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Serum Response Factor Regulates Multiple Aspects of Central Nervous System Development

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Serum Response Factor (SRF) Regulates Multiple Aspects of Central Nervous System Development

by

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Abstract of the Dissertation

Serum Response Factor (SRF) Regulates Multiple Aspects of Central Nervous System Development

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SRF is a highly evolutionary conserved activity-dependent transcription factor. Previous studies have shown that neuron-specific deletion of SRF results in deficits in tangential cell migration, guidance-dependent circuit assembly, activity-dependent gene expression, and synaptic plasticity in the hippocampus. However, very little is known in terms of whether SRF participates in earlier aspects of central nervous system development such as neuronal projection establishment, cell-fate specifications, and neural stem cell homeostasis and survival.

We report that SRF is critical for development of major axonal tracts in the forebrain. Conditional mutant mice lacking SRF in neural progenitor cells (*Srf*-Nestin-cKO) exhibit striking deficits in cortical axonal projections including corticostriatal, corticospinal, and corticothalamic tracts, and they show a variable loss of the corpus callosum. Neurogenesis and interneuron specification occur normally in the absence of SRF and the deficits in axonal projections were not due to a decrease or loss in cell numbers. Similar axonal tract deficits were also observed in mutant mice lacking SRF in the developing neurons of neocortex and hippocampus (*Srf*-NEX-cKO). These findings suggest crucial functional roles for SRF during neuronal development; SRF is specifically required in a cell-intrinsic

manner for axonal tract development but is dispensable for cell survival, neurogenesis, neocortical lamination, and neuronal differentiation.

Furthermore, we found that deletion of SRF in neural precursor cells in *Srf*-Nestin-cKO animals results in 40 - 60% loss in astrocytes as well as oligodendrocytes precursor cells at birth. Astrocytes and oligodendrocytes play crucial roles in nearly every facet of brain development and function; abnormalities in glia have important implications in neurological disorders and neurodegenerative diseases. Despite considerable knowledge on the role of several ligand-receptor complexes that regulate astrocyte and oligodendrocyte specification, the transcriptional mechanisms critical for their development in the brain remain unknown. The loss of astrocytes and oligodendrocytes is not due to cell death or increased neurogenesis. SRF-deficient NPCs exhibited normal growth rate and capacity to self-renew but were deficient in glial specification in response to several pro-astrocytic or pro-oligodendrocyte signals *in vitro*. Similarly, we observed an increase in the number of proliferative cells in the ventricular zone from embryonic day 14 to day 18, suggesting that SRF-deficient precursor cells accumulate as they fail to acquire post-mitotic glial cell-fates. In contrast, conditional SRF deletion in developing forebrain neurons (*Srf*-NEX-cKO) did not affect astrocyte differentiation, suggesting a cell-autonomous role for SRF in astrocyte specification. Mechanistically, SRF mediates astrocyte fate-choice by regulating Notch2 receptor expression in NPCs, and Notch2 receptor deletion in NPCs phenocopies the deficits in astrocyte specification. Interestingly, conditional SRF deletion in committed

astrocytes (*Srf*-GFAP-cKO) exhibited hypertrophic and gliosis morphology concomitant with a 4-6 fold increase in astrocytes throughout the brain of 4-week old mutant mice. Together, our findings show that SRF is required, but not sufficient, for astrocyte and oligodendrocyte specification in the brain; SRF is necessary in a cell-autonomous manner in NPCs to regulate astrocyte specification and it plays a critical role in committed astrocytes for proper development.

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Chapter 1: Introduction

Central nervous system development

The development of mammalian central nervous system requires a precise orchestration of temporally and regionally specific molecular events within the population of neural stem/precursor cells (NPC) to establish a highly organized and sophisticated organ comprised of three predominant cell types—neuron, astrocyte and oligodendrocyte. These molecular events are initiated and modulated sequentially by inductive extracellular cues and intracellular signaling pathways, which lead to the generation of neurons, followed by astrocytes, then oligodendrocytes in the developing cortex. In rodents, neurogenesis begins at embryonic day (E)12, peaks at E14, and gradually recedes around E17. Astrocyte specification begins at E18, peaks around P0 to P2, and is followed by oligodendrocyte formation which is first seen postnatally with peak production at P14 (Levison et al., 1993; Zerlin et al., 1995; Parnavelas, 1999).

Neurogenesis

In the mammalian cortex, the earliest proliferating NPCs constitute a single layer of pseudostratified columnar epithelium, also called the neuroepithelial cells (Gotz and Huttner, 2005). These NPCs can undergo

symmetric divisions, giving rise to two proliferative precursor daughter cells, or an asymmetric a cell division, generating a daughter cell with the same precursor fate as well as one with restricted lineage commitment such as a neuron (McConnell, 1995; Rakic, 1995). During early neurogenic phase, the initial population of neurons establishes the preplate first, then they populate the cortical plate in an inside-out fashion, and cortical neurogenesis is mostly complete during the embryonic period (Okano and Temple, 2009a). Radial glia, a population of proliferative cells that arise from the neuroepithelium after the onset of neurogenesis, are a pivotal source of neurons and provide scaffold support for cortical layering establishment (Kriegstein and Gotz, 2003; Anthony et al., 2004; Merkle et al., 2004; Englund et al., 2005). Initially, radial glial cells that reside in the ventricular zone undergo asymmetric cell division to give rise to lower level cortical pyramidal neurons; radial glia also undergo symmetrical cell division that takes place primarily at the basal level of the ventricular zone to generate intermediate progenitor cells, which are restricted to the neuronal fate, that then give rise to neurons populating the upper layer of the neocortex (Haubensak et al., 2004; Noctor et al., 2004; Englund et al., 2005). Genetic analysis in *Drosophila* first identified a number of “pro-neural genes” that are transcription factors from the basic helix-loop-helix (bHLH) class and were demonstrated to be intrinsic determinants that are necessary and sufficient to promote the commitment of precursor cells to neuronal lineage (Bertrand et al., 2002).

These pro-neural genes were found to be expressed primarily, if not exclusively, in the developing nervous system in vertebrates. For example, neurogenin 1 and 2 (Ngn1/2) are expressed in the ventricular zone only during neurogenesis (Gradwohl et al., 1996), and they function through binding onto E box domains to activate genes that promote neuronal cell-fate specification, and expression of Notch ligands — Delta and Jagged — that repress neuronal commitment in neighboring precursor cells (Fode et al., 1998; Sun et al., 2001). Furthermore, Ngn1 is capable of inhibiting glial cell-fate by sequestering CREB binding protein (CBP)/p300 from signal transducers and activators of transcription (STAT), which is a transcription factor crucial for gliogenesis (Sun et al., 2001). Another bHLH transcription factor Mash1, expressed in the olfactory epithelium, is required for olfactory neuron differentiation, and ablation of this gene results severe reduction of olfactory neurons (Cau et al., 1997). Importantly, inactivation of transcription factors that modulate neurogenesis such as Tbr2/Eomes can lead to anatomical disorders such as microcephaly and cognitive deficits (Baala et al., 2007; Arnold et al., 2008).

In early neurogenesis period, environmental signals such as bone morphogenic proteins (BMP) enhance neuronal specification by signaling through their heterotrimeric serine/threonine kinase receptors and activate downstream Smad transcription factors, allowing complex formation with CBP/p300, at a distinct site to which Ngn1 binds and resulting activation of neuronal genes expression (Li et al., 1998). Similarly, growth factors like neurotrophins and

platelet-derived growth factor (PDGF) couple with receptor tyrosine kinase (RTK) receptors, mediating the activation of SHP2-MEK-ERK Rsk signaling cascade. This promotes neurogenesis by phosphorylating C/EBP family of transcription factors that bind and transactivate neuron-specific genes, such as *βIII-tubulin* and *math1*, directly (Menard et al., 2002; Uittenbogaard et al., 2007).

Other mechanisms that instruct neurogenesis act in part by ensuring gliogenesis is repressed during the neurogenic phase. Like the Ngn1 sequestration of CBP/p300 from STAT, neuregulin-1 inhibits glial cell-fate by binding to ErB4 receptors, leading to intracellular signaling and translocation of N-CoR, a corepressor for multiple transcription factors by complexing with histone deacetylases, from cytoplasm to the nucleus. This inhibits cytokine-mediated gliogenesis by complex formation with the Notch effector recombination signal binding protein-Jκ (RBP-Jκ) and repress astrocytic genes such as *gfap* and *s100β* (Sardi et al., 2006).

Astrogenesis

The switch from neurogenesis to gliogenesis, starting with astrocyte formation, has been found to be dependent on both intrinsic competency of the precursor cells and their extrinsic environment. Culture experiments of early forebrain neural precursors have shown that they only generate neurons in the first few days and then astrocytes and oligodendrocytes – a reflection of intrinsic

programming. Similarly, cells isolated from early embryonic cerebral cortex differentiate preferentially into neurons, whereas cells from late prenatal or early postnatal stages differentiate into astrocytes and oligodendrocytes (Qian et al., 2000), suggesting their intrinsic cell-fate competency changes over-time. On the other hand, early embryonic precursor cells cultured with embryonic cortical slices differentiate into neurons, yet when cultured with postnatal cortical slices they adopt glial phenotype (Morrow et al., 2001), a result that demonstrates that the surrounding extracellular cues also play an important role.

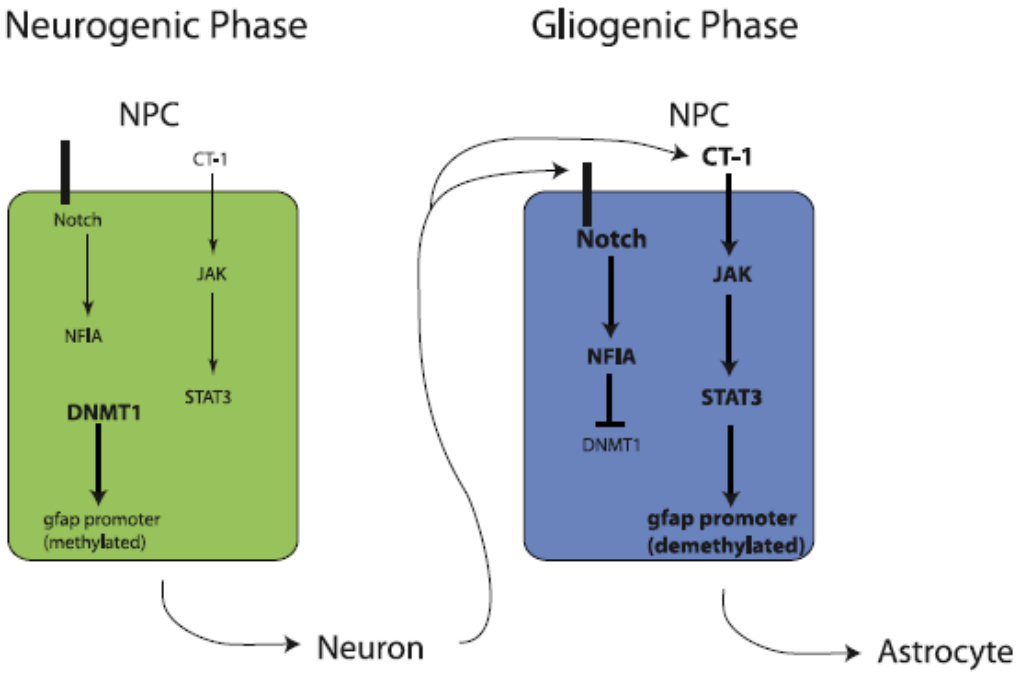
Accordingly, growth factors in the subfamily of interleukin 6 (IL6) cytokines, including ciliary neurotrophic factor (CNTF), leukemia inhibitory factor (LIF), and cardiotrophin-1 (CT-1) have been shown to be particularly potent instructors of astrogenesis via the Janus kinase-signal transducers and activators of transcription (JAK-STAT) pathway. Their interaction with LIFRβ and gp130 receptors activates intracellular JAKs, which phosphorylate STAT transcription factors, allowing their association with CBP/p300 to specifically transactivate the expression of a subset of astrocytic genes, like *gfap* and *s100β* (Johe et al., 1996; Bonni et al., 1997; Rajan and McKay, 1998; Nakashima et al., 1999; Barnabe-Heider et al., 2005). The neurotrophic cytokine CT-1, for example, demonstrates a mechanism whereby new born cortical neurons can regulate the onset of astrocyte differentiation of neighboring precursor cells through the secretion CT-1 as ligands of gp130 receptors.

The ablation of this neuron-derived factor causes perturbation of CBP/p300 resulting 50-70 % reduction of cortical astrocytes *in vivo*, while enhanced expression via *in utero* electroporation leads to precocious gliogenesis (Barnabe-Heider et al., 2005).

Signaling of BMP2/4 collaboratively promotes astrogenesis with JAK-STAT pathway during gliogenic period through binding with CBP/p300. Activation of BMP receptors phosphorylates Smad transcription factors, which then translocate to the nucleus forming a transcriptional complex with STAT3 and CBP/p300 to promote the transcription of astrocyte specific genes (Nakashima et al., 1999). BMP's positive effect on astrogenesis is reinforced further by the induction of inhibitor of DNA binding (Id)1 and Id3 that sequester bHLH Ngn1 and Mash1 from neuronal-specific promoters (Nakashima et al., 2001).

Likewise, Notch signaling also enables astrocyte differentiation through direct transcriptional modulation of astrocytic genes. Activation of Notch leads to the release of Notch intracellular domain (NICD), permitting its interaction with RBP-Jκ in the nucleus to promote the expression of proglial genes such as Hairy/enhancer of split (*hes*) and hes-related proteins (*Hesr*) that also act as inhibitors of pro-neuronal bHLHs (Louvi and Artavanis-Tsakonas, 2006). This gliogenesis role of Notch has been described widely from the peripheral nervous system (Morrison et al., 2000), the retina (Furukawa et al., 2000), to the neural precursors residing in the embryonic forebrain (Chambers et al., 2001) and adult

hippocampus (Tanigaki et al., 2001). Recently, a novel regulatory action of Notch has been discovered. In the embryo prior to the gliogenesis period, the methylation of astrocytic genes by methyl-CpG-binding protein 2 (MeCP2) results an inactive chromatin conformation that blocks the binding of STAT transcription factors (Takizawa et al., 2001b), a epigenetic form of control that inhibits the early precursor cell's responsiveness to cytokine-mediated JAK-STAT signaling. In a study by Namihira *et. al*., they showed Notch signaling, activated by neighboring neuronal precursors, is required for the demethylation of astrocytic genes via upregulation of nuclear factor-1A (NF1A), a pro-astrocytic transcription factor, that binds to the promoters of astrocyte specific genes to block methylation exerted by DNA methyltransferase I (DNMT1) (Namihira et al., 2009). This finding demonstrates Notch signaling complements cytokine-mediated JAK-STAT signaling to timely initiate astrocyte production and illustrates a concerted result involving both extrinsic cues and cell-intrinsic mechanisms.



Notch signaling cooperates with canonical JAK-STAT signaling to initiate the switch of neurogenesis to astrogenesis (Chenn, 2009).

Transcription factor regulators of astrocyte differentiation

Despite numerous insightful studies on the extracellular instructive cues that promote astrocyte differentiation beginning to emerge more than a decade ago, the intracellular transcriptional programs that specify astrocytic fate-choice still remain elusive. The study conducted by Deneen *et al.*, is one of the first to identify a family of transcription factors, NFIA and NFIB, as both necessary and sufficient for glial-fate specification.

These transcription factors antagonize neurogenesis and promote pro-gliogenic genetic programs in the ventricular zone of the spinal cord (Deneen et al., 2006). They demonstrated that this function of NFIA is mediated through the requirement of NFIA for the expression of Notch effector Hes5. Interestingly, in the absence of NFIA, Notch effectors alone could not promote glial-fate commitment. Another study by Stolt *et al*., demonstrated that Nestin-Cre driven conditional ablation of Sox9 transcription factor within neural stem cells in the spinal cord leads to defects in astrocyte and oligodendrocyte generation (Stolt et al., 2003). Although the precise mechanism by which Sox9 coordinates the switch from neurogenesis to gliogenesis remains to be determined, their finding revealed an additional transcriptional network that is a crucial component of gliogenesis specification. Furthermore, stem cell leukemia (Scl), a bHLH transcription factor, was shown in the mouse and chick embryonic spinal cord to be both necessary and sufficient for the acquisition of astrocyte cell-fate, by antagonizing Olig2-dependent generation of oligodendrocyte precursor cells (Muroyama et al., 2005). Recognizing the need to have a more comprehensive understanding of the transcriptional programs that direct astrocyte specification, a recent study conducted a genome wide screen *in silico* using the Mahoney pictorial atlas to identify potential transcription factors that modulate astrocyte differentiation based on their spatial and temporal expression patterns. This study identified Klf15 to be sufficient, but not necessary, for the genesis of precocious GFAP-positive astrocytes (Fu et al., 2009).

These advances are certainly valuable for a better understanding the genetic programs governing astrogenesis, or more broadly gliogenesis, but it remains to be established that whether these transcriptional regulators identified in the spinal cord are also equally essential for the same processes in different regions of the brain.

Astrocytes are developmentally and functionally crucial for numerous aspects, including synaptogenesis, synaptic plasticity, modulating breathing rhythm generation, controlling blood flow as a part of the blood brain barrier, internalizing and clearing Aβ deposits, responding to brain injuries and enhance neuronal survival through reactive gliosis, and mediating Aβ-induced neurotoxicity via releasing pro-inflammatory cytokines (Freeman; Barres, 2008; Gourine et al., 2010). Despite being the most abundant cell type within the rodent and human brain (Allen and Barres, 2009), astrocytes are the least well understood developmentally and molecularly.

Oligodendrocyte differentiation

Oligodendrocyte specification occurs the latest, among the 3 predominant cell-types generated within the brain, at late embryonic and early postnatal periods from precursor cells of ventricular zones of the forebrain and the spinal cord (Miller, 1996; Lee et al., 2000). Because oligogenesis is restricted regionally within the brain, various local environmental cues are critical for oligodendrocyte

development. Extrinsic growth factors, such as neurtrophin-3 (NT-3) and platelet derived growth factor (PDGF), are important for promoting the proliferation of oligodendrocyte precursor cells (OPC) (Barres et al., 1994; Robinson and Miller, 1996). Extracellular sonic hedgehog (Shh) signal in ventral telencephalon was found to be necessary and sufficient for inducing commitment to oligodendrocyte precursor in both regions (Alberta et al., 2001; Tekki-Kessaris et al., 2001). Downstream to Shh signaling, two bHLH factors, Olig1 and Olig2, are of particular importance in oligodendrocyte development. Their ectopic expression demonstrated sufficiency to promote oligodendrocyte specification (Lu et al., 2001; Zhou et al., 2001), while loss-of-function studies showed that in absence of Olig1/2 motor neurons and oligodendrocytes are largely abolished and precursor cells resort to interneuron and astrocyte lineages instead (Lu et al., 2002; Zhou and Anderson, 2002). Interestingly, subsequent studies indicate that Olig bHLH factors not only promote oligodendrocytic lineage, but they also repress astrogenesis pathways by sequestering CBP/p300 from STAT3 (Fukuda et al., 2004) and antagonize NF1 (Deneen et al., 2006).

