 2 washes in PBS. Cells were blocked for 1h at room temperature in 5% donkey serum diluted in PBS and primary antibodies were diluted in blocking solution and incubated in a humidified chamber overnight at 4ºC. Primary antibody was aspirated and cells were washed 3 times in PBS for 5 min/wash. Secondary antibodies were diluted in blocking solution and incubated for 1 h at room temperature in the dark. After two rinses in PBS, cells were counterstained with DAPI (1:5000) diluted in PBS for 10 min at room temperature. Cells were rinsed in PBS for 5 minutes and then mounted using DAKO cytomation mounting medium and visualized using an inverted fluorescent microscope (Zeiss). For E14.5 dorsal telencephalic-derived cultures, cells were counted as 4 quadrants using a light microscope. Numbers obtained from the 4 quadrants were then averaged to obtain numbers of glia (GFAP-positive cells) and neurons (Tuj1-positive cells for Experiment 1; Dcx and Calretinin-positive cells for Experiment 2).

3.3 Results and Discussion

3.3.1. P7 Hippocampal NPC Proliferation and Differentiation in Culture

In order to assess the cell intrinsic role of Cux2 in cultured hippocampal NPCs, the proliferative and differentiation capacities of this system were first characterized. After 7 days in proliferative conditions, P7 hippocampal derived neurospheres appeared to be large and healthy (i.e. reflected light under the microscope, data not shown). Cells were

transferred to differentiation conditions to test their neurogenic potential. Double immunostains were performed on fixed cells to obtain a profile of cell types present after 7 days in differentiation conditions. Each sample was stained for either Nestin/Cux2, PDGFR/Tuj1, or GFAP/Dcx. Cells were found to differentiate into GFAP-positive cells (**Figure 3.3C**), PDGFR-positive cells (**Figure 3.3B**), or remain as Nestin-positive cells (**Figure 3.3A**). However, cells did not differentiate into Dcx- (**Figure 3.3C,** green) or Tuj1-positive neurons (**Figure 3.3B,** green). An additional observation was made concerning the expression of Cux2 in cultured NPCs. Although Cux2 is thought to be exclusively expressed in the cell nucleus, it appeared to sometimes be excluded from the nucleus in proliferating neurospheres (**Figure 3.3A**). Future work is needed to address this observation and determine Cux2 localization in cell culture. Nonetheless, counting numbers of GFAP-positive glia showed that gliogenesis was not affected by Cux2 loss in culture. This finding recapitulates my *in vivo* data which showed that numbers of glia did not differ in *Cux2*$^{neo/neo}$ mutant hippocampal sections when compared to their littermate controls (**Chapter 2**). However, since cultured P7 hippocampal-derived NSCs were predominantly gliogenic, the role of Cux2 in culture neurogenesis could not be evaluated in this system.

To attempt to stimulate the formation of neurons from P7 neurospheres, the culture experiment was repeated with supplementation of a high concentration of Bone-derived Neurotrophic Factor (BDNF) (10ug/mL) in differentiation medium. Previous work has shown that BDNF is a potent differentiation factor *in vitro* which down-regulates cell proliferation and increases neurogenesis (Cheng et al., 2003; Babu et al., 2007). However, several attempts of increasing BDNF yielded similar cultures exclusively populated by glia

(data not shown). This may reflect the limited potential of NPCs derived from the maturing hippocampus, as observed by others **(**Seaberg and van der Kooy, 2002; Clarke and van der Kooy, 2011). However, it is also possible that dissections and culture conditions could be further optimized. Nonetheless, it is interesting that adult hippocampal NPCs are no longer primarily neurogenic once removed from the hippocampal niche and placed in cell culture differentiation conditions. Indeed, this phenomenon remains part of an ongoing debate as to whether the hippocampus contains multipotent NPCs or rather separate lineages of committed progenitors (See **Chapter 4** for a more detailed discussion). By contrast, NPCs derived from the SVZ remain neurogenic when placed in culture conditions (Doetsch et al., 2002). Central questions are raised as to why SVZ cells are inherently different from those in the hippocampus? What unique signals are present in the hippocampus that permit neurogenesis *in vivo* that are apparently absent from culture conditions? Interestingly, when SGZ hippocampal NPCs are transplanted into the RMS they become olfactory interneurons instead of hippocampal granule cells (Suhonen et al., 1996). Similarly, when precursor cells from the non-neurogenic spinal cord and substantia nigra are placed in the hippocampus they become hippocampal granule cells (Shihabuddin et al., 2000; Lie et al., 2002). These studies suggest that NPC fate is more dependent upon the host environment rather than on intrinsic factors. It is therefore not surprising that hippocampal NPCs behave differently in culture than within their niche environment. Indeed, the current model of the neurogenic niche frames NPCs and their environment as forming a functional unit (Kempermann, 2011) and highlights the importance of the bidirectional interaction of NPCs and the other niche factors.

3.3.2. The Role of Cux2 in Promoting Neurogenesis in Fetal NPCs

3.3.2.1. Experiment 1: Assessing the Role of Cux2 in Proliferation and Differentiation Potential of Fetal NPCs

Since embryonic NPCs from the dorsal telencephalon form neurons in culture, these cells were utilized instead of P7 hippocampal NPCs to test the cell intrinsic role of Cux2. After 7 days of proliferation, E14.5 dorsal telencephalon-derived neurospheres generally appeared to be healthy (reflected light and did not appear dark). Proliferative cultures were triturated, counted and plated either under differentiation conditions or in a second round of proliferation to test self-renewal. Differentiation of control cultures for 7 days yielded many Tuj1-positive neurons (**Figure 3.4 A,C**) as well as some GFAP-positive glia in culture (**Figure 3.4 B,C**) as expected. This confirmed that embryonic neurospheres from the dorsal telencephalon are a useful system for studying neurogenesis under defined conditions in culture. This experiment was repeated utilizing NPCs derived from *Cux2*$^{neo/neo}$ mutant dorsal telencephalon and that from littermate controls. Indeed, *Cux2*$^{neo/neo}$ mutant neurospheres produced significantly fewer Tuj1-positive neurons in culture upon differentiation (**Figure 3.5 B,C**) than did Cux2$^{neo/+}$ littermate controls (**Figure 3.5 A,C;** $p=0.025$). This finding shows that Cux2 is required for normal neurogenesis of embryonic NPCs derived from the dorsal telencephalon in culture. Whereas mutant cultures generated significantly fewer neurons, numbers of GFAP-positive glia were unchanged (**Figure 3.5 C;** $p>0.05$) indicating that Cux2 is not required for normal gliogenesis of embryonic NPCs in culture. Again, this finding is consistent with previous conclusions (**Chapter 2** and **Experiment 3.3.1**) that the role of Cux2 is specific to the neuronal lineage.