Besides positive bHLH factors, the transition to oligodendrocyte specification is also regulated intrinsically by repressive HLH Id proteins that through heterodimerization blocks the bHLH factors thereby inhibiting their transactivation of oligodendrocyte-related genes. To permit oligodendrocyte differentiation, Id2 is translocated out of the nucleus to cytoplasm prior to the onset differentiation; overexpression of Id2 confirms suppression of

oligodendrocyte generation, while ablation of Id2 results precocious oligodendrocyte differentiation (Wang et al., 2001).

In addition to transcriptional modulation, a recent study report an epigenetic regulation of oligodendrocyte lineage commitment and progression by histone deacetylases, HDAC1 and HDAC2, via interaction with Wnt signaling to increase the chromatin compaction and limit the accessibility of oligodendrocyte repressor genes, such as Id2 (Ye et al., 2009).

Oligodendrocytes are functionally indispensable for higher organism nervous system as they are responsible for the generation and maintenance of myelin that facilitates neuronal salutatory transmission as well as for providing trophic support – for example, insulin growth factor-1 (IGF-1) and glial cell line-derived neurotrophic factor (GDNF) – to promote the survival of neurons and growth of axons (Wilkins et al., 2001; Wilkins et al., 2003). Disruptions to oligodendrocyte development or health contributes to disorders such as multiple sclerosis and leukodystrophies (Baumann and Pham-Dinh, 2001).

CNS development dysregulation and disease

In the past decade numerous studies have elucidated the intricate regulatory networks encompassing extracellular signaling cues and intrinsic molecular mechanisms that direct the formation of neurons, astrocytes, and oligodendrocytes.

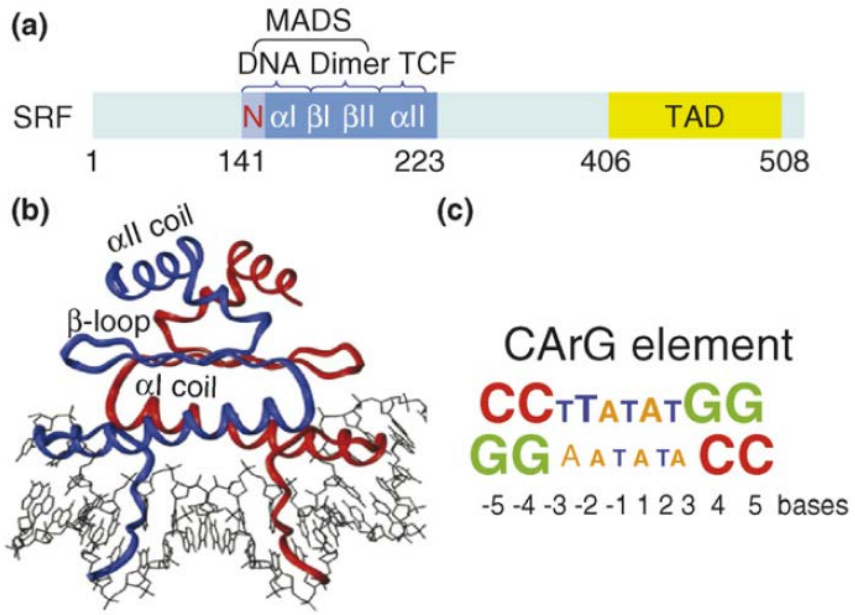
However, our knowledge in these fields remains incomplete to fully unravel the fundamental perturbations within many neurodevelopmental disorders. For example, Noonan syndrome is a genetic disorder with a frequency of 1 in 2500 births, involving missense mutations in the human ptpn11 (shp-2) gene and resulting learning disabilities and mental retardation in afflicted individuals (Noonan, 1994). A recent study reported increased astrogenesis at the expense of neurogenesis in the mouse model of mutant SHP-2, which is a tyrosine phosphatase that when mutated in the germline leads to LEOPARD syndrome and mental retardations (Gauthier et al., 2007). Further research in the direction of the regulation of cortical cell-fate decisions implicating SHP-2-related pathways and its broader functional implications may provide more insight to the biological dysregulation that lead to mental retardation in human patients. Similarly, mutations that create loss-of-function in one allele of CBP/p300, a key integrator of signaling pathways of both neurogenesis and gliogenesis, is associated with cognitive impairments and mental retardation in Rubinstein-Taybi syndrome (Josselyn, 2005). Future studies in CBP/p300-related mechanisms of cell type specification may help us better understand the molecular and cellular processes that underlie cognitive dysfunction.

Our overarching goal of studying CNS development is to better understand the molecular mechanisms that guide not only fundamental brain development, but also neurological disorders that emerge at as a result of dysregulated cell-fate and neuronal development.

Serum Response Factor

SRF is one of the first activity-dependent transcription factors to be characterized and was originally isolated based on its ability to activate the *c-Fos* gene (Treisman, 1987, 1995). Comprised of 508 amino acids in mammals, SRF is a highly evolutionarily conserved MADS-domain containing transcription factor that binds as a homodimer to the serum response element (SRE) dyad symmetry sequence ($CC(A/T)_6GG$), also referred to as a CArG domain (Treisman, 1987; Norman et al., 1988). SRF has very little structural similarity to other mammalian proteins suggesting little or no functional redundancy with other factors. This is illustrated by the early embryonic lethality (~E9.5) of SRF homozygous null mice due to its vital requirement for mesoderm formation, in a non-cell-autonomous manner (Arsenian et al., 1998; Weinhold et al., 2000). However, because the animal body is capable for developing largely normally up to E6.5 from a blastocyst, SRF is thought to be dispensable for embryonic stem (ES) cell proliferation and cell cycle progression. This was verified by a functional study of SRF-deficient ES cells that showed albeit SRF is required for the activation of immediate early genes (IEGs) – many of which are involved in the $G_0 – G_1$ cell cycle transition – SRF itself is dispensable for ES cells proliferation (Schratt et al., 2001). Furthermore, SRF was found to contribute to the regulation of apoptosis particularly during differentiation through direct targeting of the Bcl-2 expression, which is an anti-apoptotic gene (Schratt et al., 2004). Besides its importance for the aforementioned developmental processes, SRF controls the cellular structural

organization via the expression of cytoskeletal structures – such as F-actin – and focal adhesion components – including vinculin and β1-integrin (Schratt et al., 2002).



Protein structure of SRF and nucleotide sequences of the CArG element, also known as a SRE site (Niu et al., 2007).

Interestingly, SRF deletion in post-natal brain did not affect neuronal cell survival or cellular architecture *in vivo* and the adult mouse brain lacking SRF exhibit specific defects in activity-induced expression of several neuronal plasticity genes (Ramanan et al., 2005; Etkin et al., 2006). While basal synaptic transmission does not require SRF, it is a gene of important function in regulating both LTP and LTD and its absence restricted to forebrain mature neurons caused

learning and memory behavioral deficits (Ramanan et al., 2005; Etkin et al., 2006). SRF is activated by several physiological stimuli including growth factors/mitogens, glucose, serum, and neuronal activity; its activation of target genes such as IEGs and cytoskeletal genes is facilitated by a ternary complex transcription factor (TCF) (Treisman, 1994; Whitmarsh et al., 1995; Liao et al., 1997). The ternary complex consists of the SRF homodimer and an Ets-domain family transcription factor, the best characterized of which is $p62^{Elk1}$ (Elk1). Extracellular stimuli that result in SRE-mediated transcription promote both TCF-dependent and TCF-independent transcription. In the TCF-dependent mechanism, activated intracellular signaling through the MAPK cascades result in the phosphorylation of multiple serine and threonine residues on Elk1. These phosphorylation events are necessary for the TCF-dependent SRE-mediated transcription (Hill et al., 1993; Marais et al., 1993).

Functions of SRF in CNS development

Until recently, SRF function has been exclusively studied in the ES cell *in vitro* system and *in vivo* in the cardiac system, where SRF has been shown to play a critical role in orchestrating smooth and cardiac muscle differentiation (Miano, 2003; McDonald et al., 2006; Ivey et al., 2008; Niu et al., 2008). While earlier studies using cultured neurons demonstrated the importance of SRF for stimulus-dependent transcription, very little was known about its *in vivo* functions in the

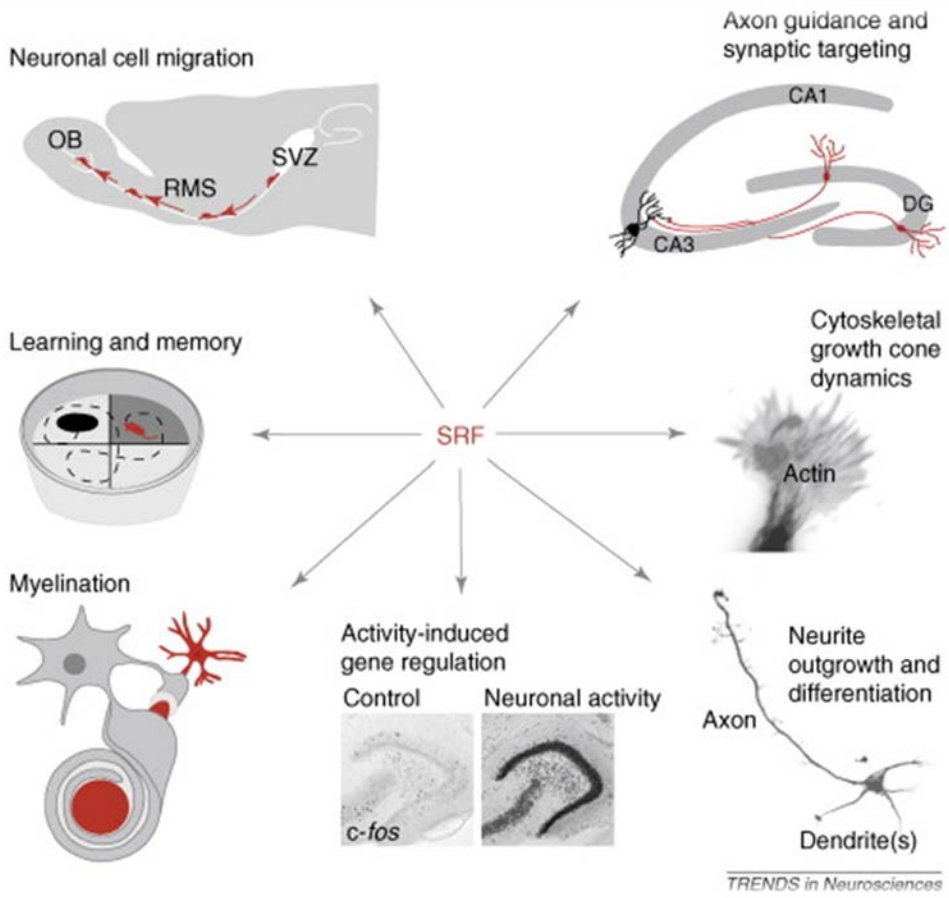
brain and elsewhere owing to the early embryonic lethality of the *Srf* null mice (Arsenian et al., 1998). To ascertain the role of SRF in neuronal development and plasticity, *Srf* conditional knockout mice were generated with SRF ablation restricted to postmitotic neuronal populations in the CNS. Deletion of SRF at late prenatal stages in the forebrain leads to defects in neuronal rostral migratory stream (RMS) migration from subventricular zone (SVZ) to the olfactory bulb (Alberti et al., 2005). The migration defects were attributed in part to alterations in actin cytoskeletal dynamics and particularly due to decreased levels of expressed and polymerized F-actin along with functional deficits in actin severing proteins, gelsolin and cofilin, in the SRF deficient neurons (Alberti et al., 2005). These mice also exhibited several phenotypic abnormalities such as poor feeding behavior, reduced size and weight and impaired locomotor activity and finally death by 3 weeks of age (Alberti et al., 2005). Postnatal neuronal deletion of SRF yielded several interesting results: (a) SRF is critical for activity-dependent expression of several neuronal plasticity genes including *c-Fos, Egr1, Egr2,* and *Arc*; (b) unlike in ES cells, SRF deletion does not affect cell survival or cellular architecture *in vivo,* and (c) while basal synaptic transmission does not require SRF, it has an important role in regulating both long-term synaptic potentiation (LTP) and synaptic depression (LTD) (Ramanan et al., 2005; Etkin et al., 2006).

Additionally, by analyzing conditional mutants in which SRF is ablated in mature neurons, a study revealed that it is necessary for hippocampal circuitry formation, mossy fiber segregation, and axonal guidance through ephrin-A and

semaphorin mediated signaling (Knoll et al., 2006). These results confirmed SRF's role as a master regulator of cytoskeletal machinery, actin dynamics, and lamellipodial and filopodial formation within the brain, as it is in many other cell types (Miano et al., 2007; Knoll and Nordheim, 2009). *In vitro* studies of SRF-deficient and inactive megakaryoblastic leukemia (MKL), a forebrain-enriched co-factor of SRF, hippocampal/cortical neurons further showed that SRF transcriptional activity is essential for neurite generation and outgrowth and dendritic complexity (Ishikawa et al.; Stern et al., 2009) This function is promoted by activin, a member of the transforming growth factor β (TGF-β) superfamily, and repressed by suppressor of cancer cell invasion (SCAI) within the nucleus. Although physiologically it is demonstrated that SRF modulates hippocampal dendritic development through reelin signaling (Stritt and Knoll), but concrete findings on which axonal tracts require SRF within the brain is still unknown. This has not been shown *in vivo* because that many researchers do not yet have conditional mutant mice of SRF that delete the gene sufficiently early, prior to neuronal development.

A recent study by Stritt *et al*., reported a novel function of SRF in modulating oligodendrocyte maturation as the authors found a reduction of mature oligodendrocytes and an increase in oligodendrocyte precursor cells when SRF is ablated specifically in neurons utilizing CaMKIIα-Cre mediated recombination. This paracrine regulation is shown to be a result of SRF's function as a repressor of expression of connective tissue growth factor (CTGF) within neurons, a

factor that inhibits neighboring oligodendrocyte precursors to mature by suppressing the insulin-like growth factor (IGF-1) signaling (Stritt et al., 2009).



Current field of knowledge of SRF's functions in CNS cellular development and functioning (Knoll and Nordheim, 2009).

Relating to neurological disorders of CNS, studies have found a link between SRF and Alzheimer's disease pathology as SRF is expressed at high levels within the vascular smooth muscle cells (VSMC) – both in patients and in mouse

models – governing cerebral blood flow and LRP-mediated amyloid β clearance (Chow et al., 2007; Bell et al., 2009). Conventionally, Alzheimer's research focused primarily on the degeneration of neurons as the origin of pathogenesis. More recently, increasing evidence shows that cerebral blood flow regulation is also very important for neuronal homeostasis and synaptic transmission – perturbation of which contributes to cognitive decline – and physiological clearance of soluble amyloid β. The aforementioned studies showed that the knockdown of SRF activity using short-hairpin RNAs normalized the contractility of SMCs and improved the hypoperfusion phenotype in animal models. Higher SRF activity was found to reduce amyloid β clearance through enhancing the expression of SREBP2, which is a repressor of the LRP1 receptor that modulates amyloid β clearance (Chow et al., 2007; Bell et al., 2009). These results are promising as they suggest further understanding of SRF function and appropriately managing its activity in cerebral vasculatures could be a therapeutic target to help Alzheimer's patients, for whom there currently are no effective treatments to slow their cognitive deterioration and disease progression.

Brain development, SRF, and what is missing?

Because existing knowledge of the functional roles of SRF within the brain were mostly derived from postnatal neuronal conditional mutant studies and *in vitro* neuronal culture studies, it is unknown whether SRF – as a highly brain-

enriched and a unique stimulus-dependent transcription factor – plays a role in regulating brain development. Specifically, is SRF required for cell-fate specifications that govern neurogenesis, astrogenesis, and oligodendrocyte differentiation? Lack of SRF undermines neurite outgrowth in culture, but is SRF necessary for neuronal innervations *in vivo* or could this effect be compensated by the much more complex environment within the brain? If so, which major tracts and axonal projections require SRF for establishment? Does SRF function similarly in NPCs as it does in ES cells? As a stimulus-dependent transcription factor, is SRF needed for the proliferation and self-renewal of NPCs? Is SRF also needed for cell survival in NPCs just as it is for ES cells undergoing differentiation?

To address these questions, we crossed our SRF conditional floxed mouse with a Nestin-Cre recombinase mouse in which Cre expression is driven by the Nestin promoter and is restricted to NPCs in the brain and spinal cord prior to cell-fate lineage commitment and the establishment of neuronal arborization. Moreover, utilizing different cell-type specific transgenic lines – including a neuronal-specific NEX-Cre line and an astrocyte-lineage specific GFAP-Cre line – as well as neurosphere culture techniques, we aim to define the cellular context of which SRF controls these processes and elucidate their underlying molecular mechanisms.

Chapter 2: The Role of SRF in Neuronal Development

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Serum Response Factor Is Required for Cortical Axon Growth But Is Dispensable for Neurogenesis and Neocortical Lamination

Abstract

Previous studies have shown that neuron-specific deletion of serum response factor (SRF) results in deficits in tangential cell migration, guidance-dependent circuit assembly, activity-dependent gene expression, and synaptic plasticity in the hippocampus. Furthermore, SRF deletion in mouse embryonic stem cells causes cell death *in vitro*. However, the requirement of SRF for early neuronal development including neural stem cell homeostasis, neurogenesis, and axonal innervations remains unknown. Here, we report that SRF is critical for development of major axonal tracts in the forebrain. Conditional mutant mice lacking SRF in neural progenitor cells (*Srf*-Nestin-cKO) exhibit striking deficits in cortical axonal projections including corticostriatal, corticospinal, and corticothalamic tracts, and they show a variable loss of the corpus callosum. Neurogenesis and interneuron specification occur normally in the absence of SRF and the deficits in axonal projections were not due to a decrease or loss in cell numbers. Radial migration of neurons and neocortical lamination were also not affected. No aberrant cell death was observed during development, whereas there was an increase in the number of proliferative cells in the ventricular zone from embryonic day 14 to day 18. Similar axonal tract deficits were also observed in mutant mice lacking SRF in the developing excitatory neurons of neocortex and hippocampus (*Srf*-NEX-cKO). Together, these findings suggest distinct roles for SRF during neuronal development; SRF is specifically required in a cell-

autonomous manner for axonal tract development but is dispensable for cell survival, neurogenesis, neocortical lamination, and neuronal differentiation.

Introduction

Neuronal development in the CNS is an intricately coordinated process that involves proliferation and maintenance of neural precursor cells (NPCs), neurogenesis, growth and extension of axons and dendrites, and structural organization within specific brain regions. At the molecular level, these processes are regulated by several extracellular cues through activation of specific transcriptional programs (Goldberg et al., 2002; Zhou and Snider, 2006). Serum response factor (SRF) is a stimulus-dependent transcription factor belonging to the Mcm1-Agamous-Deficiens-SRF-domain family of transcriptional regulators. Thus far, the roles of SRF in CNS development remain poorly understood because of early embryonic lethality of SRF-null mice (Arsenian et al., 1998). Recent studies using conditional SRF mutant mice have begun to elucidate the importance of SRF in CNS development and adult function. Perinatal neuron-specific deletion of SRF results in several developmental abnormalities, including defects in tangential neuronal migration along the rostral migratory stream, deficits in axon guidance within the hippocampal mossy fiber circuitry, hippocampal lamination and dendritic complexity of hippocampal pyramidal neurons, and ultimately resulting in lethality by 3 weeks of age (Alberti et al., 2005; Kno¨ll et al., 2006; Stritt and Kno¨ll, 2010). In contrast, mice carrying

postnatal forebrain-specific deletion of SRF are viable and fertile, and do not exhibit any of the above developmental abnormalities (Ramanan et al., 2005; Etkin et al., 2006). Instead, these mice exhibit specific deficits in activity-dependent expression of several immediate early genes (IEG), including *c-Fos*, *Egr-1*, and *Arc*, in the hippocampus and neocortex (Ramanan et al., 2005). SRF ablation does not affect basal synaptic transmission but disrupts both early and late phases of LTP and LTD in hippocampus and in cultured cerebellar Purkinje neurons (Ramanan et al., 2005; Etkin et al., 2006; Smith-Hicks et al., 2010). Interestingly, SRF loss does not affect neuronal cell survival and maintenance (Ramanan et al., 2005). Defects in activity-dependent transcription and synaptic plasticity are the likely underlying causes of learning and memory deficits observed in these mice (Etkin et al., 2006; Johnson et al., 2011).