Interestingly, E14.5 mutant cultures displayed other qualitative differences when compared to their littermate controls. Differentiating Tuj1-positive neurons were often still attached to neurospheres and failed to gain typical bipolar neuronal morphology in *Cux2*$^{neo/neo}$ mutant cultures (**Figure 3.5 B).** Where organized neuronal networks with arborizations appeared to form in control cultures (**Figure 3.5 A**), Tuj1-positive cells in mutant cultures appeared disorganized and clumped (**Figure 3.5 B**). These defects in dentritic arborizations in mutant cultures are particularly interesting since neuronal function is largely regulated by dendrites and spines (Parrish et al., 2007). Given that Cut genes have been previously shown to modulate dendritic branching and dentritic spine morphologies in the upper layer neurons of the cortex (Cubelos and Nieto, 2010) this may be one explanation for our observations. Indeed, in the cortex the functions of Cux1 and Cux2 are additive and complement one another in order to determine the characteristics of the dendritic tree as well as the final synaptic strength (Cubelos and Nieto, 2010). Interestingly, similar results have been obtained for Sox2 mutant cultures (Cavallaro et al., 2008). Upon differentiation, Sox2 mutant cultures did not develop extensive arborizations as were present in control cultures and the number of mature neurons generated was reduced. Also, numbers of glia were not affected (Cavallaro et al., 2008) highlighting the specific role for Sox2 in neuronal development.

To assess the proliferative capacities of control versus mutant cultures, neurospheres that had been plated for a second round of proliferation at equal densities were counted. Numbers of neurospheres did not differ between control and mutant cultures (**Figure 3.6 C; p=0.57**) suggesting that Cux2 is not required for normal proliferation of fetal-derived neurospheres in culture. Overall, this experiment shows that

Cux2 is important for normal embryonic neurogenesis in culture and is not required for gliogenesis. This result also demonstrates that self-renewal capacity does not differ in mutant cultures after second passaging. However, this experiment did not address any potential differences in self-renewal of neurospheres between the first and second passages.

3.3.2.2. Experiment 2: Characterizing Normal Embryonic NPC Behavior as a Model Culture System

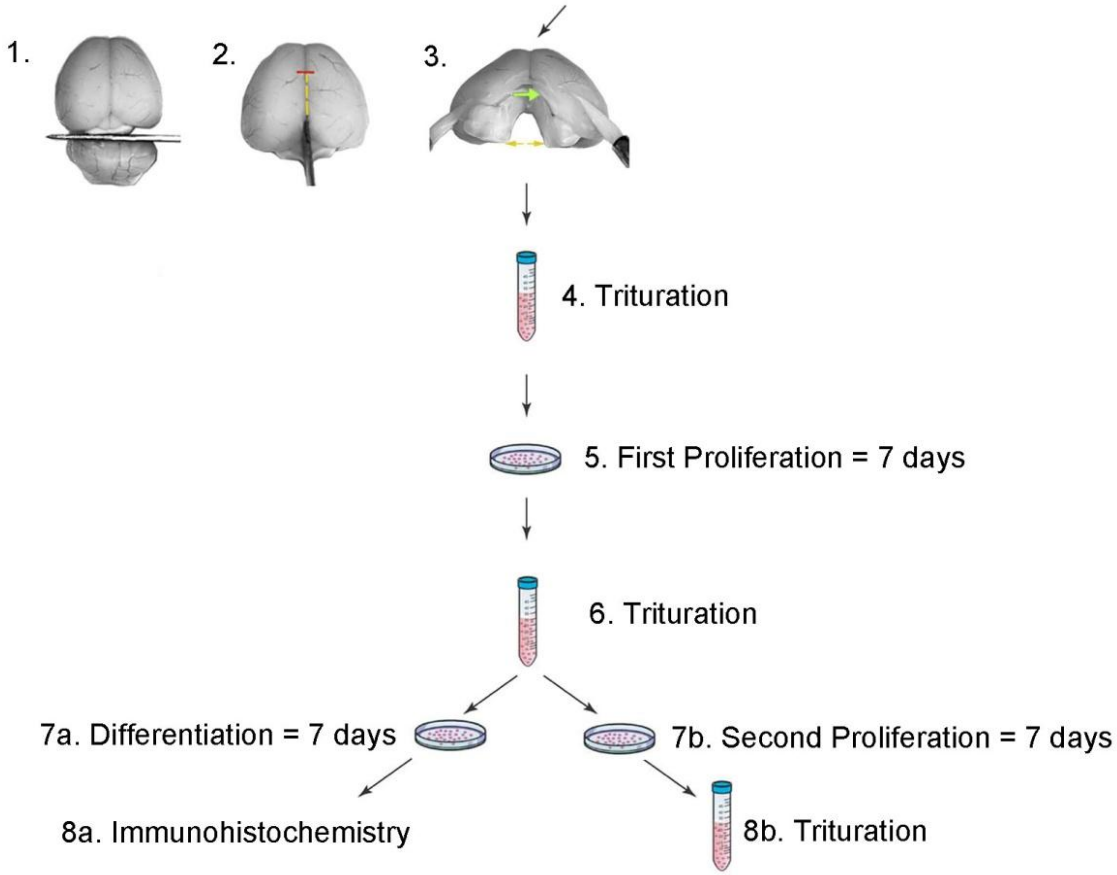
In order to further characterize embryonic dorsal telencephalic-derived neurospheres as a model system for the study of culture neurogenesis, the culture experiment was repeated using 2 litters of control E14.5 mice (n=14). It has been proposed that the size of neurospheres may reflect their "stemness" in that larger neurospheres are more like stem cells in their self-renewal properties than small neurospheres (Walker et al., 2008). Since self-renewal capacity decreases with each passage (Bull and Bartlett, 2005), I hypothesized that smaller neurospheres would be produced after the second passage than with the first. Although I found that neurospheres were significantly smaller after the second passaging as predicted (**Figure 3.7 B**; $p>0.0001$), neurospheres were also significantly more numerous (**Figure 3.7 A**; $p<0.0001$). This finding may indicate that second passaging triturates neurospheres more easily and so more individual cells (and not clumps of cells) are present than with the first passage. However, since neurospheres have a tendency to fuse (Singec et al., 2006; Jessberger et al., 2007), sphere size should not be used as the sole indicator of stemness.

While the previous experiment revealed that E14.5 Cux2 mutants generate fewer Tuj1-positive neurons in culture, the next aim was to further characterize normal neuronal

differentiation in this system. To determine the relative proportion of neuroblasts to immature neurons after 7 days of differentiation, the numbers of Dcx-positive neuroblasts were compared to the numbers Calretinin-positive immature neurons in control cultures. Dcx-positive neuroblasts were significantly more numerous than the calretinin-positive immature neurons (**Figure 3.8 A-D;** p=0.001). Interestingly, calretinin-positive immature neurons were also positive for the more immature marker Dcx (**Figure 3.8 D**) indicating that most neurons are just beginning to mature after 7 days under differentiaton conditions. Differentiation for a longer period of time may yield more maturing neurons. Future work will address whether control and mutant cultures have different relative proportions of several neuronal markers including Dcx and Calretinin. Cux2 may be required to *efficiently* promote neurogenesis and so a cell-type profile analysis such as this could reveal differences in progression along the neuronal differentiation pathway.

To more fully characterize the cellular identity of Cux2 positive cells in culture immunostains were completed for markers Nestin, Sox2, GFAP, and Dcx with Cux2 after 7 days of proliferation (**Figure 3.9**). Cux2 appeared to colocalize with NPC markers Nestin (**Figure 3.9 A-C**), Sox2 (**Figure 3.9 D-F**) and GFAP (**Figure 3.9 G-I**) and also with the neuroblast marker Dcx (**Figure 3.9 J-L**). This suggests that Cux2 may be more broadly expressed in neurons in embryonic cultures than in the postnatal hippocampus since previous work showed that Cux2 is only weakly expressed in Dcx-positive cells (**Chapter 2**). Future work could directly compare Cux2 localization in cultured hippocampal NPCs with cultured embryonic NPCs to determine differences in expression.

Figure 3.1. P7 mouse hippocampal dissection and cell culture experimental outline. The cerebellum was removed using a sterile razor blade **(1)**. An opening was made along the longitudinal fissure (diagonal arrow) to expose the hippocampus **(2)**. The hippocampus was removed from each hemisphere with a stab blade in a rolling motion **(3)** and placed in a falcon tube for trituration **(4)**. Cells were cultured under proliferation conditions for 7 days **(5)** followed by re-tituration **(6)**, counting and plating for either differentiation **(7a)** or challenged with a second round of proliferation **(7b)**. Cells were stained to reveal cell type **(8a)** or triturated and counted to quantify proliferation **(8b**).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5. First Proliferation = 7 days
6. Trituration
7a. Differentiation = 7 days
7b. Second Proliferation = 7 days
8a. Immunohistochemistry
8b. Trituration

Figure 3.1.

Figure 3.2. Location of the embryonic dorsal telencephalon dissected to obtain NPCs for culture. (A) Sagittal view of mouse embryo after removal from pregnant female. **(B)** Representative depiction of the dorsal telencephalon (red) in a single cerebral hemisphere. Dissected tissue was treated as in the experimental outline for P7 mouse hippocampal NSCs in Figure 3.1. Representative diagram adapted from Hutton and Pevny, 2008.

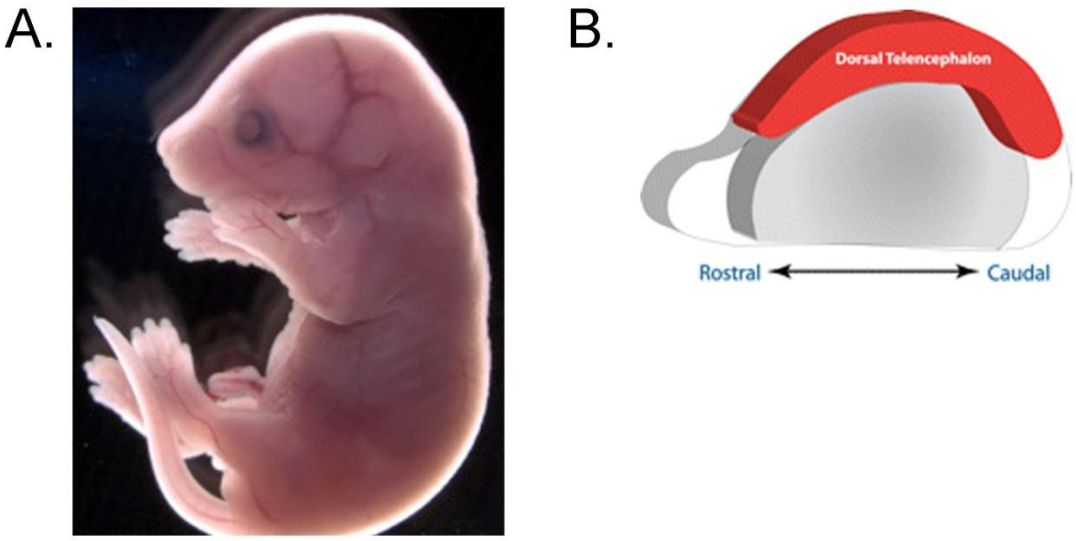

Figure 3.2.

Figure 3.3. P7 hippocampal derived neurospheres differentiate into glia but not into neurons. Representative images of cells after 7 days of differentiation immunostained with Nestin (red) and Cux2 (green; **A**), oligodendrocyte marker, PDGFR(red) and neuronal marker Tuj1 (green; **B**) and immature neuronal marker Dcx (red) and glia marker GFAP(green; **C**). Cells counterstained with DAPI. (n=9 different control cultures).

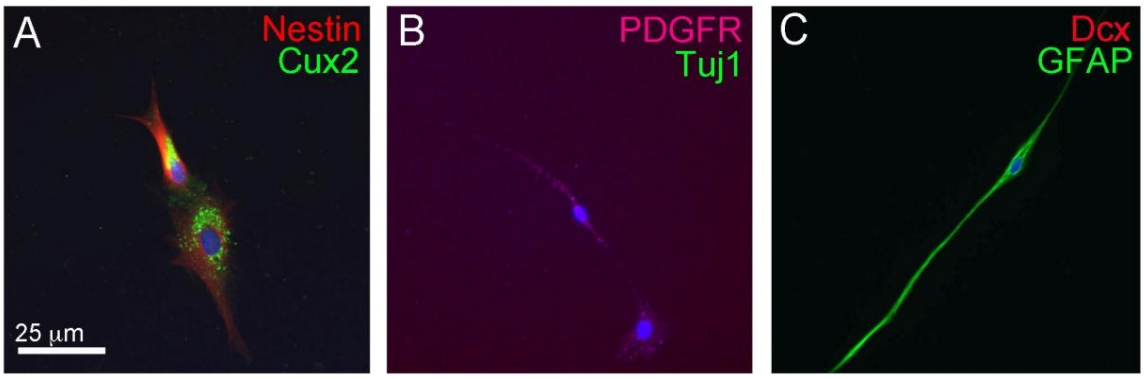

Figure 3.3.

Figure 3.4. E14.5 dorsal telencephalon neuropheres from Cux2$^{neo/+}$ mouse after 7 days of differentiation. The majority of cells were positive for neuronal marker Tuj1 (green) (**A, C**) and others for glial marker GFAP (red) (**A, B, C**). Images were captured using Zeiss fluroescent microscope. Cells counterstained with DAPI nuclear stain (Blue).