Despite these advances, the role of SRF in neural progenitor cell homeostasis, neurogenesis, and neuronal maturation during early brain development remains unknown. In this study, we conditionally deleted SRF in NPCs using a nestin-cre transgenic line to investigate earlier developmental roles of SRF. *Srf*-Nestin- cKO mutants exhibited neonatal lethality along with several abnormalities in brain architecture. Closer analysis revealed that loss of SRF affected the development of major CNS axonal fiber tracts. However, neurogenesis, neuronal subtype specification, and neuronal survival were unaffected. Similarly, *Srf*-NEX-cKO mutant mice, lacking SRF only in postmitotic glutamatergic neurons in the neocortex and hippocampus, also

exhibited defects in axonal projections suggesting a cell-autonomous role of SRF in axon growth *in vivo*. Contrary to previous findings, neocortical lamination occurs normally in both these lines of mutant mice.

Last, examination of NPCs revealed an accumulation of precursors in *Srf*-Nestin-cKO mutants suggesting that SRF plays an important role in NPC homeostasis. Thus, our study reveals a critical role for SRF in NPC maintenance and axon outgrowth during CNS development.

Materials and Methods

Animals. Srf f/f mice (control) were maintained as a homozygous colony as previously described (Ramanan et al., 2005). The *Srf* f/f were crossed to the nestin-Cre transgenic mouse strain (Tronche et al., 1999) (The Jackson Laboratory, Stock # 003771) to generate *Srf* f/_; Nestin-Cre double heterozygous mice. The double heterozygous mice were bred to *Srf* f/f mice to obtain *Srf* f/f; Nestin-Cre (*Srf*-Nestin-cKO) mutant mice in the expected Mendelian ratio. Similarly, *Srf* f/f mice were bred to the NEXcre transgenic mice to generate *Srf* f/f; NEX-Cre (*Srf*-NEX-cKO) mice (Goebbels et al., 2006). The *Srf*-NEX-cKO mice were viable and were bred to *Srf* f/f mice to propagate the colony. For experiments that required embryos of various developmental stages, we set up timed pregnancies with the day following detection of a vaginal plug being identified as embryonic day 0.5 (E0.5). All experiments were approved by the

Animals Studies Committee, Division of Comparative Medicine, Washington University School of Medicine, St. Louis, MO.

Immunohistochemistry. Immunohistochemistry was performed as previously described (Ramanan et al., 2005). Briefly, postnatal day 0.5 (P0.5) and older animals were fixed by transcardial perfusion. The brains were cryopreserved in 30% sucrose, frozen, and stored at -80°C until use. For staining, 12–16 µm cryosections were made and incubated in blocking/permeabilization solution containing 3% normal goat serum and 0.3% Triton-X in PBS. Embryos until E18.5 were drop-fixed in 4% PFA followed by cryopreservation in 30% sucrose. The following primary antibodies were used: NeuN (1:1000, Millipore Bioscience Research Reagents), SRF (1:1500, Santa Cruz Biotechnology), 2H3/Neurofilament (1:1000, DSHB), activated-Caspase 3 (1:1500, Millipore), Tbr2/EOMES (1:50, Santa Cruz Biotechnology), Tbr1 (1:250, Santa Cruz Biotechnology), Cux1 (1:5000, Santa Cruz Biotechnology), 40E-C/Vimentin (1:50, DSHB), somatostatin (SST; 1:600, Santa Cruz Biotechnology), calbindin (1:2000, Sigma-Aldrich), parvalbumin (1:1000, Sigma-Aldrich), Gad-6 (1:500, DHSB), p-histone H3 (1:500, Sigma-Aldrich), Ki67 (1:500, BD Biosciences), and Sox2 (1:100, Santa Cruz Biotechnology).

The following secondary antibodies were used: anti-goat Cy3 (1:300, Jackson ImmunoResearch), anti-mouse Alexa Fluor 594 and Alexa Fluor 488 (1:500, Invitrogen), and anti-rabbit Alexa Fluor 488 and Alexa Fluor 594 (1:500, Invitrogen).

TUNEL. Embryonic and neonatal brains were perfused with 4% PFA and cryoprotected in 30% sucrose. Samples were sectioned at 12–16µm. Before staining, sections were permeabilized with 0.1 M citrate buffer, pH 6.0, at 80°C for 30 min. Slides were rinsed with PBS and immersed in 0.1M Tris-HCl containing 3% BSA and 20% bovine serum for 30 min at room temperature. Finally, 50–100 _l of terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) reaction mixture (Roche) was added per slide and incubated at 37°C in a humidified chamber in dark to complete the staining.

In situ hybridization. In situ hybridization was performed as previously described (Ramanan et al., 2005). The cDNA clones of *Cux2*, *Klf6*, *Lhx5*, *Lmo4*, *Nfix*, *Nr4a2*, and *Sox5* for riboprobes were generously provided by Paul Gray, Washington University School of Medicine, St. Louis, MO. Both sense and antisense riboprobes were synthesized and hybridized and sense strand probes did not produce any signal above that of the background.

Cell counts. High-magnification (10x or 20x) images of 10 nonconsecutive bregma axis-matched sections were taken using a Nikon 80i epifluorescence microscope. A universal threshold determined by signal to background ratio was applied to all images from control and knockout samples. Positive cells based on their nuclear staining were counted using Analyze Particle function with constraints on the particle size in pixels (300–2000 pixels) and circularity of the particle (0.4 –1.0) in ImageJ software. Total number of counts

per area in pixel square was computed and converted to counts per square micrometers based on the magnification of the image.

DiI labeling. Tiny crystals of 1,1-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI) similar in size were placed on the surface of the motor and the visual cortices (ipsilateral hemisphere) of neonatal *Srf*-Nestin-cKO, *Srf*-NEX-cKO, and control littermate brains, using an insect needle pin. Control and knock-out littermate brain pairs were positioned next to each other to ensure crystal placements were as comparable as possible. Samples were incubated in 37°C for 2–4 weeks and then sectioned coronally, sagittally, or horizontally at 100_mthickness using a vibratome. Sections were collected as floating sections and mounted serially on glass microscope slides using Vectashield mounting medium with DAPI (Vector Laboratories). For DiI staining of thalamocortical axons in sections, glass beads (250 µm, acid-washed, Supelco) were coated with DiI (2 mg of DiI in 100 ml of methylene chloride to coat 300 mg of glass beads). A single DiI-coated bead was placed in the ventral thalamus of 100 µm paraformaldehyde-fixed coronal section and incubated for 3 weeks at 37°C.

Quantification of axonal projections. DiI-labeled corticostriatal projections in 2–3 slices were measured for projection length using ImageJ to track and record the absolute length in pixels and then converted to micrometers. For measuring the target innervation of corticothalamic projection, DiI-labeled thalamic area was measured using ImageJ in square pixels and then converted to

square micrometers. Mean of projection length or target innervation area of comparable sections from three pairs of control and mutant animals was calculated to quantify for the difference between control and mutants.

Nissl staining. Fresh frozen brains were sectioned at 20 μm and mounted on gelatin-coated glass slides. After overnight drying, slides were immersed in 0.5% cresyl violet in water for 10 min; rinsed in H2O; dehydrated serially in 50%, 75%, 95%, and 100% ethanol (2 min each), followed by two rinses in xylenes (3 min each); and then coverglass mounted with permount histology mounting medium.

Statistical analyses. The mean, SD and SEM for cell counts were calculated from images, which were sampled serially to encompass a structural region. Pups of either sex (n = 3–5) from at least two different litters were used in all experiments. Statistical significance between control and mutant pair was determined by Student's t test.

Results

Ablation of SRF in *Srf*-Nestin-cKO mutant brain

To determine the role of SRF in neurogenesis and CNS development, we deleted SRF using a nestin-Cre transgenic mouse line, in which Cre-mediated recombination has been shown to occur ~E9.5 to E11.5 (Tronche et al., 1999). *Srf*-Nestin-cKO animals were born in the expected Mendelian ratio, but the mutant mice did not survive beyond P1 due to unknown reasons.

The *Srf*-Nestin-cKO mice were physically indistinguishable from control littermates (*Srf* f/f) but exhibited neonatal hemorrhage starting ~12–16 h after birth (Fig. 1*A*). In the *Srf*-Nestin-cKO mutant mice, SRF deletion begins_E12.5 and is complete by E14.5 in the brain and spinal cord as determined by immunohistochemistry (data not shown). SRF loss was also confirmed by immunoblotting using whole brain lysates from neonatal control and *Srf*-Nestin-cKO mice (Fig. 1*B*). A closer examination of spatial deletion of SRF at P0.5 in *Srf*-Nestin-cKO brains by immunostaining using anti-SRF antibody demonstrates loss of SRF in all regions examined, including the neocortex, hippocampus, striatum, thalamus, and hypothalamus (Fig. 1*C*, data not shown). Coimmunostaining for NeuN, a marker of mature neurons, and SRF showed absence of SRF expression in neurons throughout the brain (Fig. 1*D*, data not shown).

To analyze the consequence of SRF ablation on brain morphology, we performed nissl staining of P0.5 *Srf*-Nestin-cKO and control brains. The mutant mice exhibited enlarged lateral ventricles and the corpus callosum was present only in the most rostral part of the brain (Fig. 1*E*). In the *Srf*--Nestin-cKO mutant brain, several of the white matter tracts were notably absent or greatly diminished compared with control mice. In the rostral forebrain, the anterior commissure was absent and the lateral ventricular zones were also enlarged in *Srf*-Nestin-cKO mice (Fig. 1*E*). More caudally, the stria medullaris, mammilothalamic tract, internal capsule, and anterior commissure were also markedly diminished in

Srf-Nestin-cKO mice compared with those in control littermates (Fig. 1*E*). The hippocampus was smaller and the dentate gyrus was more compact while the CA1 pyramidal cell layer was disorganized (Fig. 1*F*).

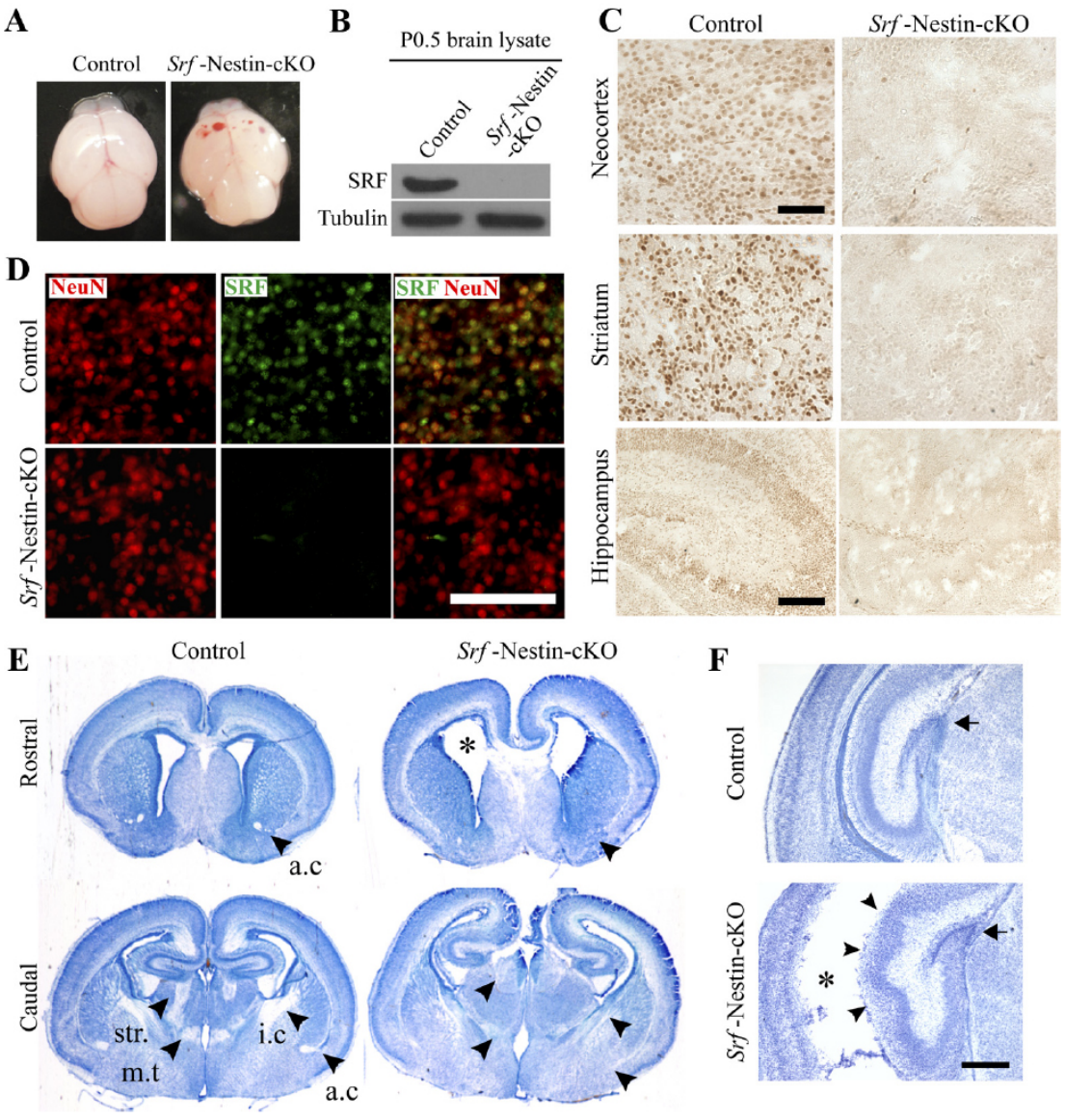

Figure 1. SRF deletion in *Srf*-Nestin-cKO mutants. ***A***, *Srf*-Nestin-cKO mice exhibited neonatal hemorrhage starting 12–16 h after birth while the brains of

control littermates were normal. The mutant mice died by 18 –24 h. The weight of the brains was comparable. ***B***, Immunoblotting using whole brain lysates shows complete loss of SRF in P0.5 *Srf*-Nestin-cKO mice. ***C***, Immunostaining using a SRF-specific antibody shows loss of SRF expression in forebrain regions including the neocortex, striatum, and hippocampus. Scale bars: Neocortex, 50μm; others, 150μm. ***D***, Co-immunofluorescence staining for SRF and the neuron-specific marker, NeuN, shows deletion of SRF in neurons of *Srf*-Nestin-cKO mice compared with control littermates. Scale bar, 30μm. ***E***, Forebrain nissl staining reveals several abnormalities in the mutant brains. Compared with control littermates, *Srf*-Nestin-cKO mice exhibit enlarged lateral ventricles (asterisk) and greatly diminished anterior commissure (a.c), stria medullaris (str.), mammilothalamic tract (m.t), and internal capsule (i.c). ***F***, Closer examination of the mutant hippocampus shows that the dentate gyrus is more compact (arrow) and cellular lamination of CA3 and CA1 fields are more aberrant (arrowheads) than those of control littermates. Asterisk indicates enlarged lateral ventricle in the caudal forebrain. Scale bar, 150μm.

SRF is required for proper establishment of cortical axonal innervations

Given these deficits in white matter tracts, we further analyzed axon growth in *Srf*-Nestin-cKO mice. Immunostaining using anti-neurofilament antibody (anti-2H3) revealed striking loss of entorhinal-hippocampal or perforant path innervations in the hippocampus of *Srf*-Nestin-cKO mutants

(Fig. 2*A*, *n* = 3 animals). The entorhinal-hippocampal pathway, which is important for hippocampal plasticity (Bliss and Gardner-Medwin, 1973), is comprised of the axonal projections primarily from the entorhinal cortex layer II/III neurons that innervate all fields of CA1 pyramidal neurons, granular neurons of the dentate gyrus, and subicular neurons in the hippocampus (Witter et al., 2000). Furthermore, we found a substantial reduction in corticostriatal innervations, which arise mainly from layer V cortical pyramidal neurons in the *Srf*-Nestin-cKO mutants compared with control littermates (Fig. 2*B*, *n*= 3 animals). As observed with nissl staining, anti-2H3 staining also revealed a lack of or deficits in anterior commissure, fasciculus retroflexis, and internal capsule in *Srf*-Nestin-cKO mice compared with control mice (Fig. 2*C*, *n* = 3 mice). We also performed anti-β-III tubulin (anti-Tuj1) immunostaining of sagittal brain sections to visualize the corticospinal tract projections of pyramidal neurons in layer V of the motor cortex. We observed significantly less abundant corticospinal projections through the internal capsule and cerebral peduncle in the *Srf*-Nestin-cKO brains as compared with control littermates (data not shown).