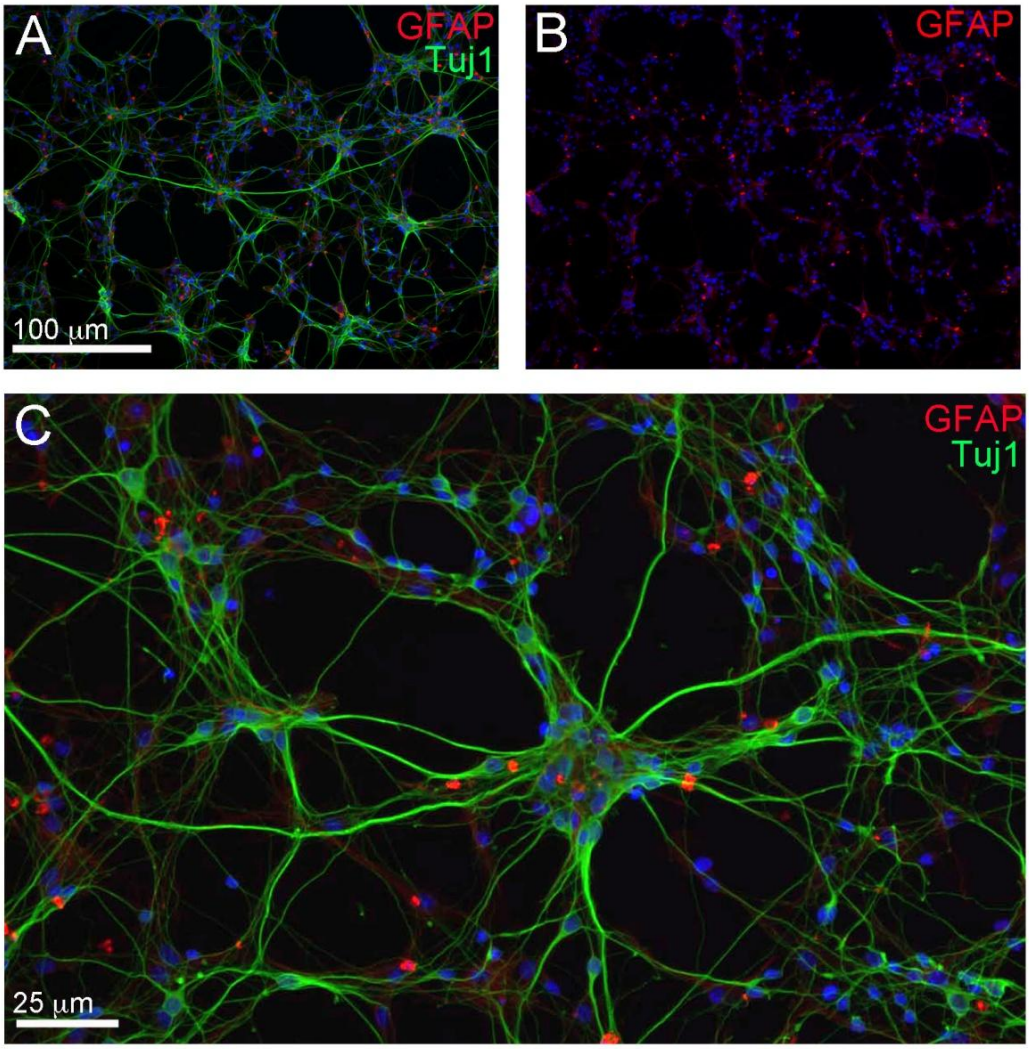

Figure 3.4.

Figure 3.5. E14.5 dorsal telencephalon neurospheres after 7 days of differentiation. Tuj1-positive neurons (green) were significantly reduced in *Cux2*$^{neo/neo}$ cultures (**B**) compared to their *Cux2*$^{neo/+}$ littermate controls (**A, C**; n=8, p=0.0246) whereas numbers of GFAP-positive cells (red; **A, B**) were unchanged (**C**). Cells counterstained with DAPI. Error bars represent ± SEM.

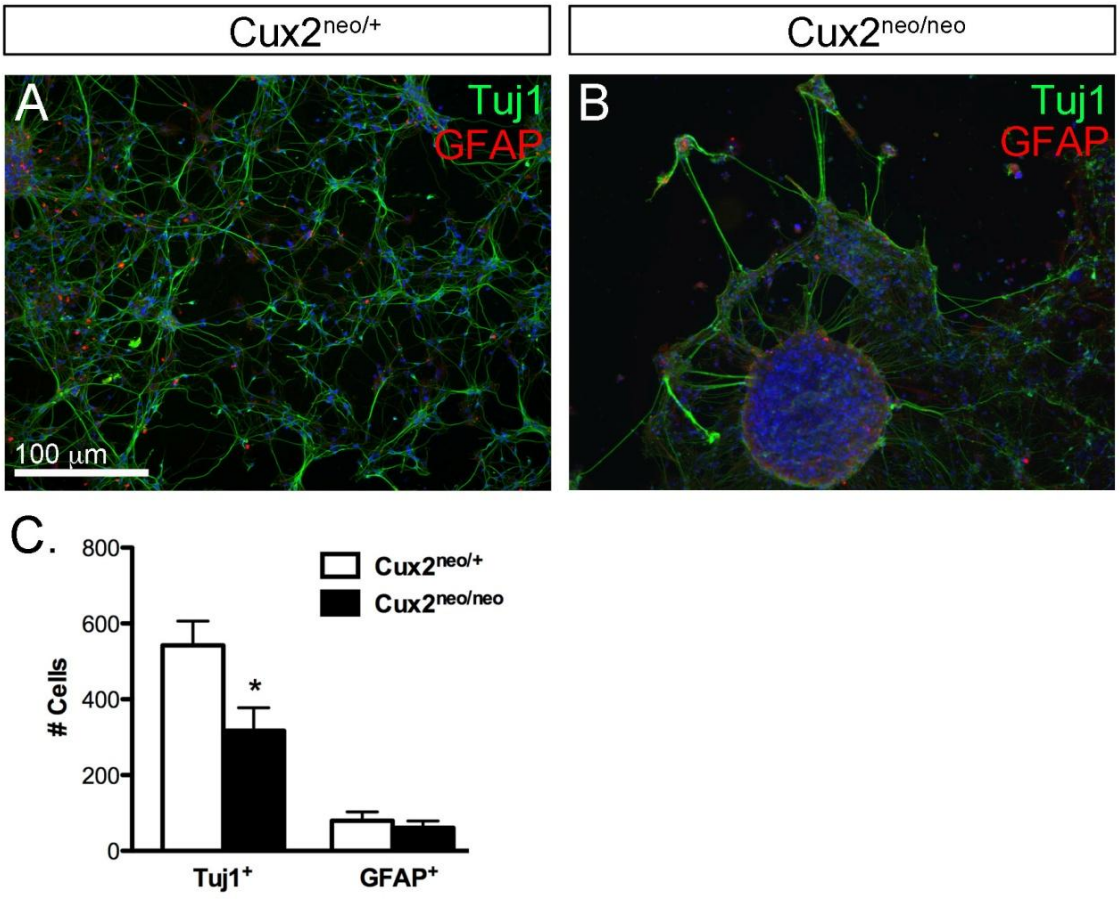
Cux2neo/+
Cux2neo/neo
A
Tuj1
GFAP
100 μm
B
Tuj1
GFAP
C.
800
600
400
200
0
Cells
Cux2neo/+
Cux2neo/neo
*
Tuj1+
GFAP+

Figure 3.5.

Figure 3.6. E14.5 dorsal telencepthalon neurospheres after a second round of proliferation. (**A**) *Cux2*$^{neo/+}$ neurospheres did not differ in number compared to (**B**) *Cux2*$^{neo/neo}$ cultures (**C**; n=8, p=0.57) after the second round of proliferation in culture for 7 days. Error bars represent mean ± SEM.

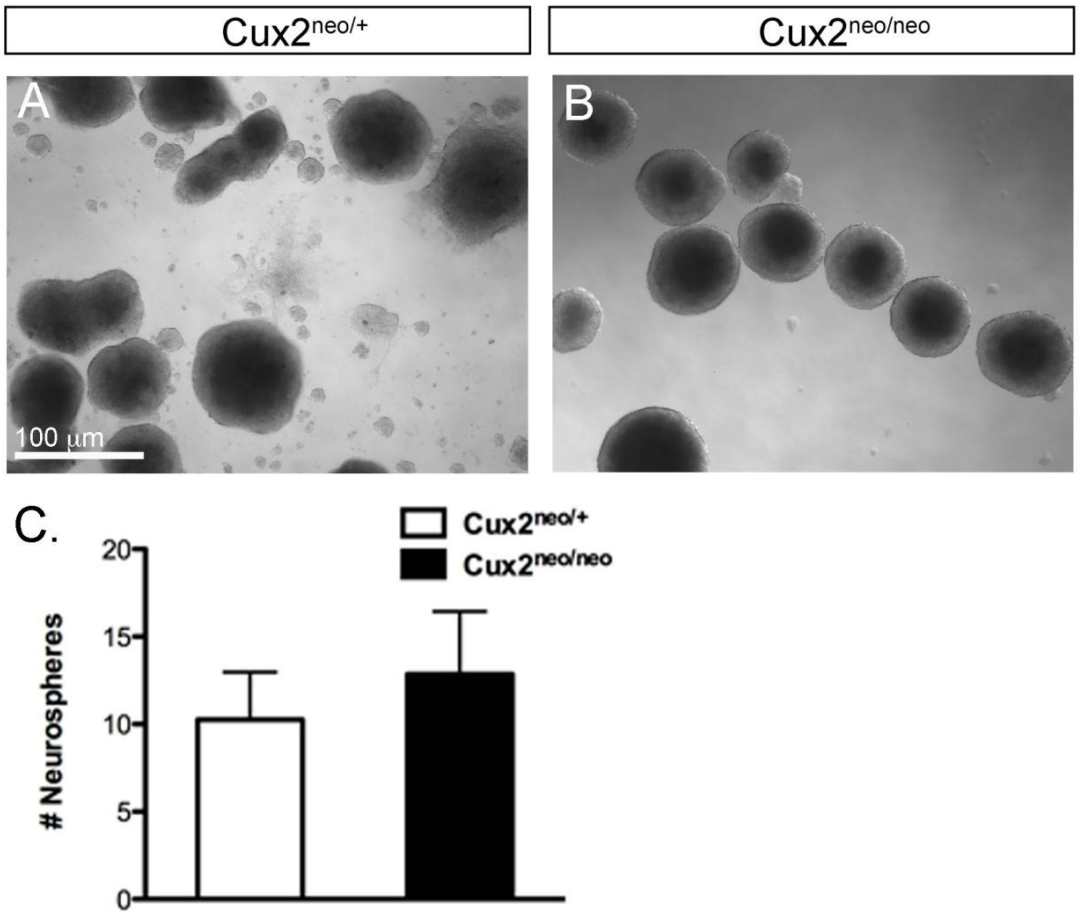
Cux2neo/+
Cux2neo/neo
A
B
100 μm
C.
Cux2neo/+
Cux2neo/neo
Neurospheres
20
15
10
5
0

Figure 3.6.

Figure 3.7. E14.5 neurosphere proliferation after first and second passages.

The number of proliferating neurospheres was significantly increased after the second passage (**A**; n=14, $p<0.0001$) but neurospheres were significantly smaller after the second passage (n=14, $p<0.0001$; **B**). Error bars represent mean ± SEM.

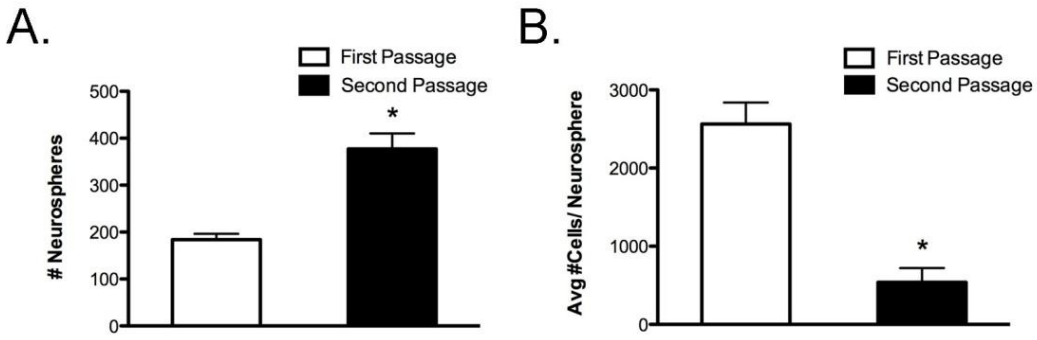
A.
First Passage
Second Passage
Neurospheres
500
400
300
200
100
0
*
B.
First Passage
Second Passage
Avg #Cells/ Neurosphere
3000
2000
1000
0
*

Figure 3.7.

Figure 3.8. E14.5 neurospheres (from Experiment #2) in control mice after 7 days of differentiation. The number of Dcx-positive cells (green) was significantly greater (**D**; n=8, p=0.0022) than the number of calretinin-positive immature neurons (**B**). Most Calretinin-positive immature neurons (red) were also positive for Dcx (green **A, B**). Dcx-positive cells also out-numbered cells that were double positive for both Dcx and Calretinin (**C**; p=0.0008). Error bars represent mean ± SEM.

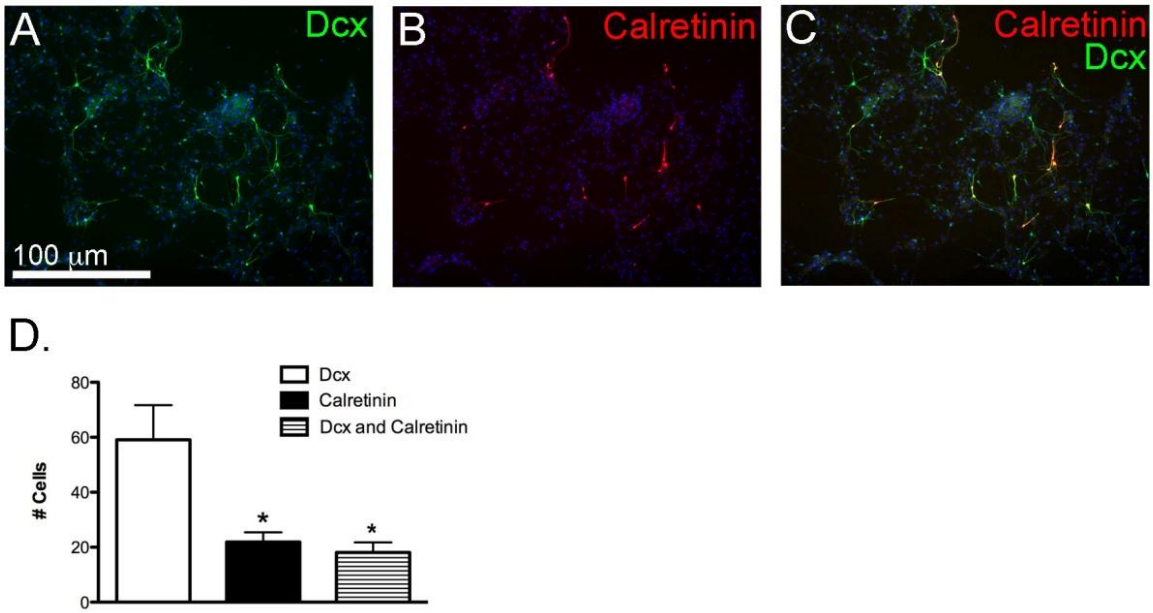
A
Dcx
100 μm
B
Calretinin
C
Calretinin
Dcx
D.
Dcx
Calretinin
Dcx and Calretinin
Cells
80
60
40
20
0
*
*

Figure 3.8.

Figure 3.9. E14.5 dorsal telencephalon neurospheres after 7 days of proliferation. (**A-C**) Nestin (red) was often co-expressed with Cux2 (green). (**D-F**) Sox2 (red) co-localized with Cux2-positive cells (green) in an interesting pattern (white box). (**G-H**) GFAP (red) rarely labeled Cux2-positive cells (green) and (**I-K**) the majority of Dcx-positive cells (red) co-labeled with Cux2 (green). Cells counterstained with DAPI.