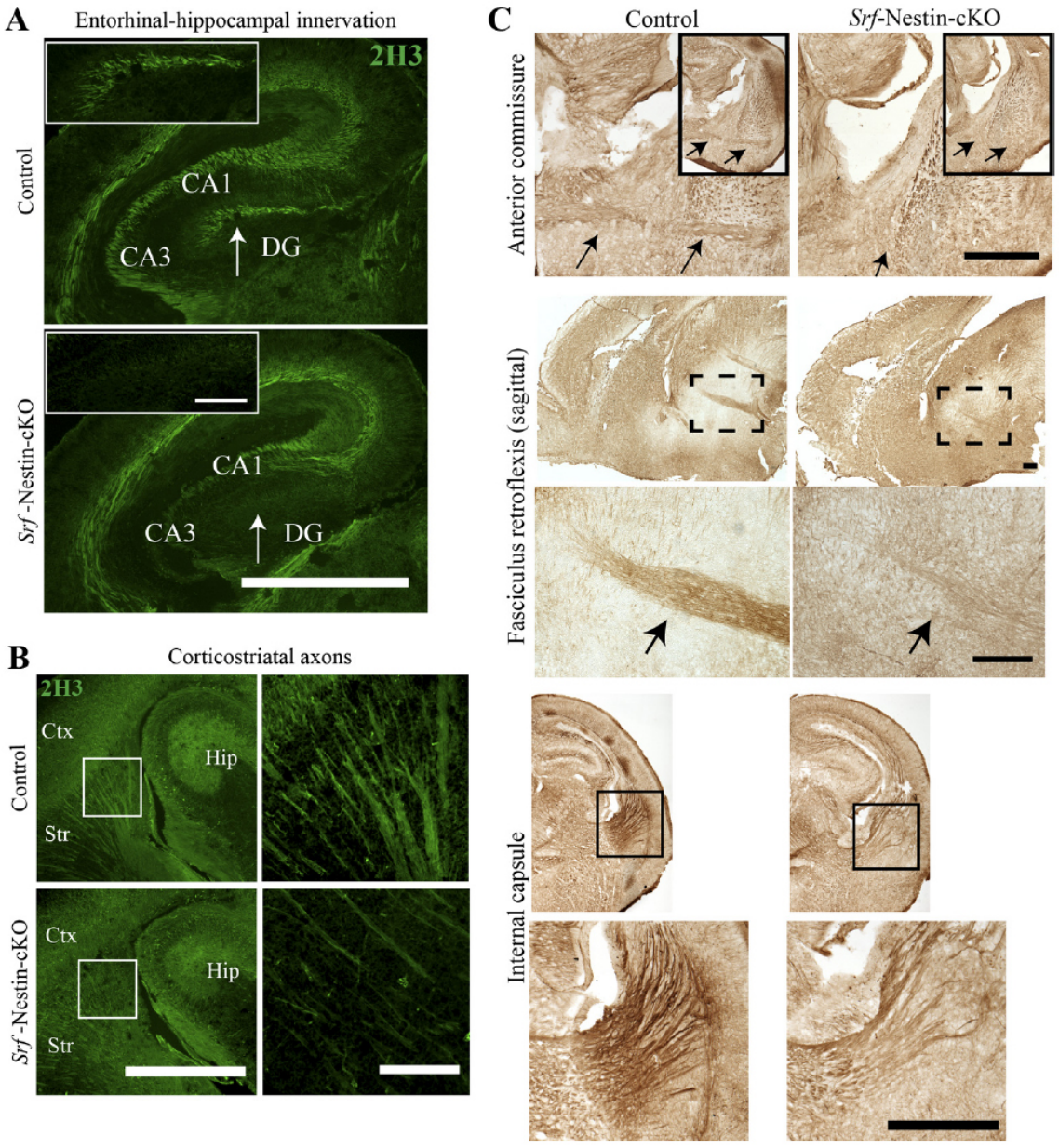

Figure 2. *Srf*-Nestin-cKO mutant mice exhibit axonal growth deficits. ***A***, Neurofilament immunostaining using anti-2H3 antibody reveals absence of entorhinal-hippocampal innervation in the hippocampus of *Srf*-Nestin-cKO mice. Inset shows the magnified view of the region indicated by the arrow. ***B***, Examination of neurofilament expression in striatum shows less abundant

corticostriatal projections in *Srf*-Nestin-cKO brains than in control littermates, *n* = 3 mice. Right, Magnified views of the boxed regions in ***B***, showing sparse axonal projections in the *Srf*-Nestin-cKO mice compared with control littermates. ***C***, Anti-2H3 neurofilament staining shows absence or highly reduced anterior commissure (arrows), fasciculus retroflexis (arrows), and internal capsule. Enlarged view of the boxed region is shown for each fiber tract. Scale bars: ***A*** and ***C***, large panels, and ***B***, left, 500μm; ***A***, insets, and ***B,*** right, 100μm. DG, Dentate gyrus; Ctx, neocortex; Str, striatum; Hip, hippocampus.

In addition to immunostaining, we used DiI labeling to visualize axonal projections *in vivo*. DiI crystals were placed on the surface of the brain corresponding to the motor and visual cortices of one hemisphere (Fig. 3*A*). To ensure the comparisons between control and mutant brain sections are made accurately, we cross-examined all coronal serial sections from the anterior to the posterior forebrain. As observed for anti-neurofilament immunostaining (anti-2H3), DiI labeling showed significant deficits in the corticostriatal projections in *Srf*-Nestin-cKO brains. In the anterior forebrain region of control mice, the projections from the cortical neurons clearly innervated the striatum. However, in *Srf*-Nestin-cKO brains these projections mostly terminate in the lateral corpus callosum and fail to reach their targets in the striatum (Fig. 3*B*,*D*; projection length: control, 460 ± 13 μm; *Srf*-Nestin-cKO, 248± 55μm; *n*= 2 mice; $p < 0.05$).

In the posterior forebrain of control mice, we observed robust corticothalamic projections innervating the thalamus. Strikingly, these corticothalamic innervations were absent in *Srf*-Nestin-cKO brain (Fig. 3*C*,*D*; projection area: control, 20,875 ± 1127 μm2; *Srf*-Nestin-cKO, 1619 ± 2894 μm2; $p < 0.01$). Furthermore, we also observed lack of innervations to the dorsal hippocampal commissure in the *Srf*-Nestin-cKO mice (Fig. 3*C*). DiI labeling further confirmed the deficits in anterior commissure and corpus callosum observed using anti-2H3 immunostaining (Fig.*3 E*,*F*). We then asked whether projections to the cortex were also affected in the mutant mice. DiI-coated beads placed in the ventral thalamus revealed striking deficits in thalamocortical projections in *Srf*-Nestin-cKO mutant mice compared with control (Fig. 3*G*, $n = 2$ mice). The axonal projection deficits in the *Srf*-Nestin-cKO mutant mice were consistent in all animals analyzed. Together, these observations suggest that SRF plays a critical role in the formation of axonal tracts during neural development.

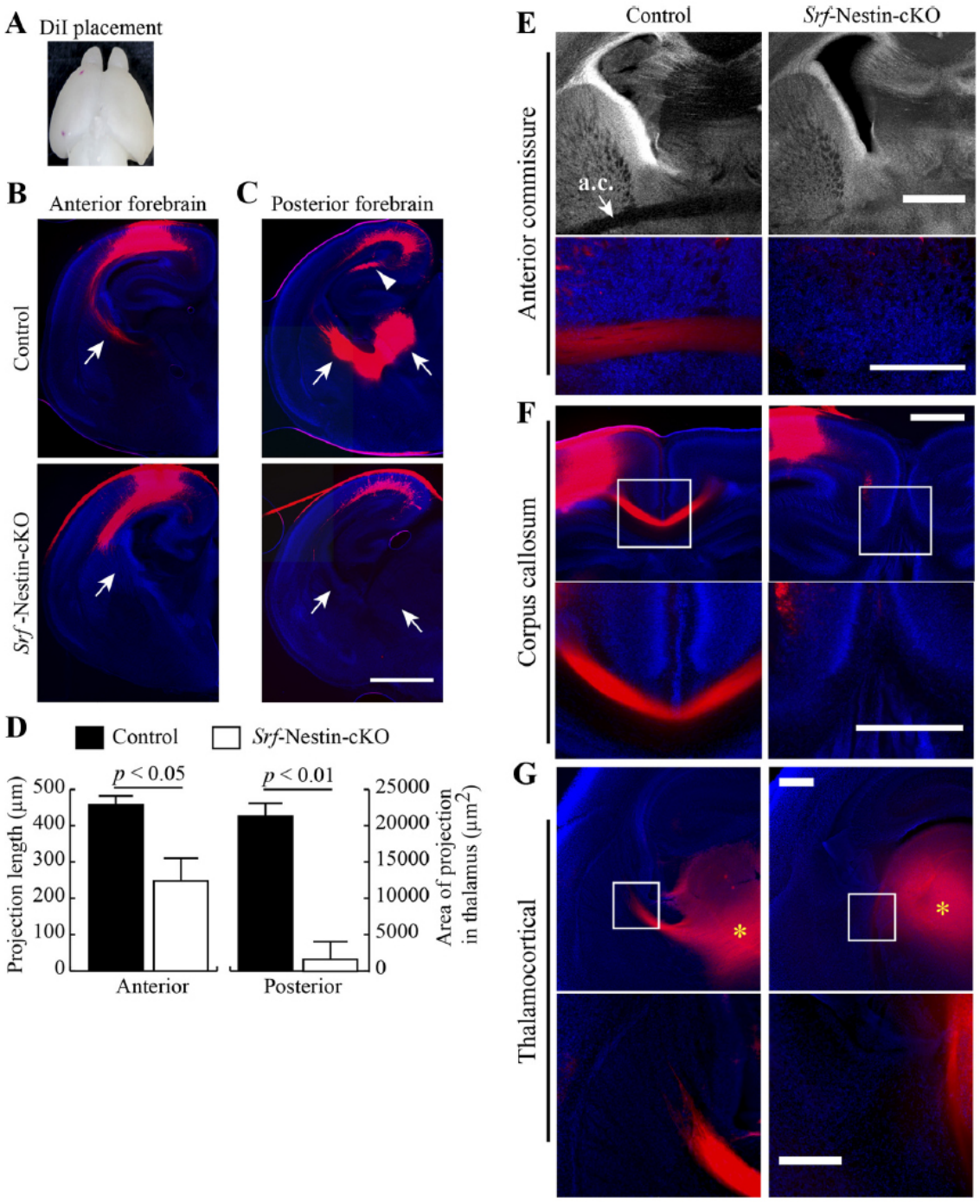

Figure 3. Corticostriatal, corticothalamic, and thalamocortical projections are impaired in *Srf*-Nestin-cKO mice. ***A***, DiI-labeled P0.5 brain showing the position of the DiI crystals. DiI crystals were placed on the surface of the brain in motor

and visual cortices of control and *Srf*-Nestin-cKO mice. ***B***, Coronal section of the anterior forebrain shows robust corticostriatal projection (arrows) into the striatum in the control, but not in *Srf*-Nestin-cKO brain. Instead, innervations mostly terminate in the corpus callosum in *Srf*-Nestin-cKO mice. ***C***, Examination of the posterior forebrain reveals a striking absence of corticothalamic projection (arrows) and lack of innervation into the dorsal hippocampal commissure (arrowhead) in the mutant brain. ***D***, Quantification of axon length from the position of the DiI crystal in the anterior region (***B***) and the area of target innervation in the posterior region (***C***) of the brain are shown (n = 3 mice). ***E***, ***F***, DiI labeling shows absence of anterior commissure (***E***) and corpus callosum (***F***) in*Srf*-Nestin-cKO mice compared with control littermates. Scale bar, 200μm.***G***, DiI labeling from the thalamus reveals deficits in thalamocortical projections in *Srf*-Nestin-cKO mutant mice while robust projections are seen in control littermates. Asterisk indicates position of the DiI-coated bead. Scale bar, 100μm.

Defects in neuronal projections are not a result of cell death or defects in neurogenesis

The deficits in axonal projections could result from a requirement of SRF for axon growth or from cell loss as a result of apoptosis. A previous study has shown that SRF is required for the survival of mouse embryonic stem cells and that SRF promotes cell survival by regulating the expression of the antiapoptotic gene, *Bcl-2* (Schratt et al., 2004).

Also, SRF has been shown to promote survival of neocortical neurons under conditions of trophic deprivation *in vitro* (Chang et al., 2004). To determine whether deficits in axonal projections are due to cell loss during development, we first assessed cell death using TUNEL assay and immunostaining for activated-caspase 3 expression at P0.5. We did not find any increase in the number of TUNELpositive and activated caspase-3-positive cells in the brains of *Srf*-Nestin-cKO brains as compared with control littermates (Fig. 4*A*). To ascertain whether increased cell death could have occurred earlier during brain development, we analyzed the brains of control and *Srf*-Nestin-cKO mice at E14.5, E16.5, and E18.5. We did not observe any difference in cell death at any of these stages between *Srf*-Nestin-cKO and control littermates, suggesting that SRF-deletion does not cause apoptotic cell death during brain development (Fig. 4*B–D*).

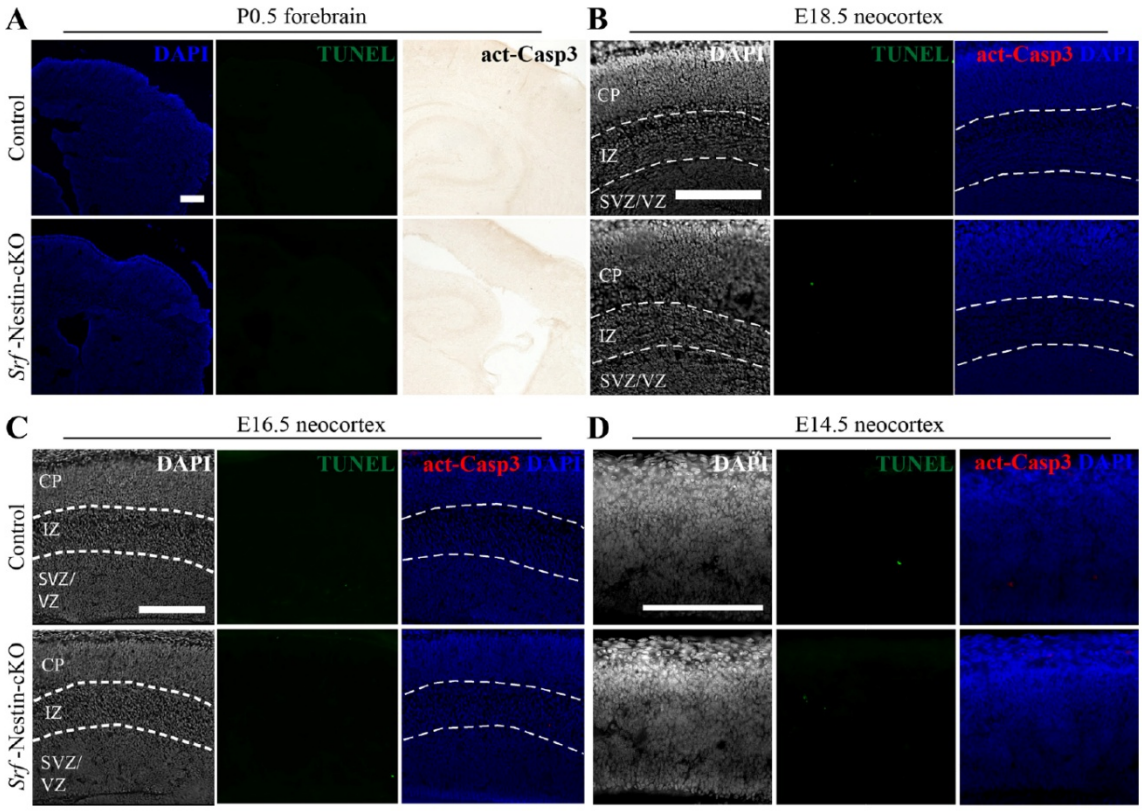

Figure 4. Loss of SRF does not cause apoptotic cell death during brain development. ***A***, TUNEL cell death assay and immunostaining for cleaved activated-caspase3 (act-Casp3) show no detectable elevation of apoptotic cell death *in vivo* in the absence of SRF at birth. Scale bar, 100μm. ***B–D***, Both TUNEL assay and immunostaining against activated-Caspase3 at E18.5, E16.5, and E14.5 indicate no significant elevation in the number of apoptotic cells in *Srf*-Nestin-cKO neocortex. CP, Cortical plate; IZ, intermediate zone; SVZ, subventricular zone; VZ, ventricular zone. Scale bar, 100μm.

Although we did not see any increased cell death in the brains of *Srf*-Nestin-cKO mice during development, it is possible that the deficits in axonal tract formation observed in these mutant mice could be due to deficits in the total number of neurons generated. To investigate the effect of SRF loss on neurogenesis, we determined the number of neurons in control and *Srf*-Nestin-cKO brains by immunostaining for NeuN, a marker for mature neuronal cell nuclei. We found that the total number of NeuN positive cells was similar in both *Srf*-Nestin-cKO and control littermates (Fig. 5*A–D*; neocortex: control 100 ± 4.5% and knock-out 108.95 ± 6.2%; striatum: control 100 ± 3.5% and knock-out 105.2 ± 4.4%; thalamus: control 100 ± 6.0% and knock-out 106.1 ± 2.7%; hippocampus: control 100 ± 5.9% and knock-out 103.5 ± 8.0%; and dentate gyrus: control 100 ± 7.6% and knock-out 92.8 ± 3.1%. Data shown are mean ± SEM as a percentage of mean; n = 3 mice). Although the dentate gyrus in *Srf*-Nestin-cKO mice appeared smaller, it had a higher cell density than that of control animals and there were no appreciable differences in total neuronal numbers (Fig. 5*C*, data not shown).

We also analyzed the number of intermediate neuronal precursors (INPs), as identified by Tbr2 (or Eomes) expression, and found no statistically significant difference in the total number of INPs within the neocortex of *Srf*-Nestin-cKO mice and control littermates (Fig. 5*E*; control 100 ± 6.5% and knock-out 96 ± 5.8%; $p < 0.001$; $n \pm 3$ mice).

Together, these results demonstrate that SRF is not required for cell survival and neurogenesis and that the deficits in axonal projections observed in *Srf*-Nestin-cKO brains reflect a specific requirement for SRF for axon growth.

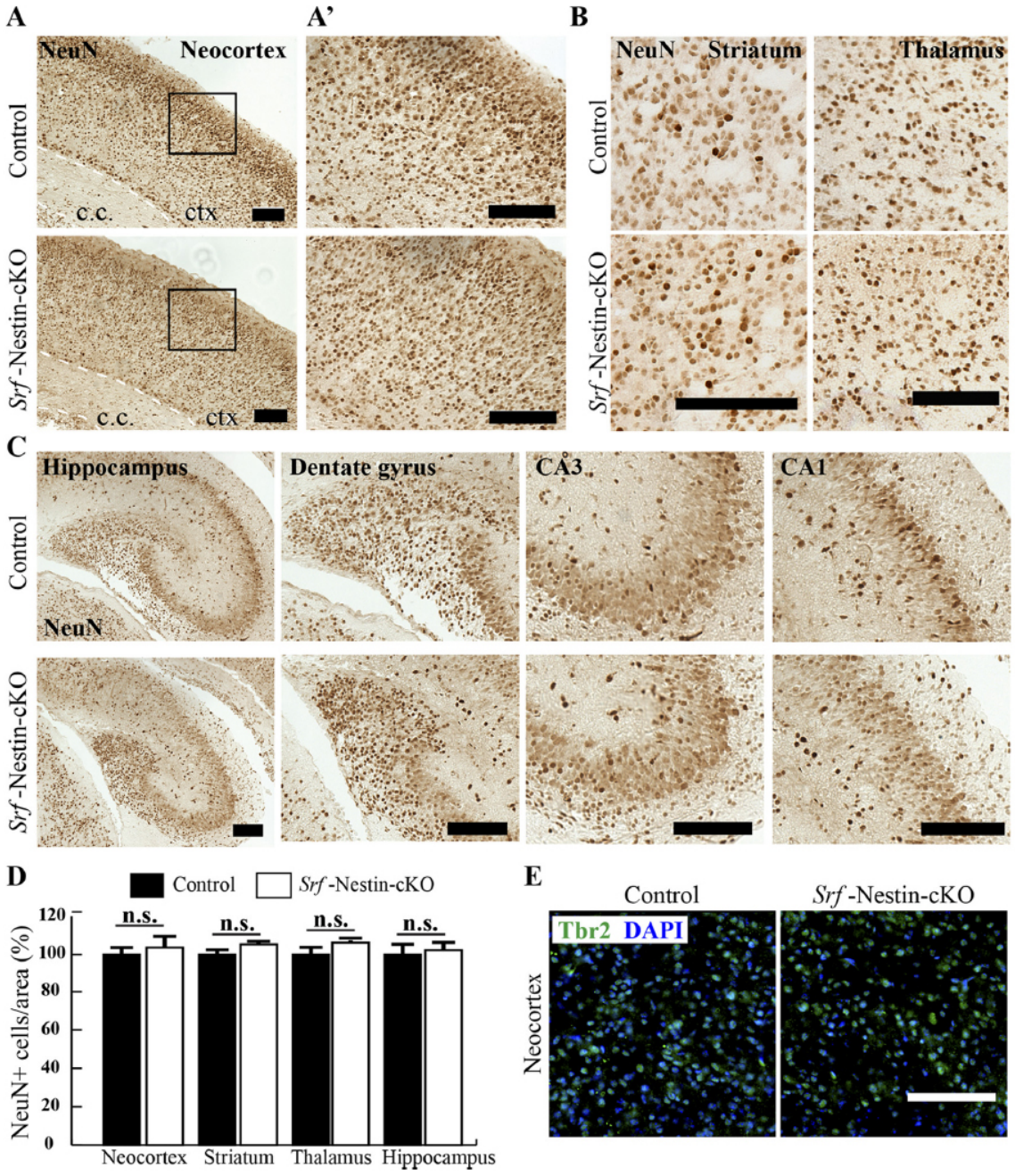

Figure 5. Loss of SRF does not affect neurogenesis. ***A***, *Srf*-Nestin-cKO mutants exhibit no significant changes in the number of neurons generated, as indicated by NeuN immunostaining, in the neocortex. ctx, Neocortex; c.c, corpus callosum. ***A'***, Magnified view of boxed regions shown in ***A***. ***B***, ***C***, Immunostaining for NeuN in the striatum and thalamus (***B***) and hippocampus (***C***) of control and *Srf*-Nestin-cKO mice. Although the dentate gyrus is more compact in *Srf*-Nestin-cKO mice, there is no significant difference in the number of cells compared with control mice. Scale bars: ***A–C***, 100 μm. ***D***, Quantification of total number of NeuN-positive cells in neocortex, striatum, thalamus, and hippocampus. Error bars represent SEM. Student's *t* test analysis between control and *Srf*-Nestin-cKO animals shows no statistically significant differences. ***E***, Immunostaining for Tbr2 (or Eomes), a marker for intermediate neuronal progenitors, showed no difference in total number of committed neuronal precursors in *Srf*-Nestin-cKO mice and control littermates. Scale bar, 50μm.