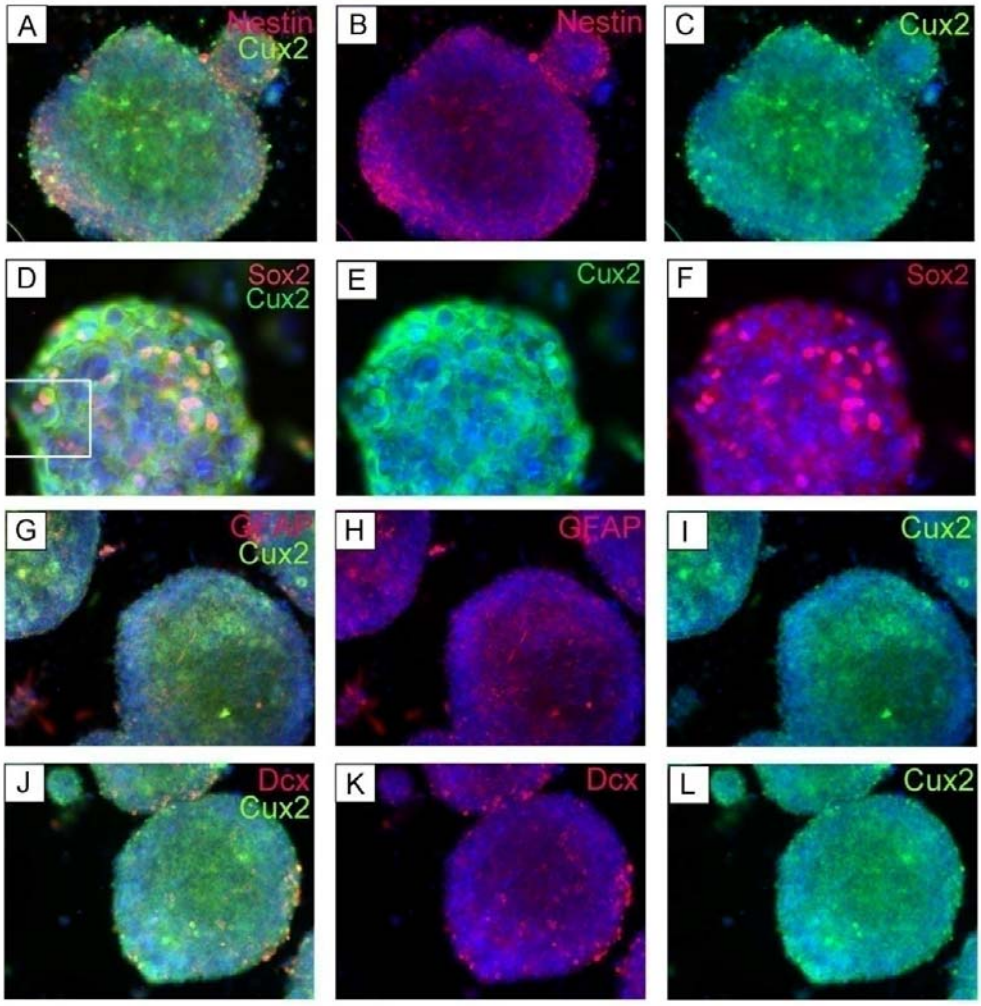
A
Nestin
Cux2
B
Nestin
C
Cux2
D
Sox2
Cux2
E
Cux2
F
Sox2
G
Cux2
H
GFAP
I
Cux2
J
Dcx
Cux2
K
Dcx
L
Cux2

Figure 3.9.

CHAPTER 4: DISCUSSION

4. Discussion

The major findings presented in this study demonstrate that Cux2 is a novel transcription factor involved in postnatal hippocampal neurogenesis. *Cux2*$^{neo/neo}$ mice showed reduced neurogenesis in the form of significantly fewer calretinin-positive immature neurons, Tbr2-positive transit amplifiers and Dcx-positive neuroblasts **(Figure 4.1.)**. Importantly; however, Cux2 was primarily not expressed in these neuronal cell types in the DG. Instead, Cux2 was predominantly co-expressed with Nestin and Sox2 in Type 1 and/or Type 2a NPCs in the SGZ. Since *Cux2* loss did not affect gliogenesis, Cux2 appears to be specific to the neuronal lineage and may be an important driver of neuronal differentiation. Indeed, regulation in glia and neuronal lineages appears to be largely independent (Steiner et al., 2004) and some evidence suggests that the hippocampus contains separate committed progenitors for the neuronal or glial lineages (Clarke and van der Kooy, 2011). While this is a matter of debate in the current literature, Cux2 may serve as a novel marker for NPCs committed to the neuronal fate. This is particularly important for our understanding of adult neurogenesis given that it is presently unclear when a progenitor is first committed to becoming a neuron in the hippocampus. Indeed, the identification of such a marker would be a milestone for understanding postnatal hippocampal neurogenesis (Bohlen and Halbach, 2007) and may eventually be useful in therapeutic strategies.

In addition to identifying a role for Cux2 in postnatal hippocampal neurogenesis, this study also demonstrates that Cux2 is important for *in vitro* neurogenesis of embryonic NPCs from the dorsal telencephalon. *Cux2*$^{neo/neo}$ mutant cultures generated significantly fewer Tuj1-positive neurons compared to their littermate controls, recapitulating the

proneuronal role of Cux2 identified in the postnatal hippocampus. While initially not the main focus of this study, these embryonic results are important for our understanding of Cux2 function. These findings show a critical role for Cux2 (i) in an additional system to the hippocampus, (ii) in development, and (iii) independent of the niche in cell culture conditions. As in the postnatal hippocampus, Cux2 loss did not affect gliogenesis in embryonic cultures, further highlighting the proneuronal role for Cux2. Despite the obvious differences between the postnatal hippocampal niche and embryonic cultures, Cux2 remains specific to the neuronal lineage in both systems. Indeed, a similar comparison of adult versus embryonic NPCs was used to characterize the role of Sox2 using mutant cultures (Cavallaro et al, 2008).

While this study highlights a specific role for Cux2 in neuronal differentiation, it also shows how Cux2 loss affects numbers of NPCs in the postnatal hippocampus. After a short acute pulse of BrdU in the postnatal mouse, *Cux2*$^{neo/neo}$ mutants had significantly more BrdU-positive cells in the DG than in littermate controls. Co-immunostains revealed that the majority of BrdU-positive cells were Nestin or Sox2-positive NPCs suggesting that Cux2-mutants appear to have increased proliferation. Greater proliferation of NPCs may reflect one of two realities. One possibility is that NP proliferation is somehow enhanced when Cux2 is not present. The other, and more probable conclusion, is that NPCs are simply unable to differentiate and are thus kept in a state of proliferation. Indeed, differentiation programs and cell-cycle exit must be coordinated in order to produce the correct number of differentiated cell types (Kintner, 2002). Cux2 is coupled with cell-cycle exit in development of the spinal cord (Iulianella et al., 2008). Cux factors bind to the promoters of cell cycle inhibitory proteins such as p27^{kip1}, to regulate their expression

(Iulianella et al., 2008; Sharma et al., 2009; Ledford et al., 2002) and have a dual role in both proliferation and neuronal differentiation in spinal cord NPCs. *In vitro* however, proliferation did not differ between Cux2 control and mutant embryonic neurospheres. This may suggest that the role of Cux2 does not become evident until the neurospheres are challenged with differentiation conditions. In other words, differences do not become evident until control cultures freely differentiate while mutant cultures appear to be withheld from differentiation. Similarly, previous work using Sox2 hypomorphic mutant mice showed that neurosphere numbers were unchanged in mutant cultures of adult SVZ NPCs (Cavallaro et al., 2008). It was only upon differentiation that mutant cultures showed deficits in the neurogenesis. Further, Sox2 mutant cultures of E14.5 forebrain neurospheres showed similar deficits in neurogenesis (Cavallaro et al., 2008). Current work in our lab is utilizing Chromatin Immunoprecipitation to determine direct interactions of Cux2 with other proteins. This should help to determine how Cux2 is acting in both neuronal differentiation and progenitor maintenance.

Beyond the functional role for Cux2 in neurogenesis, this is also the first study to characterize Cux2 expression within the hippocampal niche. Immunostaining with blood vessel marker PECAM initially showed that Cux2 positive cells are closely associated with the vasculature. Indeed, there is emerging importance of the vasculature in the hippocampal neurogenic niche for both progenitor maintenance and neuronal differentiation (Tavazoie et al., 2008). New neurons are born close to blood vessels within the hippocampus (Palmer et al., 2000) and Cux2-positive cells reside in areas rich in NPCs of the SGZ. Indeed co-immunostainings with typical NPC markers Nestin and Sox2 revealed that Cux2 is predominantly found in these Type 1/Type 2a NPCs. Although other

regulatory proteins, such as Neurogenin 2 (Ngn2) and NeuroD1 have been shown to regulate hippocampal neurogenesis (Roybon et al., 2009), they are principally expressed in Tbr2-positive transit-amplifying NPCs (Ozen et al., 2007; Roybon et al., 2009). Since Cux2 is expressed in the less differentiated Type 1/Type2a NPCs, Cux2 may function upstream of these bHLH proneurogenic factors in the postnatal brain. This demarcates Cux2 as a transcription factor specific to the neuronal lineage expressed at the beginning of the neuronal differentiation programme. Such a role for Cux2 in the postnatal brain would be consistent with the expression of Cux2 in spinal cord progenitors which acts upstream of NeuroD1 to activate a neurogenic program (Iulianella et al., 2008). Cux2 may now serve as one of the only known markers of basal NPCs which are committed to the neuronal lineage in the postnatal DG.

Comparing Cux2 expression in the DG of mice of various postnatal ages, demonstrated that Cux2 expression was greatest at the 3 week time-point. Interestingly, these maximal levels of Cux2 expression correlate with the timing of maximal postnatal neurogenesis (Altman and Bayer, 1990). Indeed, most postnatal hippocampal neurogenesis in the mouse takes places within the first few weeks of life after which it declines (Seki and Arai, 1995; Kuhn et al., 1996). For this reason **Chapter 2** focused on mice at 3 weeks of age to evaluate the role of Cux2 in postnatal hippocampal neurogenesis.

While many neuronal cells born during adult neurogenesis are actually eliminated before they become integrated in the neuronal network of the hippocampus, these cells do not lack functional significance. Newly born cells can still respond to excitatory stimuli and may thus serve an important function even if they only survive for a transient period of time and do not become fully integrated. Indeed, hippocampal NPCs *in vitro* have been

shown to sense neighboring neuronal activity and respond by entering the neuronal differentiation program (Deisseroth et al., 2004; Babu et al., 2009). Furthermore, when slice preparations were stimulated, by the entorhinal cortex, 3 week old immature neurons were easily activated (Mongiat et al., 2009). Schmidt-Hieber and colleagues demonstrated that newborn cells show the greatest synaptic plasticity a few weeks after they are born at which time they are critical for hippocampus-dependent learning tasks (Farioli-Vechioli et al., 2008). While studies such as these suggest an important role for newly generated neuronal cells, the importance of different stages of adult neurogenesis has not been fully characterized. Indeed regulation of neurogenesis can take place at many levels along the neuronal differentiation pathway. We have identified the importance for Cux2 in Type 1/and or Type 2a early NPCs in directing neurogenesis in the mouse hippocampus.

It is important to consider that the process of *de novo* neurogenesis itself takes approximately 3 weeks from neuronal birth to functional integration. Newborn neurons express immature neuron marker, Dcx, for roughly three weeks prior to final maturation (van Praag et al., 2002; Carleton et al., 2003; Jessberger and Kempermann, 2003). This means that any *de novo* neurons produced during postnatal neurogenesis would not have become integrated into the circuitry of the hippocampus at the 3 week time point in the postnatal brain. Therefore, we would not expect to see much difference in numbers of mature neurons of the hippocampus at this time point. Indeed, this is reflected in our DAPI counts of total cell numbers in Control versus *Cux2* mutant mice which did not differ. This was further reinforced using Nissl staining that showed that the numbers of mature neurons, specifically, do not differ between control and $Cux2^{neo/neo}$ hippocampi at 3 weeks of age.

An important caveat to consider regarding our mouse model; however, is that the hypomorphic mutation has been present in mutant mice throughout development. If Cux2 plays a developmental role in the formation of the hippocampus, any differences noted at the postnatal stage may be due to differences in NPCs that have carried over from development. Work is currently being conducted in our lab to address the potential role of Cux2 in the development of the DG using the same Cux2$^{neo/neo}$ mouse line that has been used in this study. To account for the possible complication of Cux2 being important for hippocampal development, the present study focused on *de novo* postnatal neuronal cell type markers for immature neurons including Dcx, Tbr2, and Calretinin. These neuronal markers were selected in order to capture the process of postnatal neurogenesis and not neurons that had already matured during development. Indeed, a similar study was performed using a Sox2 hypomorphic mutant mouse line to query the role of Sox2 in adult neurogenesis (Cavallaro et al., 2008). Cux2 consistently shows proneuronal roles at various ages, in various tissues including the developing spinal cord and cortex (Iulianella et al., 2008; Cubellos et al., 2008). Nonetheless, our study is the first to characterize Cux2 expression in the postnatal DG and to show that it is involved in normal hippocampal neurogenesis. Furthermore, we provide support for Cux2 as a novel marker for NPCs that are committed to neuronal differentiation.

To more clearly address the cell autonomous role of Cux2, NPCs were dissected from the adult hippocampus and cultured independent of the niche environment. However, since we found that these cells were primarily gliogenic in culture we could not compare numbers of neurons between controls and *Cux2*$^{neo/neo}$ mutants. It is interesting that hippocampal NPCs are gliogenic in culture while those from the SVZ are still neurogenic