SRF is not required for projection neuron subtype specification and cortical lamination

We next asked whether SRF was required for neuronal subtype specification. The excitatory projection neurons reside in defined layers of the neocortex and make intracortical, subcortical, or subcerebral connections (Molyneaux et al., 2007). A number of transcription factors, including *Fezf2* and *Ctip2*, are critical for specification of cortical projection neuron subtypes and their

loss results in absence of subcerebral and subcortical projections (Arlotta et al., 2005; Chen et al., 2005; Molyneaux et al., 2005; Chen et al., 2008). Therefore, it is possible that the lack of cortical projections observed in *Srf*-Nestin-cKO mutants could be attributed to a switch in projection neuron identity, a result that could affect neocortical lamination. To study this, we probed for expression of Tbr1, a deep-layer neuronal marker, and Cux1, which is specifically expressed in neocortical layers 2–4 (Hevner et al., 2001; Ferrere et al., 2006). Immunostaining of P0.5 brains revealed no differences in the layer-specific expression patterns of both Tbr1 and Cux1 in *Srf*-Nestin-cKO mice and control littermates (Fig. 6*A*,*B*). Additionally, we also probed for expression of other neocortical layer-specific transcription factors by *in situ* hybridization (Gray et al., 2004). Expression patterns of several transcription factors, including *Cux2*, *Klf6*, *Lhx5*, *Lmo4*, *Nfix*, *Nr4a2*, and *Sox5*, which specify the identity and position of projection neuron subtypes, were similar between *Srf*-Nestin-cKO mice and control littermates (Fig. 6*C*, data not shown). These results indicate that there are no changes in the establishment of layer-specific neuronal subtypes and neocortical lamination at P0.5 in the brains of *Srf*-Nestin-cKO mice.

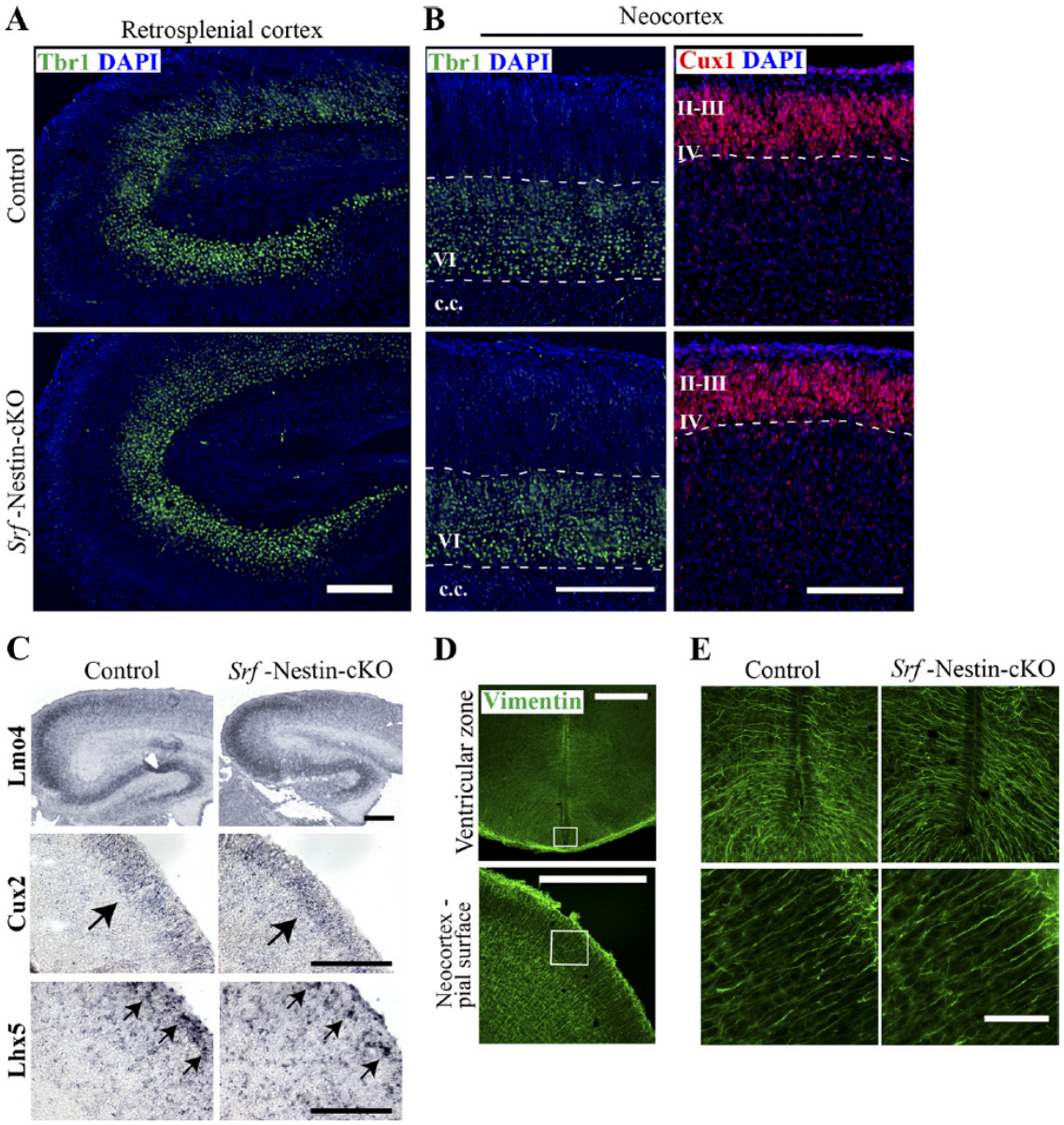

Figure 6. SRF is dispensable for neocortical lamination and projection neuron subtype specification. ***A***, Immunostaining for *Tbr1* expression, a marker of deep layer neurons in the neocortex, shows normal layer VI lamination in *Srf*-Nestin-cKO and control brains. Shown here is the retrosplenial cortex. ***B***, Immunofluorescence staining for expression of Tbr1 and Cux 1 (a marker for

superficial layer neurons) in the neocortex shows that both layer 2/3 and layer VI neurons are specified and positioned normally in knock-out mice. c.c., Corpus callosum. ***C***, *In situ* hybridization of layer-specific transcription factors, including *Lmo4*, *Cux2*, and *Lhx5*, shows that neocortical lamination and the specification of those subtypes are normal in the absence of SRF. Arrows point to Cux2- or Lhx5-expressing upper layer neurons. Scale bars: ***A***, ***C***, 200μm; ***B***, 100μm. ***D***, Immunostaining using anti-vimentin antibody, expressed in radial glial processes, show that the structural integrity of radial glia is normal in *Srf*-Nestin-cKO brain. ***E***, Magnified images of boxed regions in ***D*** show normal appearance of radial glial processes in *Srf*-Nestin-cKO mice compared with control mice. Scale bar, 20μm.

The proper lamination of the neocortex in *Srf*-Nestin-cKO mice suggested that radial migration of neurons is not affected in the absence of SRF. We found that SRF is also deleted in radial glial cells in *Srf*-Nestin-cKO mice (data not shown). We immunostained P0.5 brains using anti-vimentin antibody, which labels radial glial processes, and found no gross alteration in the abundance of radial glial processes or the integrity of projections within multiple regions examined including the ventricular zone, neocortex, hippocampus, and corpus callosum (Fig. 6*D*,*E*, data not shown). Together, these findings suggest that loss of SRF does not affect radial glial projections and thereby, the radial migration of neurons and lamination of neocortex.

SRF is not required for interneuron subtype specification

Given the role of SRF in mediating differentiation and development of several cell types in other tissues, we next sought to determine whether SRF is required for specification of interneurons. Interneurons, which show astonishing differences in their electrophysiological, morphological, and molecular properties, are primarily derived from the medial and caudal ganglionic eminences during brain development and then migrate to populate the neocortex as the brain matures (Marín and Rubenstein, 2003; Wonders and Anderson, 2006). Since *Srf*-Nestin-cKO mutants do not survive beyond P1, we restricted our analysis to interneuron populations in the striatum. We used immunostaining for anti-Gad-6 to identify the expression of GAD, an enzyme that synthesizes GABA neurotransmitters in all interneurons (Fig. 7*A*). We found no significant difference in the overall numbers of interneurons between control and *Srf*-Nestin-cKO mice (Fig. 7*A*,*C*; control 100 ± 6.8% and knock-out 98.8 ± 10.8%). Next, we analyzed for different interneuron subtypes, including SSTpositive, parvalbumin-positive, and calbindin-positive cells. We observed no difference in their numbers between *Srf*-Nestin-cKO mutants and control littermates (Fig. 7*B*,*C*; SST+ cells: control 100 ± 7.3% and knock-out 92.6 ± 7.5%; parvalbumin+ cells: control 100 ± 6.4% and knock-out 117.8 ± 11.2%; calbindin+: control 100 ± 7.8% and knock-out 116.0 ± 10.1%).

Together, these findings demonstrate that, in addition to projection neuron subtype specification, the establishment of interneuron subtypes occurs normally in the absence of SRF during neuronal lineage commitment.

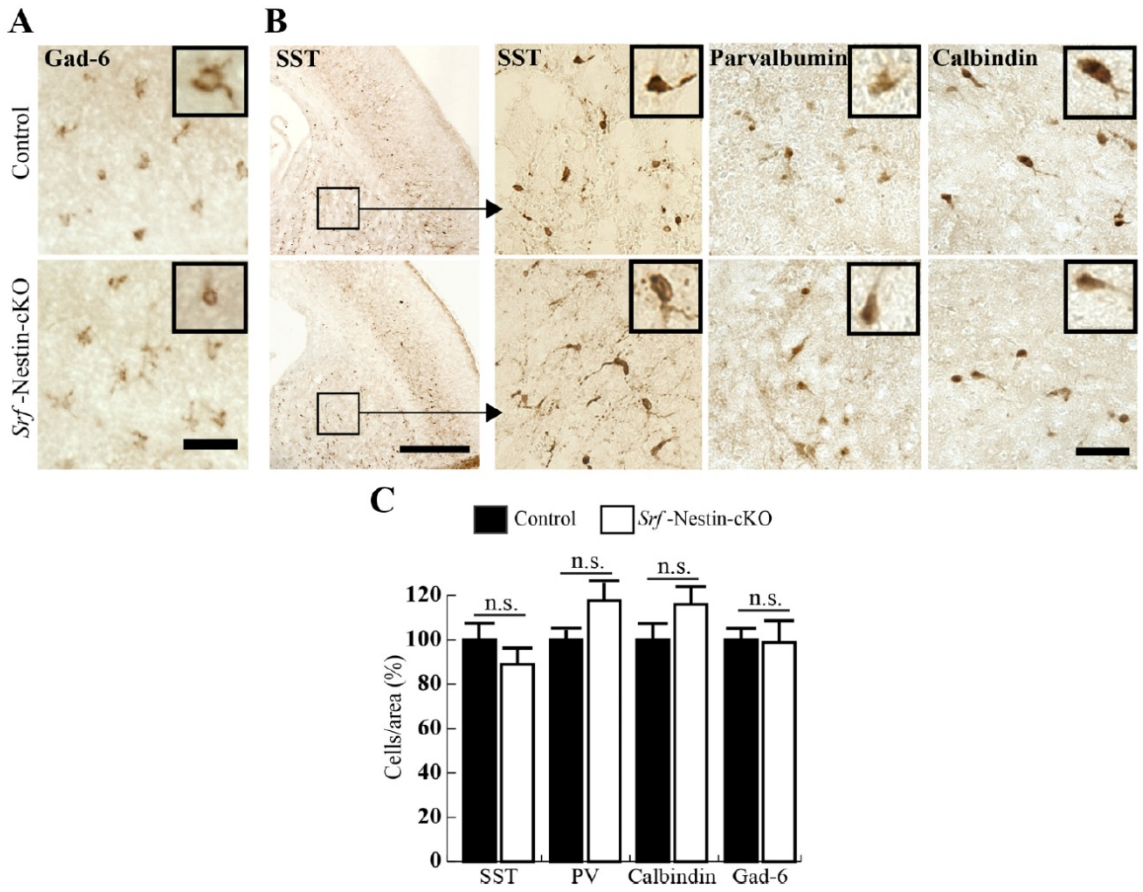

Figure 7. SRF is not required for interneuron subtype specification. ***A***, Immunostaining for Gad-6, which labels all GABAergic interneurons, showed normal expression of Gad-6 in *Srf*-Nestin-cKO and control mice. Inset shows enlarged image of a single stained neuron. ***B***, Immunohistochemistry staining showing expression of SST, parvalbumin, and calbindin, which label unique subtypes of interneurons, suggests no apparent change in the population of

interneurons in brains of *Srf*-Nestin-cKO mice. The striatal region from control and mutant is shown as magnified images. Inset shows enlarged image of a single stained neuron. ***C***, Quantification of cell numbers for different interneurons subtypes for ***B***. Student's *t* test showed no statistically significant difference in the number of each interneuron subtype between paired control and knock-out animals. Error bars represent SEM.

Neural precursor cell population increases in *Srf*-Nestin-cKO mutant mice

Quiescent cells that rest in the G0 state can be induced by extracellular stimuli to express immediate early gene (IEG) programs (Herschman, 1991). A number of transcription factor-encoding IEGs, such as *cFos*, *c-Myc*, *Egr-1*, and *JunB*, and are then responsible for activating gene cascades that enable cell progression to the G1 state (Greenberg and Ziff, 1984; Lau and Nathans, 1985). SRF-mediated transcription was demonstrated to be necessary for inducing IEG expression in embryonic stem cells and in neurons (Norman et al., 1988; Schratt et al., 2001; Ramanan et al., 2005). Sequestration of functional SRF was also shown to impede rat embryonic fibroblast and myoblast proliferation, but not self-renewal of embryonic stem cells (Gauthier-Rouvie`re et al., 1991; Soulez et al., 1996). We therefore assessed whether SRF deletion affects NPC growth and maintenance *in vivo*. Proliferating progenitor cells are identified by the expression of phospho-histone H3, which is a modification event that occurs specifically during cell division events of both mitosis and meiosis (Hans and Dimitrov, 2001).

Surprisingly, we observed an increase in phosphohistone H3-positive cells in the proliferative subventricular zone (SVZ) of *Srf*-Nestin-cKO brains at both E14.5 and E18.5 (Fig. 8*A–D*). Quantitative analyses of the number of phospho-histone H3-positive cells per area of the parameter of the SVZ revealed a 20% and 80% increase in mutants at E14.5 and E18.5, respectively (Fig. 8*E*; E14.5 p-histone H3 normalized: control 100 ± 7.3% and knock-out 120.7±7.8%; and E18.5 p-histone H3 normalized: control 100 ± 3.0% and knock-out 180.6 ± 4.2%). We confirmed the increase in proliferative progenitor cell numbers using two additional markers: Ki-67, which is expressed by cells in the cell-cycle phases G1, S, and G2 and in mitosis; and Sox2, a transcription factor expressed in NPC. At E14.5, immunohistochemistry staining of Ki-67 in *Srf*-Nestin-cKO brains showed an increase in NPC numbers per square micrometer in the SVZ and the neocortex compared with control (Fig. 8*E*,*F*; Ki-67 normalized: control 100 ± 18.0% and knock-out 140 ± 6.3%). Similarly, neocortex and SVZ in *Srf*-Nestin-cKO showed markedly increased numbers of Sox2-expressing cells compared with those in control littermates (Fig. 8*F*). These observations suggest that loss of SRF affects NPC homeostasis during development without affecting NPC survival.