when taken out of the niche environment. Why are NPCs from the hippocampus different? Why are culture conditions sufficient for neuronal differentiation of SVZ NPCs but insufficient for those from the SGZ? Interestingly, the two neurogenic regions have been shown to respond differently to external stimuli such as pathological conditions and treatment with neurotrophic factors (Li and Zhao, 2008; Zhao et al., 2008). Also, some genetic players serve different functions in the SVZ than in the DG such as Mash1/Asc11 (Parras et al., 2007; Jessberger et al., 2008) and FXR2. Fragile X relative protein 2 (FXR2), for example regulates neurogenesis in the DG through reduction of Noggin expression (Gu et al., 2011). However, this regulation does not occur in the SVZ highlighting an example of differential regulation between the two neurogenic regions. Also specific to the DG is the expression of Prox1 where it has a stage-specific role in adult hippocampal neurogenesis (Karalay et al., 2011). Indeed, several different transcription factors are expressed and play differential roles in the neurogenic zones and future work will examine the role of Cux2 in the adult SVZ. Beyond regulators of neurogenesis, the nature of NPCs themselves may differ in the DG and the SVZ in terms of their multipotency and self-renewal capacities (Kempermann et al., 2011). In fact, it has been suggested that unlike the SVZ, hippocampal NPCs may be a heterogenous population in that some are predestined for the neuronal lineage while others are exclusively committed to the glial lineage (Clarke and Van der Kooy, 2011). It has also been shown that NPCs in the SGZ may have limited self-renewal and are unable to proliferate indefinitely (Bull and Bartlett, 2005). Using *in vitro* single cell assays, single cells from the adult DG produced small, unipotent, and non-self-renewing neurospheres (Clarke and Van der Kooy, 2011). However, given the clear importance of the niche environment *in*

vivo, it is difficult to extrapolate *in vitro* findings to the living brain. Just because cells are unipotent in culture does not mean that they behave similarly in the brain. Indeed, *in vivo* fate analyses showed that Sox2-positive NPCs are in fact multipotent and self-renewing in the adult hippocampus (Suh et al., 2007) highlighting the opposing conclusion to the *in vitro* results. Similarly, our findings that postnatal hippocampal NPCs are primarily gliogenic in culture demonstrates a stark difference with the *in vivo* reality where NPCs were found to readily differentiate into neurons in the hippocampus.

It is currently an area of intense research as to whether external niche cues or rather cell intrinsic factors are more important for fate determination. Transplantation studies seem to suggest that the niche environment is more influential than cell intrinsic programmes. For example, when hippocampal NPCs are transplanted to the RMS of the olfactory system, they become olfactory neurons instead of hippocampal granule cells. Similarly, when precursor cells are extracted from the non-neurogenic spinal cord and substantia nigra and implanted in the hippocampus they differentiate into hippocampal granule cell neurons (Shihabuddin et al., 2000; Lie et al., 2002) and do not become neurons when placed in the non-neurogenic neocortex. While the results of our study cannot directly support either side of the NPC debate of the hippocampus, our findings suggest that Cux2 may be a novel marker for NPCs committed to the neuronal fate in the DG. Future work could develop sorting strategies by which only Nestin/Sox2-positive NPCs that express Cux2 are sorted by FACs. These Cux2-positive NPCs could be cultured and lineage-traced in order to and compare to the fate of NPCs that do not express Cux2. If the hippocampus contains a mixed population of NPCs, Cux2 may be particularly useful for delineating neuronally committed progenitors, or conversely, those that have become

committed to the neuronal differentiation programme after a division event of truly multipotent NPCs **(Figure 4.1B)**.

While cell culture experiments provide a powerful model system with which to address the cell intrinsic role of a transcription factor, one must be cautious when extrapolating findings back to the *in vivo* brain. Although therapeutic strategies to date have mostly aimed at NPC transplantation, a more likely approach may be to stimulate the endogenous system that is already intact in the living brain. Nonetheless, we need to first characterize the molecular players that govern postnatal neurogenesis before we can begin to design new therapeutic targets to combat injury and disease. Beyond potential new therapies; however, adult neurogenesis provides us with an important example of brain plasticity and serves as a relatively new frontier waiting to be discovered. Our understanding of adult neurogenesis is therefore of intrinsic importance to our understanding of basic principles guiding brain development and function.

Figure 4.1. Summary of hypothesized Cux2 function.

(A) *Cux2* loss resulted in decreased neuronal differentiation and increased numbers of Type1/2a NPCs. **(B)** Cux2 is required for normal neuronal differentiation and Cux2 may be a novel marker for Type 1/2a NPCs in the postnatal DG upon commitment to the neuronal differentiation program. Figure obtained from Dr. Iulianella.

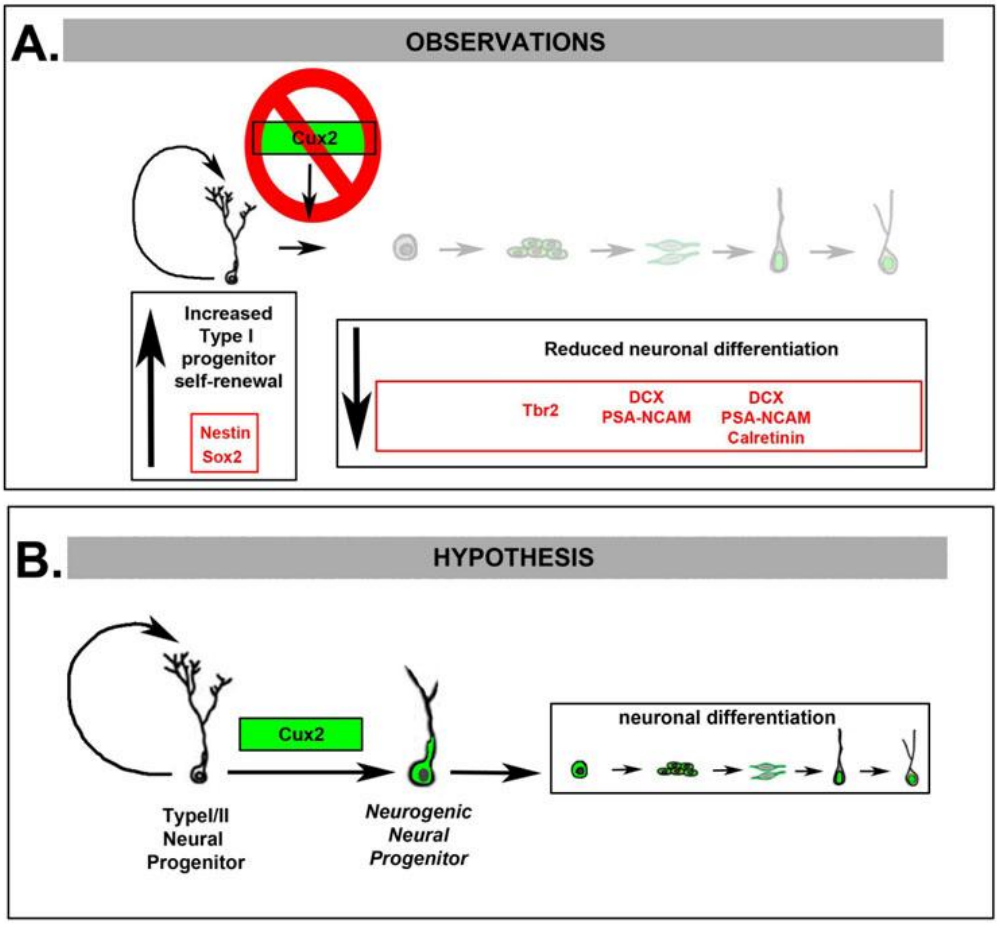
A.
OBSERVATIONS
Cux2
Increased
Type I
progenitor
self-renewal
Nestin
Sox2
Reduced neuronal differentiation
Tbr2
DCX
PSA-NCAM
DCX
PSA-NCAM
Calretinin
B.
HYPOTHESIS
Cux2
neuronal differentiation
Typel/II
Neural
Progenitor
Neurogenic
Neural
Progenitor

Figure 4.1.

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