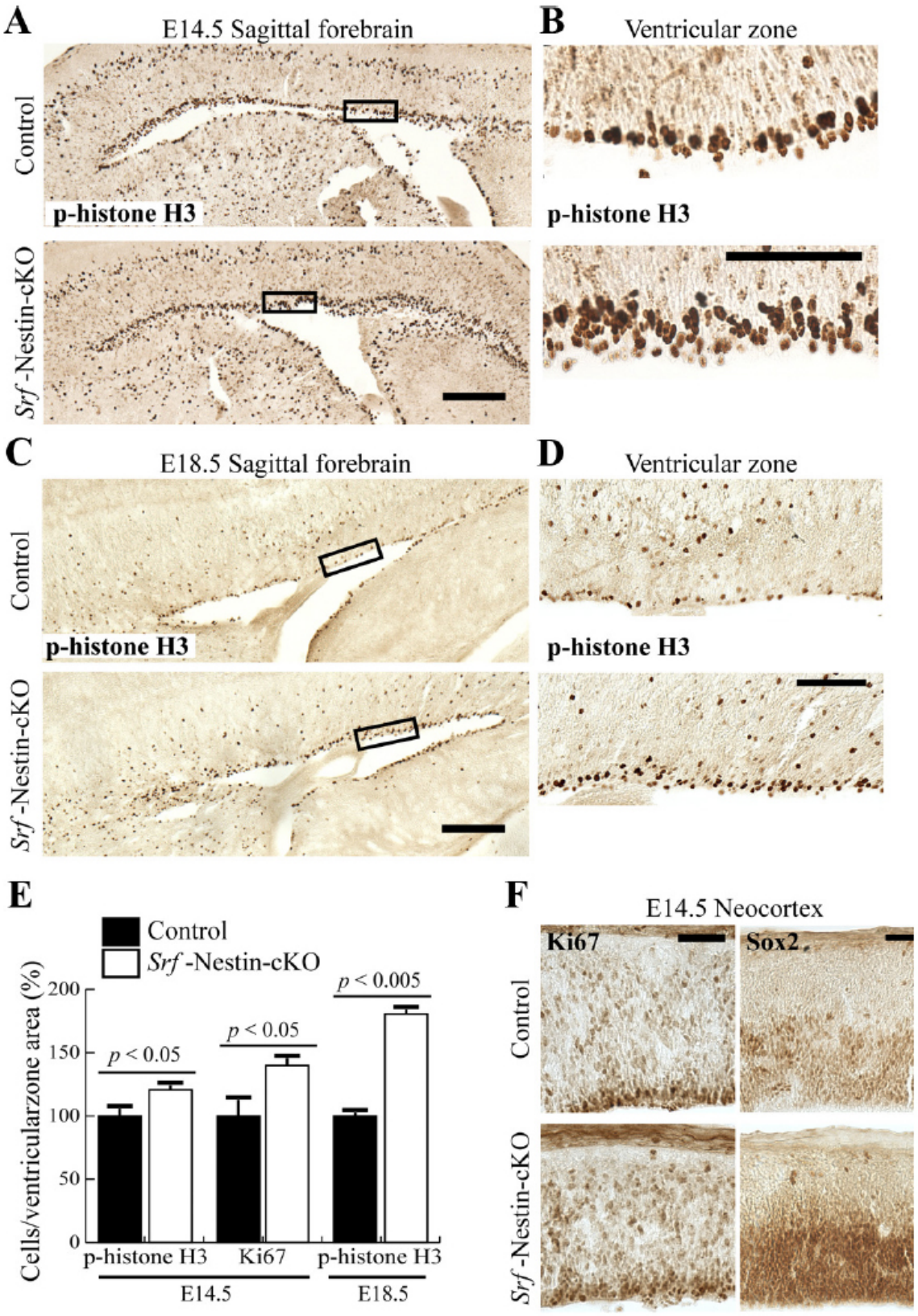
A
E14.5 Sagittal forebrain
Control
p-histone H3
Srf -Nestin-cKO
B
Ventricular zone
p-histone H3
C
E18.5 Sagittal forebrain
Control
p-histone H3
Srf -Nestin-cKO
D
Ventricular zone
p-histone H3
E
Control
Srf -Nestin-cKO
Cells/ventricularzone area (%)
200
150
100
50
0
p < 0.05
p < 0.05
p < 0.005
p-histone H3
Ki67
p-histone H3
E14.5
E18.5
F
E14.5 Neocortex
Ki67
Sox2
Control
Srf -Nestin-cKO

Figure 8. Loss of SRF results in an increase in the number of neural precursor cells. ***A***, Proliferating NPCs are identified in the control and *Srf*-Nestin-cKO forebrains using anti-p-histone H3 antibody at E14.5. ***B***, Magnification of the boxed regions in ***A***. ***C***, Comparison of NPC populations at E18.5. ***D***, Magnified view of the boxed regions in ***C***. ***E***, Cell count analyses of the number of p-histone-H3- and Ki67-positive cells at E14.5 indicate a statistically significant increase in the number of proliferating cells in the ventricular zone of *Srf*-Nestin-cKO mice. The difference in relative numbers of NPCs between the control and mutant brain is more pronounced at E18.5. ***F***, Immunostaining for two additional proteins, Ki67, a marker of cells in the active phase of cell cycle, and Sox2, a marker of neural precursor cells, was used to visualize proliferating cells in the neocortex at E14.5. Mutant brains not only display more Ki67-positive cells but also show a broadened layer of Sox2-positive cells. Scale bars, 50μm.

Conditional deletion of SRF in developing forebrain neurons

Our analyses of the *Srf*-Nestin-cKO mice suggested that SRF plays a specific role in the development of axonal tracts without affecting neurogenesis, neuronal survival, and neuronal subtype specification. However, since SRF deletion occurs in all neural precursor cells before cellular differentiation occurs, it is possible that axon growth defects in *Srf*-Nestin-cKO mice could be due to a non-cell autonomous requirement of SRF for axon growth. To ascertain whether SRF is required cell autonomously for axon growth, we generated a neuron-

specific deletion of SRF using the NEX-Cre transgenic mouse. In the NEX-Cre mouse, cre expression is controlled by the onset of expression of the *NEX* gene (also known as *Math2* or *NeuroD6*), an early neuronal basic helixloop- helix gene expressed specifically in differentiating neurons (Schwab et al., 1998; Goebbels et al., 2006). Cre recombinase-mediated excision in the NEX-Cre mouse has been shown to take place starting at ~E11.5 and is restricted only to the glutamatergic neurons in the neocortex and hippocampus, whereas cre expression is not observed in the interneurons and glial cells (Brockschnieder et al., 2004; Goebbels et al., 2006; Kashani et al., 2006). The *Srf*-NEX-cKO mice were born in the expected Mendelian ratio but unlike the *Srf*-Nestin-cKO mice, these mice did not exhibit neonatal lethality and grew to adulthood. We first confirmed deletion at P0.5 by immunostaining and found that SRF deletion was restricted to the neocortex and hippocampus but not in the striatum and other regions of the brain, consistent with previous findings (Fig. 9*A—D*, data not shown). We next asked whether lamination occurs normally in *Srf*-NEX-cKO mice. We performed immunostaining for laminar-specific markers, Cux1 and Tbr1, on P21 brains. We did not find any deficits in neocortical lamination in *Srf*-NEX-cKO mice as compared with control littermates (Fig. 9*E*). At birth, the lack of defects in lamination in *Srf*-NEX-cKO mice was similar to that observed for *Srf*-Nestin-cKO mutant mice (data not shown). Together, these findings suggest neocortical lamination is properly established in the absence of SRF.

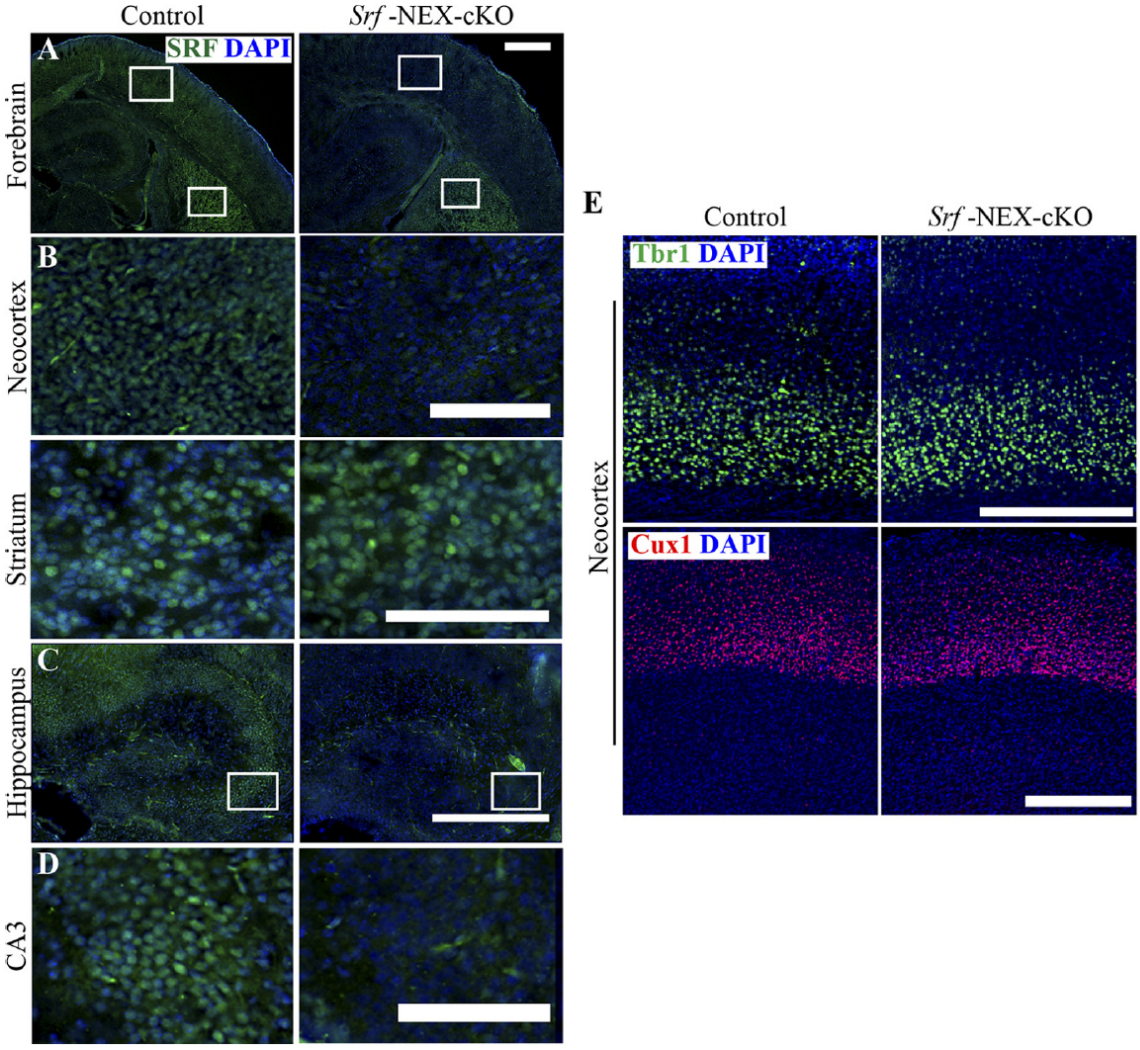

Figure 9. Ablation of SRF in *Srf*-NEX-cKO. ***A***, Immunofluorescence staining at P0.5 using anti-SRF antibody shows that SRF is deleted in the neocortex but not in the striatum of *Srf*-NEX-cKO mutants. ***B***, Magnified views of boxed regions in ***A***. ***C***, SRF expression is also abolished in the hippocampus in mutants. ***D***, A magnified view of CA3 neurons of control and *Srf*-NEX-cKO mice. ***E***, Immunostaining of P21 brains using anti-Tbr1 and anti-Cux1 shows normal lamination of neocortex in *Srf*-NEX-cKO mice and control littermates. Scale bars, 50μm.

SRF mediates cortical neuron target innervation cell autonomously

We next analyzed axonal projections in *Srf*-NEX-cKO mice at P0.5 using DiI labeling. Two weeks following DiI labeling, sagittal sections of *Srf*-NEX-cKO mouse brains revealed greatly diminished corticospinal projections, and cortical motor neuron axons passing through the internal capsule were less abundant with very few projections reaching the cerebral peduncle (Fig. 10*A,A',A"*; *n* = 3 mice). Comparison of parallel serial sections from the lateral side to the medial region of the forebrain between control and *Srf*-NEX-cKO mice showed a severe reduction in corticospinal projections in the mutant neocortex (Fig. 10*B*; *n* = 3 mice). We then examined horizontal sections of control and *Srf*-NEX-cKO brains after 6 weeks of DiI labeling. Onthe ventral side of the brain, we observed that the intracortical and corticostriatal connections in *Srf*-NEX-cKO mice were less abundant and shorter than those observed in control littermates. In particular, a region of the thalamus is clearly innervated in the control brain; however, this innervation is less prominent in the *Srf*-NEX-cKO brain (Fig. 10*C*; *n* = 3 mice). In the medial region of the brains, similar to that observed in 2-week-old sagittal sections, it was evident that corticospinal innervations through the striatum to the cerebral peduncle were greatly reduced overall in *Srf*-NEX-cKO mutants compared with control littermates (Fig. 10*D*). We further examined serial coronal sections of control and *Srf*-NEX-cKO brains at 4 weeks after DiI labeling. In rostral sections, DiI tracing showed that callosal innervations that form the corpus callosum are diminished and shorter in the *Srf*-NEX-cKO brains (Fig. 10*E*).

We found less abundant corticostriatal projections in the *Srf*-NEX-cKO brain (Fig. 10*E*,*F*; projection area: control, 205,737 ± 496 μm2; *Srf*-NEX cKO, 12,148 ± 427 μm2, *n* = 3 mice), an observation that is consistent with that made in brains of *Srf*-Nestin-cKO mice using anti-2H3 immunostaining. Toward the caudal end of the forebrain, retrograde DiI labeling revealed that the corticothalamic connections, which are important relays of sensory information between the visual cortex and the lateral geniculate nucleus of the thalamus, were also less robustly established in the *Srf*-NEX-cKO mice compared with control mice (Fig. 10*E*,*F*; projection area: control, 41,690 ± 1643 μm2; *Srf*-NEX-cKO, 21,229 ± 1207 μm2, *n* = 2 mice). These observations were consistent in all the mutant mice analyzed.

These neuroanatomical tracing experiments demonstrate that SRF plays a critical cell-autonomous role in regulating axonal growth and establishment of axonal projections *in vivo*.

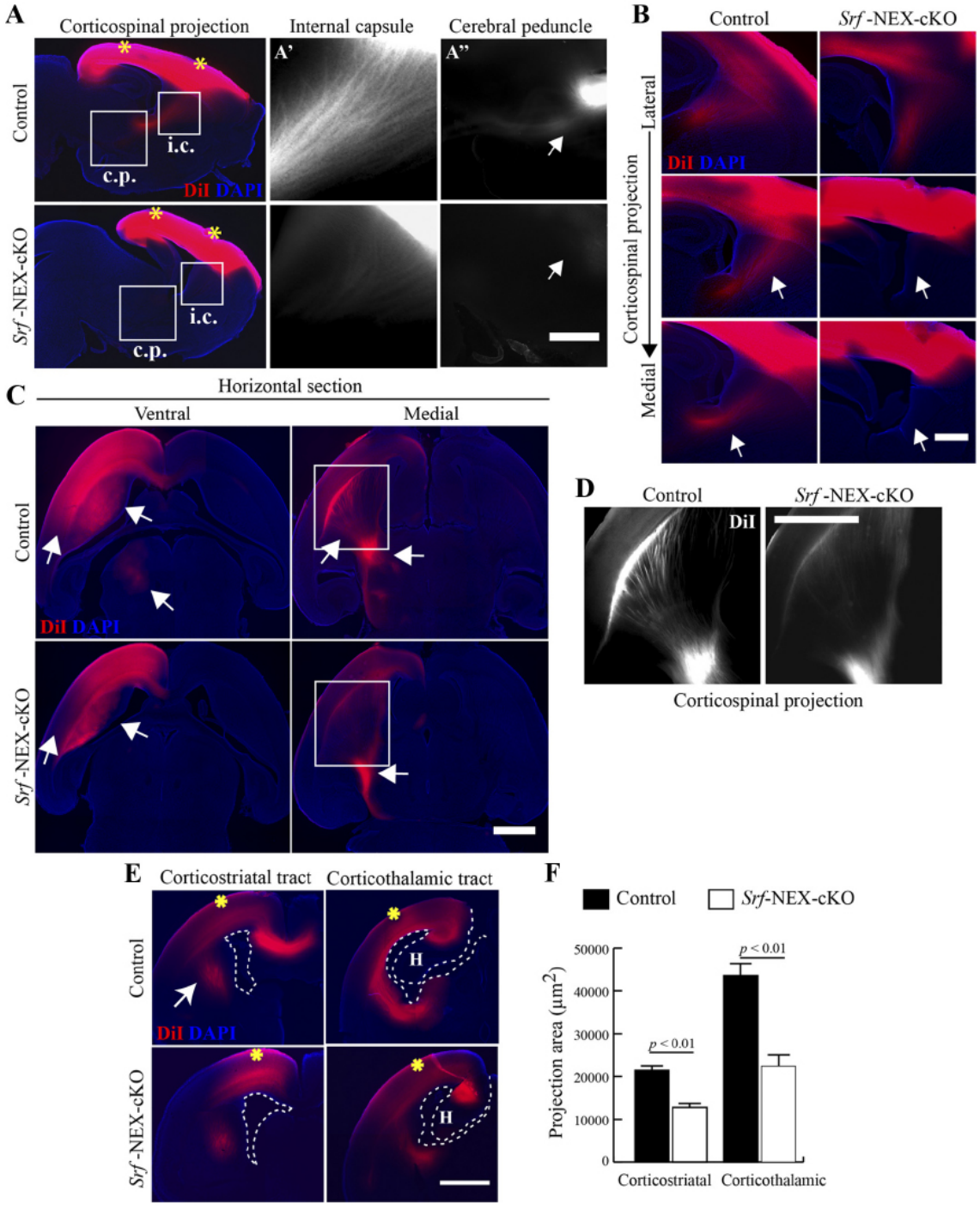

Figure 10. DiI labeling shows impairment in axonal projections in *Srf*-NEX-cKO mutants. ***A***, DiI crystals were placed on the brain surface in the regions of the motor and the visual cortices (indicated by asterisks) in P0.5 *Srf*-NEX-cKO

knock-out and control littermates. Two weeks after labeling, brains were sectioned sagittally. Impaired corticospinal innervation was observed in the knock-out brain. Magnifications of the internal capsule (i.c) and cerebral peduncle (c.p) regions are shown in ***A'*** and ***A"***. Projections through the cerebral peduncle are seen in the brains of control but *Srf*-NEX-cKO mice.***B***, Serial sagittal sections from lateral to medial regions of the brain show lack of corticostriatal projections (arrows) in *Srf*-NEX-cKO mice. No misguided axons were observed in the mutant mice. ***C***, After 6 weeks of labeling, control and *Srf*-NEX-cKO brains were sectioned horizontally. Arrows show diminished projections within the neocortex, corticostriatal projections, and innervations to the thalamus in the mutant. Medial horizontal section shows impaired projections through the internal capsule and the cerebral peduncle. ***D***, Magnified views of the boxed regions in ***C*** showing the corticospinal projections. ***E***, Coronal sections from caudal regions of the brain reveal diminished corticostriatal as well as corticothalamic tracts (arrows). Asterisks indicate sites of crystal placement; dotted lines outline the ventricular zone and the hippocampus (H). ***F***, Quantification of area of innervation by corticostriatal and corticothalamic axons in ***E*** (n = 3 mice).

Discussion

Neuronal development in the CNS involves several critical stages including neurogenesis and maturation of neurons, growth and extension of axons, and structural organization within the brain. Our current understanding of the role

of SRF in neuronal development comes mainly from studies using mice carrying conditional neuron-specific deletion of SRF in late gestation or in postnatal brain. However, the requirement of SRF for early stages of neuronal development remains unknown. In the present study, we show that conditional deletion of SRF in neural precursor cells (*Srf*-Nestin-cKO) results in severe deficits in the development of major axonal projections in the forebrain, including corticospinal, corticothalamic, corticostriatal, and thalamocortical tracts along with a variable loss of the corpus callosum. Axonal deficits were seen as early as E14.5 in the *Srf*-Nestin-cKO mice and there was little or no cell death during development.

Interestingly, there was a significant increase in the number of proliferating cells in the ventricular zone in *Srf*-Nestin-cKO mice. Conditional deletion of SRF in embryonic forebrain neurons (*Srf*-NEX-cKO) also resulted in severe deficits in major axonal projections. Neurogenesis, radial neuronal migration in the neocortex, neocortical lamination, and neuronal subtype specification were unaffected by SRF loss. Together, these findings suggest that SRF is required in a cell-autonomous manner for axon growth and extension.

Similar to that observed in mice with prenatal and postnatal deletion of SRF, SRF is dispensable for neuronal survival. Our study identifies a specific role for SRF in promoting axon growth during neuronal development without affecting neurogenesis and neuronal differentiation. Previous studies have shown that neuron-specific SRF deletion during late gestation in the brain causes deficits in terminal targeting of mossy fiber axons in the hippocampus, while SRF loss in

developing sensory neurons in the peripheral nervous system affects NGF-dependent terminal arborization and target innervation (Kno¨ll et al., 2006; Wickramasinghe et al., 2008). However, proximal axon growth in the peripheral nervous system is not affected while the role of SRF in axon growth in the CNS remains unknown. We found that deleting SRF in neural precursor cells results in severe deficits in axon growth and targeting of cortical axon projections. Observations made in cultured hippocampal neurons have shown that SRF is required for contact-mediated axon repulsion (Kno¨ll et al., 2006). We did not observe any mistargeted axonal tracts in the brains of *Srf*-Nestin-cKO mice, suggesting that the lack of target innervation seen in the SRF mutant mice is primarily due to defects in axon growth and not due to defects in axon guidance.Wefound similar axonal growth defects when SRF was deleted in developing postmitotic neurons in neocortex and hippocampus in *Srf*-NEX-cKO mutant mice. Unlike *Srf*-Nestin-cKO mice, the *Srf*-NEX-cKO mice survived to adulthood, and in preliminary observations, we found that adult *Srf*-NEX-cKO mice exhibited clasping of limbs in a dystonic manner when subjected to the tail suspension test, which is suggestive of motor dysfunction (Carter et al., 1999; Yamamoto et al., 2000). Furthermore, consistent with previous observations, we also found that SRF-deficient neurons exhibit highly attenuated axon growth in culture (Kno¨ll et al., 2006) (C. Li and N. Ramanan, unpublished observations).

The similarities in deficits in axon growth in the brains of *Srf*-Nestin-cKO and *Srf*-NEX-cKO mice suggested that SRF-dependent transcription plays a cell-intrinsic role in axon growth.

The molecular mechanisms underlying SRF-dependent axon growth remain poorly understood. One mechanism by which SRF potentially regulates axon growth is through association with specific cofactors. We found that blocking the functions of the Ternary Complex Factor-family cofactors of SRF, including Elk-1, does not affect axon growth in cultured neurons (C. Li and N. Ramanan, unpublished observations). However, we and others have found that blocking the functions of myocardin-family cofactors, MKL1 (also known as MAL/MRTF-A) and MKL2 (MRTF-B), by dominant-negative or knockdown approaches or by gene deletion attenuates axon growth *in vitro* (Kno¨ll et al., 2006; Shiota et al., 2006; Wickramasinghe et al., 2008; Mokalled et al., 2010) (C. Li and N. Ramanan, unpublished observations). Mutant mice that lack both MKL1 and MKL2 in the brain exhibit deficits in dendritic growth in the neocortex and hippocampus as assessed by MAP2 and Golgi staining (Mokalled et al., 2010). However, the effect of MKL1/MKL2 loss on axon growth *in vivo* has not been reported in these mice. In the peripheral nervous system, SRF has been shown to function downstream of NGF signaling to regulate terminal arborization of axons and target innervation (Wickramasinghe et al., 2008).

Furthermore, NGF signaling to SRF is dependent on both ERK/MEK and MAL/MKL1 signaling pathways. The findings from the peripheral nervous system raise an interesting question as to which extracellular signals might stimulate SRF-dependent transcription during axon growth in the brain. Currently we lack sufficient knowledge on the nature of the extracellular signals and the identities of SRF target genes critical for axon growth in the CNS. It is likely that SRF functions downstream of growth factors such as BDNF to regulate axon growth. SRF could also regulate axon growth by regulating the expression of components of the actin cytoskeleton, including β-actin, γ-actin, paxillin, vinculin, and talin (Schratt et al., 2002). In fact, previous studies including our own have shown that β-actin expression is reduced in SRF knock-out neurons (Alberti et al., 2005;Ramananet al., 2005;Kno¨ll et al., 2006), and it was hypothesized that reduction in actin levels was one of the underlying causes for neurite outgrowth deficits observed in SRFdeficient neurons. However, overexpression of actin was found to be insufficient to rescue the growth deficits of SRF-null neurons (Kno¨ll et al., 2006; Stern et al., 2009). Since SRF regulates the expression of several cytoskeletal proteins (Schratt et al., 2002), it is possible that the neuronal growth deficits exhibited by SRF-deficient neurons could be due to a breakdown in cytoskeletal apparatus critical for growth and extension.

We did not observe any increased cell death in the brains of *Srf*-Nestin-cKO and *Srf*-NEX-cKO mutant mice during development. There was also no noticeable difference in neuronal cell numbers in older *Srf*-NEX-cKO mice, and

this is consistent with our previous findings that SRF deletion does not result in cell death or neurodegeneration in the CNS (Ramanan et al., 2005). Interestingly, SRF deletion in neural precursor cells did not cause apoptotic cell death, a phenotype that contrasts with observations made in SRF-deficient embryonic stem cells (Schratt et al., 2004). SRF-deficient mouse ES cells exhibited apoptotic cell death both *in vitro* and *in vivo* (Schratt et al., 2004). Our findings suggest that SRF is dispensable for survival of NPCs both *in vitro* and *in vivo* (our unpublished observations). In contrast, we observed an increase in the total number of p-histone-H3 and Sox2-positive cells in *Srf*-Nestin-cKO mice.

A recent elegant study showed that SRF deletion in neurons affects oligodendrocyte differentiation in a paracrine manner (Stritt et al., 2009). Consistent with this observation, we also observed a decrease in Olig2+ cells at birth in *Srf*-Nestin-cKO mice (our unpublished observations). Therefore, a likely explanation for the increase in NPC numbers in *Srf*-Nestin-cKO mice is that SRF loss in NPCs affects oligodendrocyte differentiation, thereby resulting in an increase in undifferentiated neural precursor cells. Together, these observations suggest that there are distinct requirements for SRF in ES cells and in NPCs for cell survival.

Previous studies have shown that SRF has a profound role in regulating cell-type specific gene expression that underlies the development of many cell types. A number of tissue-specific inactivation studies later elucidated essential functions of SRF for the development of cardiac muscle cells (Niu et al., 2005,

2008; Zhao et al., 2005), the differentiation of smooth muscles (Miano et al., 2004; Parlakian et al., 2004), and the normal proliferation and differentiation of keratinocytes (Koegel et al., 2009). We observed no differences in total number of NeuN-positive cells in *Srf*-Nestin-cKO mice, suggesting that SRF is dispensable for neurogenesis in the brain.We also found that neuronal subtype specification and both interneuron and neocortical lamina-specific neuron identities were properly established in the absence of SRF.

The findings that SRF-deficient neurons negatively influence oligodendrocyte differentiation suggest that SRF-dependent transcription can promote cell-type specification in the brain (Stritt et al., 2009). SRF deletion in developing neurons has been shown to affect tangential cell migration along the rostral migratory stream (Alberti et al., 2005). We also observed similar tangential migration deficits in the *Srf*-Nestin-cKO mice (our unpublished observations). However, we found that radial migration of neurons in the neocortex was not affected and neocortical lamination was established normally in both *Srf*-Nestin-cKO and *Srf*-NEX-cKO mutant mice. Our observations differ from those of a recent study in which cortical lamination was shown to be affected in mice carrying neuron-specific deletion of SRF (Stritt and Kno¨ll, 2010). In this study, calbindin-positive cells were reduced in SRF-mutant neocortex while immunostaining for the neurofilament protein SMI-32, which also labels a subpopulation of cortical neurons (Campbell and Morrison, 1989) in layers III and V, showed mislocalization of Smi-32-positive cells between layers III and V.

Calbindin-positive interneurons are mainly generated in the medial ganglionic eminences before they tangentially migrate to populate the neocortex (Marín and Rubenstein, 2003; Wonders and Anderson, 2006), and we did not find any change in the total numbers of striatal calbindin-positive cells in *Srf*-Nestin-cKO mice at birth. In this study, we used both *in situ* hybridization and immunohistochemistry for several transcription factors that are expressed in specific cortical layers during mouse development (Gray et al., 2004). We did not find any lamination defects in the neocortex of either *Srf*-Nestin-cKO or *Srf*-NEX-cKO mice at P0.5 or in 3-week-old *Srf*-NEX-cKO mice. If SRF is critical for neocortical lamination, then this phenotype should be more severe in the *Srf*-Nestin-cKO mice, since SRF is deleted in all major cell types in the brain starting at E12.5 (our unpublished observations).

Based on our observations, we conclude that SRF loss in neural precursor cells and in developing neurons does not affect layering of the neocortex. We also found that SRF deletion in radial glial cells in *Srf*-Nestin-cKO mice did not affect their morphology, suggesting that SRF-dependent transcription is not required for extension of radial glial processes.

Our study identifies specific roles for SRF during neuronal development. SRF plays a critical role in neural precursor cell homeostasis and in the formation of major axonal tracts in the brain. SRF is dispensable for neurogenesis and cell survival but contrary to recent findings, SRF is not required for neocortical lamination.

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Chapter 3: SRF in Astrocyte and Oligodendrocyte Specification

This chapter is adapted from a revised manuscript for the Journal of Neuroscience, to be submitted in April, 2012.

A Critical Cell-intrinsic Role for Serum Response Factor in Glial Specification in the CNS

ABSTRACT

Astrocytes and oligodendrocytes play crucial roles in nearly every facet of nervous system development and function including neuronal migration, synaptogenesis, synaptic plasticity, myelination and maintenance. Previous studies have widely characterized the signaling pathways important for astrocyte differentiation and unveiled a number of transcription factors that guide oligodendrocyte differentiation in the CNS. However, the identities of the transcription factors critical for astrocyte specification in the brain remain unknown. Here we show that deletion of the stimulus-dependent transcription factor, serum response factor (SRF) in neural precursor cells (*Srf*-Nestin-cKO) results in nearly 60% loss in astrocytes and 50% loss in oligodendrocyte precursors at birth. Cultured SRF-deficient neural precursor cells (NPCs) exhibited normal growth rate and capacity to self-renew. However, SRF-deficient NPCs generated fewer astrocytes and oligodendrocytes in response to several lineage-specific differentiation factors. These deficits in glial differentiation were rescued by ectopic expression of wild type SRF in SRF-deficient NPCs. Interestingly, ectopic expression of a constitutively active SRF, (SRF-VP16) augmented astrocyte differentiation in the presence of pro-astrocytic factors. However, SRF-VP16 expression in NPCs had an inhibitory effect on oligodendrocyte differentiation as previously reported. In contrast, mice carrying conditional deletion of SRF in developing forebrain neurons (*Srf*-NEX-cKO) did not exhibit any deficits

in astrocytes in the brain. Together, our observations suggest that SRF plays a critical cell-autonomous role in NPCs to regulate astrocyte and oligodendrocyte specification both *in vivo* and *in vitro*.

INTRODUCTION

Astrocytes play multiple critical roles in brain development and functioning (Barres, 2008), and abnormalities in astrocyte development have been implicated in neurological disorders including epilepsy, neurodegenerative disorders and brain tumors (Lobsiger and Cleveland, 2007; Oberheim et al., 2008). On the other hand, oligodendrocytes are essential for myelination (Hirano, 1968; Emery, 2010), and dysregulated myelination causes multiple sclerosis and leukodystrophy (Emery, 2010). It is of critical importance to elucidate the molecular mechanisms regulating astrocyte and oligodendrocyte specification and development in the brain.

The onset of astrocyte specification begins towards the end of neurogenesis (Okano and Temple, 2009b). In the CNS astrogenesis is regulated by several ligand-receptor complexes (Freeman, 2010). Widely studied cytokines among them include ciliary neurotrophic factor (CNTF), leukemia inhibitory factor (LIF), and cardiotrophin-1 (CT-1), which activate JAK/STAT pathway to promote astrocyte differentiation (Johe et al., 1996; Bonni et al., 1997; Rajan and McKay, 1998; Barnabe-Heider et al., 2005). Activation of BMP2/4 signaling, which results in phosphorylation of SMAD transcription factors, also regulates the expression of astrocyte-specific genes through interactions with STAT3 (Nakashima et al., 1999). However, perturbation of JAK-STAT signaling caused only a 45-70% reduction in astrocytes *in vivo*, suggesting contributions from other pathways. Notch is another widely studied pathway with gain-of-function studies

showing that Notch receptors play a permissive role in NPCs promoting astrocyte differentiation (Morrison et al., 2000; Tanigaki et al., 2001; Gaiano and Fishell, 2002).

Transcriptional control of astrocyte specification has been studied mainly in the spinal cord. Ablation of Sox9 causes deficits in both astrocyte and oligodendrocyte generation (Stolt et al., 2003), whereas nuclear factor I-A/B (NFIA/NFIB) and, bHLH transcription factors, stem cell leukemia (SCL) and KLF15 were found to be necessary and/or sufficient for astrocyte specification (Muroyama et al., 2005; Deneen et al., 2006; Fu et al., 2009).

Oligodendrocyte specification occurs at late embryonic and early postnatal periods (Miller, 1996; Lee et al., 2000). Extracellular sonic hedgehog (Shh) signal in ventral telencephalon is necessary and sufficient for inducing the commitment to oligodendrocyte precursors (Alberta et al., 2001; Tekki-Kessaris et al., 2001). Downstream to Shh signaling, two bHLH transcription factors, Olig1 and Olig2, are particularly important for oligodendrocyte development (Lu et al., 2001; Zhou et al., 2001; Lu et al., 2002; Zhou and Anderson, 2002). Besides, members of SRY-related HMG box family (Sox) family of transcription factors, namely Sox9, Sox10, and Sox17, have were found pivotal for oligodendrocyte specification (Stolt et al., 2002; Stolt et al., 2003; Sohn et al., 2006). Ying Yang 1 (YY1) was one of the few transcription factors discovered within the brain to be crucial for oligodendrocyte differentiation (He et al., 2007).

Despite these advances in mapping the molecular machineries for astrocyte and oligodendrocyte specification, the identities of regulatory transcription factors important for glia development in the brain is far from complete. SRF is a stimulus-dependent transcription factor required for differentiation and development of several different cell types including keratinocytes, cardiac and smooth muscle cells (Miano et al., 2004; Parlakian et al., 2004; Niu et al., 2005; Verdoni et al., 2010). Within the CNS, SRF regulates axon growth, tangential neuronal migration, activity-dependent gene expression, synaptic plasticity and learning and memory (Ramanan et al., 2005; Etkin et al., 2006; Knoll et al., 2006; Stern et al., 2009; Johnson et al., 2011; Lu and Ramanan, 2011a). Here, we report a previously unidentified role for SRF in astrocyte and oligodendrocyte differentiation in the brain.

MATERIALS AND METHODS

Animals. *Srf* $^{f/f}$ mice (control) were maintained as a homozygous colony as described (Ramanan et al., 2005). The *Srf*-Nestin-cKO and *Srf*-NEX-cKO were generated as described (Lu and Ramanan, 2011a) using a Nestin-Cre transgenic mouse line (Tronche et al., 1999) and a NEX-Cre line (Goebbels et al., 2006). The *Srf* $^{f/+}$;Nescre double heterozygous mice did not exhibit any discernible phenotype. Pups of either sex (n=3-5) from at least two different litters were used in all experiments. All experiments were approved by the Animals Studies Committee, Division of Comparative Medicine, Washington University School of Medicine, St. Louis, MO.

Immunostaining, cell counts and statistical analyses. Immunohistochemistry was performed as described (Ramanan et al., 2005). Primary antibodies used were: Aldh1L1 (1:100, NeuroMab), S100β (1:1000, Sigma-Aldrich), GFAP/G-145 (1:1000, Sigma-Aldrich), SRF (1:1500, Santa Cruz), Nestin (1:200, DHSB), Tuj1/β-tubulin III (1:1000, Sigma-Aldrich), APC (1:1000, Millipore), β-gal (1:1500, Aves Lab), O4 (1:750, Millipore), Olig2 (1:1000, Sigma-Aldrich), Olig1 (1:50, NeuroMab), activated-Caspase 3 (1:1500, Millipore), and Sox2 (1:100, Santa Cruz). Secondary antibodies used were: anti-goat Cy3 (1:300, Jackson ImmunoResearch), anti-mouse Alexa Fluor (AF) 594 and AF488, and anti-rabbit AF488 and AF 594 (1:500, Invitrogen). Biotinylated anti-rabbit and anti-mouse secondary antibodies (1:500, Vector Labs) were used along with ABC-*Elite* or

VIP staining kits (Vector Labs). Cell count and statistical analyses were performed as previously described (Lu and Ramanan, 2011).

Western blotting. 25 µg of total protein prepared from neurospheres was immunoblotted by standard procedures. Primary antibodies were anti-SRF, 1:5000 (Santa Cruz), anti-tubulin, 1:10,000 (Sigma-Aldrich). Secondary antibodies include anti-HRP (1:20,000; anti-mouse, Jackson ImmunoResearch and anti-rabbit, Invitrogen). Chemiluminescence detection was done using Immobilon reagent (Millipore).

Neurosphere culture. Neurosphere cultures and culture media were prepared as described (Dasgupta and Gutmann, 2005). Neonatal cortices were dissected and trypsinized at 37°C for 15 min. Cells were mechanically dissociated by pipetting and incubated in 10% fetal calf serum medium for 10 min and pelleted by centrifugation. Dissociation medium was used to wash the cells before resuspending in growth medium containing 1:4000 dilution of 20 µg/ml FGF and EGF. Samples were at cultured 250,000–500,000 cells per 60-mm dish at 37°C with 5% CO_2 for 4 days.

NPC proliferation analysis. *Srf* $^{f/f}$, *Srf* $^{f/+}$;NesCre, and *Srf* $^{f/f}$;NesCre mice-derived neurospheres were dissociated by trypsin digestion and seeded singly in 24-well plate with fresh growth medium. Every 12hr, neurospheres from triplicate wells

were collected, dissociated, and cell counts determined. Cell growth was monitored over 84 hours, and results plotted with sample mean and SEM. The experiment was repeated thrice using NPCs generated from animals from different litters.

Secondary neurosphere analysis. NPCs from control and *Srf*-Nestin-cKO brains were grown as neurospheres. After 4 DIV, neurospheres were dissociated and seeded as single cells. About 50-70 single NPCs from each background were plated in 96-well plate and grown in fresh growth medium. The number of new neurospheres was monitored every 24 hr and over 5 days to measure the percentage of cells that gave rise to a secondary neurosphere. The experiment was repeated twice using animals from different litters.

NPC differentiation. Neurospheres were trypsinized and washed with dissociation medium and were plated at 150,000 cells/well in 24-well plates in differentiation medium (growth medium without FGF and EGF). To enrich for astrocytes, the following pro-astrocytic growth factors and cytokines were supplemented either individually or collectively: CNTF, 100 ng/ml; LIF, 40 ng/ml; CT-1, 50 ng/ml; BMP-2, 30 ng/ml; IL6, 20 ng/ml; sIL6R, 25 ng/ml; and JAG-1, 500 ng/ml. Cells were fixed using 4% sucrose in 4% paraformaldehyde (PFA) in phosphate buffered saline and immunostained 4 days after induction. To enrich for oligodendrocytes, PDGF, 10 ng/ml, and trtriiodothyronine (T3), 30 ng/ml,

were added to trypsinized NPCs. The percentage of OPCs generated was analyzed at 2 days post-induction and the percentage of differentiation oligodendrocytes derived was analyzed at 4 days post-induction.

Generation of MSCV virus. The retroviral murine stem cell virus vector encoding GFP (MSCV-GFP) was obtained as gift from Dr. David Gutmann Lab at Washington University School of Medicine (Dasgupta and Gutmann, 2005). We cloned wildtype SRF (SRFwt) and a constitutively active variant of SRF (SRF-VP16) into MSCV-GFP separately. Viruses were produced by using Fugene HD (Roche) to introduce the MSCV constructs and the complementary T-helper (gift of Dr. David Gutmann Lab) into 293T cells and collecting filtered viral medium at 48 hours and 72 hours. Infections were accomplished by providing 80% viral supernatant and 20% fresh NPC growth medium to NPCs for 2 days and then switching to 100% NPC growth medium for another 3 – 4 days before trypsinizing the neurospheres for differentiation induction. The empty MSCV-GFP construct was used as a control for analyzing the effects of MSCV-SRFwt and/or MSCV-SRF-VP16 in rescue experiments.

RESULTS

SRF ablation results in reduced astrocyte numbers *in vivo*

SRF has been shown to regulate cell-fate specification in a cell-autonomous manner in several organ systems in mice (Miano et al., 2004; Parlakian et al., 2004; Sandbo et al., 2009; Verdoni et al., 2010) but whether SRF has similar roles in the nervous system has remained unexplored. To address this, we conditionally deleted SRF within NPCs using a Nestin-cre transgenic mouse line (*Srf*-Nestin-cKO) (Lu and Ramanan, 2011). The *Srf*-Nestin-cKO mice died neonatally and did not exhibit any defects in cell survival, neurogenesis or neuronal subtype specification (Lu and Ramanan, 2011). Since neurogenesis was unaffected in *Srf*-Nestin-cKO mice, we examined astrocytes at P0.5 by immunostaining for the astrocyte marker, GFAP. We found that *Srf*-Nestin-cKO mice exhibited nearly 60% reduction in astrocytes in multiple brain regions including neocortex, hippocampus, corpus callosum, and thalamus (Fig 1*A* and data not shown). We next assessed SRF deletion in astrocytes. Co-immunostaining for GFAP and SRF revealed that SRF is robustly expressed in astrocytes of control mice but not in *Srf*-Nestin-cKO mice (Fig. 1*B*). We also confirmed that reduced astrocytes in *Srf*-Nestin-cKO mice were not due to diminished GFAP expression. Immunostaining brain sections from *Srf*-Nestin-cKO and control mice using two other astrocyte markers, Aldh1L1 (Cahoy et al., 2008) and S100β, showed that control mice had significantly more astrocytes in

several brain regions including neocortex and hippocampus, compared to *Srf*-Nestin-cKO mice (Fig 1*C,D,E,F*; n=5 mice).

Our recent findings revealed that *Srf*-Nestin-cKO mice did not exhibit any deficits in neurogenesis or increased apoptosis during development (Lu and Ramanan, 2011). However, there was an increase in proliferative precursor cells in the subventricular zone in *Srf*-Nestin-cKO mice (Lu and Ramanan, 2011), which is possibly a result of impairment in glial lineage commitment. Together, these findings suggest that the decrease in astrocytes in neonatal *Srf*-Nestin-cKO mice reflects a critical requirement of SRF for astrocyte differentiation *in vivo*.

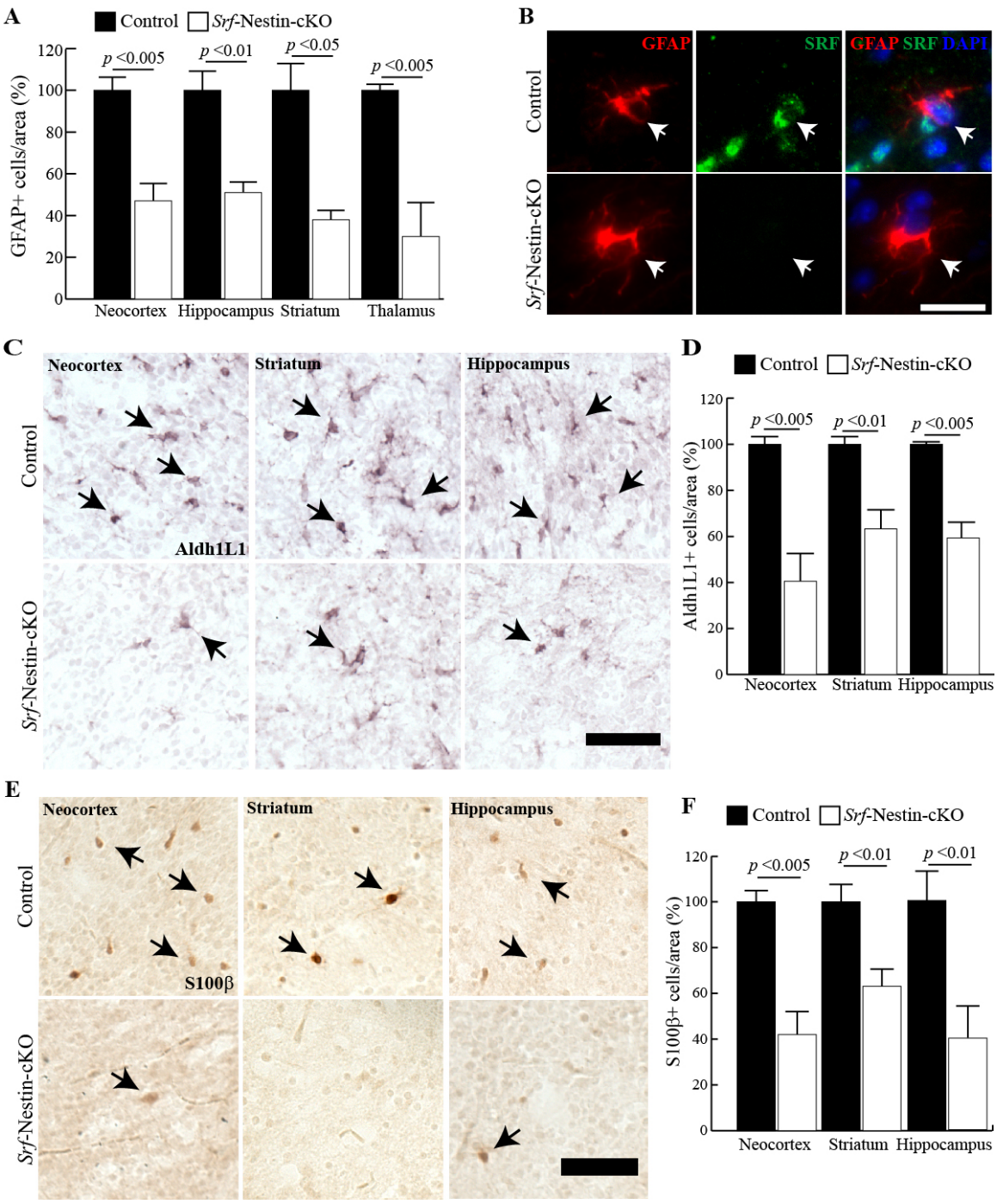

Figure 1. SRF is required for astrocyte specification *in vivo*. **(A)** Astrocytes were immunostained using anti-GFAP antibodies. Cell count analyses from several brain regions including neocortex, hippocampus, striatum and thalamus, indicate

a significant reduction in astrocytes in *Srf*-Nestin-cKO mice compared to control littermates (n=5 mice). **(B)** Co-immunofluorescence staining for SRF and GFAP shows robust SRF staining in astrocytes in control mice but not in *Srf*-Nestin-cKO mice (n=4 mice). Scale bar, 10µm, shown here is hippocampus. **(C)** Aldh1L1 immunostaining of P0.5 brain sections from control and *Srf*-Nestin-cKO mice shows astrocytes in several brain regions. Scale bar, 50 µm. **(D)** Quantification of cell counts from (C) (n=5 mice). **(E)** Immunostaining for S100β+ astrocytes in control and *Srf*-Nestin-cKO mutant mice. Scale bar, 50 µm. **(F)** Quantification of cell counts in (D) (n=5 mice).

SRF-deficiency in neural precursor cells impairs astrocyte specification *in vitro*

To study the role of SRF in astrocyte specification further, we used the neurosphere culture system (Dasgupta and Gutmann, 2005). NPCs cultured from P0.5 cortices of control and *Srf*-Nestin-cKO brains formed neurospheres in 2-3 days in the presence of EGF and FGF as mitogens and were physically indistinguishable from one another (Fig. 2*A*, 3*A* and data not shown). Co-immunostaining for NPC marker, Nestin, and SRF showed that neurospheres from control mice exhibited strong immunoreactivity for both SRF and Nestin. In contrast, neurospheres from *Srf*-Nestin-cKO mice were positive for Nestin but lacked SRF (Fig. 2*A*).

Immunoblotting of total protein isolated from control and SRF-deficient neurospheres further confirmed SRF deletion in NPCs from *Srf*-Nestin-cKO mice (Fig. 2*B*). Likewise, SRF was completely ablated in NPCs isolated from cortices of E12.5 *Srf*-Nestin-cKO mice (data not shown). We next analyzed the effect of SRF deletion on NPC proliferation and found that SRF-deficient and SRF-heterozygous NPCs had rates of proliferation comparable to NPCs from control mice (Fig. 2*C*). We analyzed the capacity of SRF-deficient NPCs to self-renew by assessing their ability to form clonal secondary neurospheres from single NPCs. SRF loss did not affect the capacity of NPCs to form secondary neurospheres (Fig. 2*D*). These findings demonstrate that SRF deletion in NPCs does not affect NPC growth rate and capacity for self-renewal *in vitro*.

NPCs are multipotent and are capable of differentiating into neurons, astrocytes and oligodendrocytes upon mitogen withdrawal (Gritti et al., 1999; Ahmed, 2009). We found that SRF-deficient NPCs were capable of generating neurons, astrocytes and oligodendrocytes as identified by the expression β-Tubulin III in neurons, GFAP in astrocytes, and the co-expression of APC (adenomatous polyposis coli) and O4 in differentiated oligodendrocytes (Fig. 2*E*). Similar to *in vivo* observations, SRF-deficient NPCs generated significantly fewer astrocytes as well as differentiated oligodendrocytes whereas similar number of neurons was generated compared to wild type NPCs (Fig. 2*F*). These results confirm that SRF is dispensable for neurogenesis but is critical for glial specification.

As previous studies elegantly demonstrated that SRF plays a functional role in neurons modulating neighboring oligodendrocyte maturation in a paracrine manner (Stritt et al., 2009), our results could indicate that the reduction in oligodendrocytes generated by SRF-deficient NPC is a consequence of impairment neuronal SRF-mediated oligodendrocyte maturation and/or a cell intrinsic requirement of SRF for oligodendrocyte specification.

Lu and **Ramanan, Figure 2**

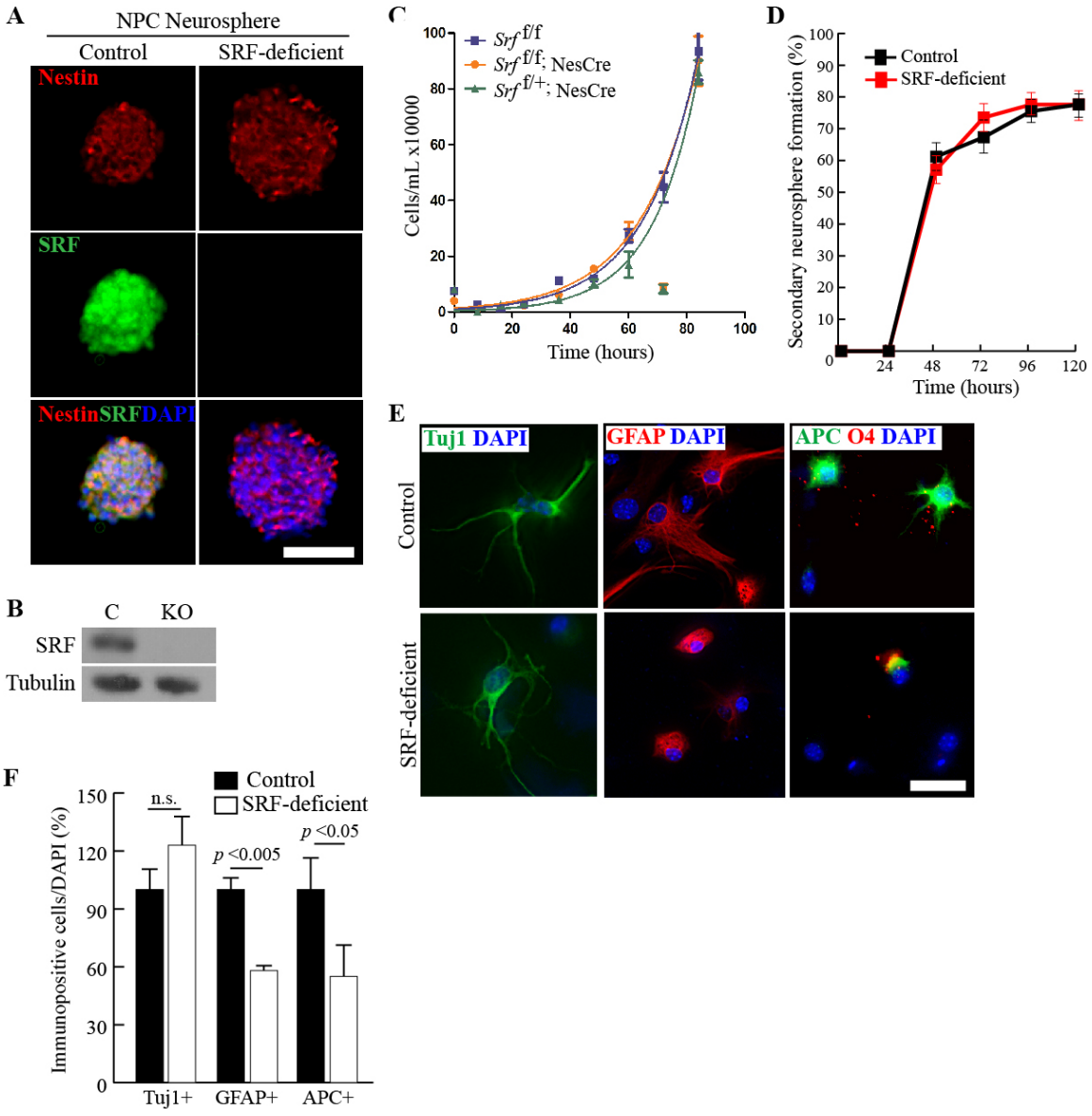

Figure 2. SRF-deficient neural precursor cells exhibit normal proliferation and self-renewal. **(A)** NPCs, identified by Nestin expression, were isolated from forebrains of P0.5 control and *Srf*-Nestin-cKO mice and cultured as neurospheres for 4 DIV. Only neurospheres from control mice, but not *Srf*-Nestin-cKO mice, robustly express SRF. Scale bar, 50 μm. **(B)** Immunoblotting of total protein

from cultured NPCs shows absence of SRF in neurospheres from *Srf*-Nestin-cKO (KO) brains but not control brains (C). **(C)** NPCs from P0.5 control, *Srf*-Nestin heterozygous and *Srf*-Nestin-cKO mice were grown as neurospheres, which were collected at the indicated times and cell count determined. SRF-deficient NPCs exhibit comparable growth rates to that of control and SRF heterozygous cells (n = 3). **(D)** NPCs growing as neurospheres were trypsinized and the percentage of single cell NPC that formed a clonal neurosphere was determined at the indicated time points. The ability of single SRF-deficient NPCs to generate secondary neurospheres was comparable to that of control NPCs (n = 3). **(E)** NPCs from control and *Srf*-Nestin-cKO mice are multipotent and could differentiate into neurons (Tuj1+), astrocytes (GFAP+) and mature oligodendrocytes (APC and O4+) upon mitogen withdrawal. Astrocytes and oligodendrocytes derived from SRF-deficient NPCs are less elaborate than those from control NPCs. Scale bar, 10 μm. **(F)** Cell count analyses from (E) show a significant decrease in astrocytes and oligodendrocytes, but not neurons, derived from SRF-deficient NPCs 4 days post-induction.

SRF-deficient NPCs fail to respond to pro-astrocytic stimuli

We tested the ability of SRF-deficient NPCs to generate astrocytes in the presence of known pro-astrocytic ligands. Following mitogen withdrawal and in the presence of CNTF and LIF, wild type NPCs differentiated predominantly into astrocytes at 4 DIV with characteristic stellar morphology along with less than

0.5% neurons and oligodendrocytes (Fig. 3*A, A', A"*, and data not shown). In contrast, SRF-deficient NPCs generated significantly fewer astrocytes compared to wild type NPCs and a large proportion of cells remained spherical, precursor-cell-like (Fig. 3*A', A"*). Furthermore, SRF-deficient astrocytes, the minor population of cells that does initiate GFAP expression, did not develop elaborate astrocytic process and the stellate morphology that were exhibited by wild type astrocytes.

We also tested the ability of other cytokines and pro-astrocytic ligands, including cardiotrophin-1 (CT-1), interleukin-6 (IL-6), bone morphogenetic protein (BMP-2) 2, and Jagged-1 (Jag-1) (Kahn and De Vellis, 1994; Marz et al., 1999; Nakashima et al., 2001; Ochiai et al., 2001; Takizawa et al., 2001a; Yanagisawa et al., 2001; Grandbarbe et al., 2003; Barnabe-Heider et al., 2005; Namihira et al., 2009). When cultured in the presence of one of above ligands, SRF-deficient NPCs generated consistently and significantly fewer astrocytes compared to control NPCs (Fig. 3*B,C*), suggesting that SRF is broadly required for astrocyte specification, rather than being necessary for mediating a particular pro-astrocyte signaling transduction. We next asked whether these cytokines and growth factors added together were capable of overcoming the impairment in astrocyte differentiation exhibited by SRF-deficient NPCs. Immunostaining for anti-GFAP and anti-S100β at 4-days post-induction showed that SRF loss attenuated astrocyte specification even in the presence of multiple pro-astrocytic stimuli (Fig. 3*D,E*).

Since deletion of SRF in NPCs does not lead to an increase or impairment in neurogenesis but rather a decrease in astrocytes and oligodendrocytes, we sought to determine the fate of NPCs remaining in culture. Immunostaining for Sox2, a transcription factor expressed specifically by NPCs, showed that compared to 63% of control cells, 76% of SRF-deficient cells were Sox2+ at 4 days post-induction (Fig. 3*D,E*). This result suggests that in the absence of SRF, NPCs are less able of responding to pro-astrocytic signals and consequently, more of them remain in the precursor-cell state. Furthermore, we did not observe statistically significant increases in the number of precursor cells in these cultures following astrocyte induction (data not shown), a result that suggests that the NPCs are not actively proliferating in the absence of mitogens and hence the percentage of GFAP+ cells is not skewed lower because of an increase in the pool of proliferating SRF-deficient precursor cells. This increase in Sox2+ cells in SRF-deficient cultures was similar to that observed in the brains of P0.5 *Srf*-Nestin-cKO mice, which exhibited an increase in p-histone-H3+, Ki67+, and Sox2+ cells along the subventricular zone and in neocortex (Lu and Ramanan, 2011a).

Lu and **Ramanan, Figure 3**

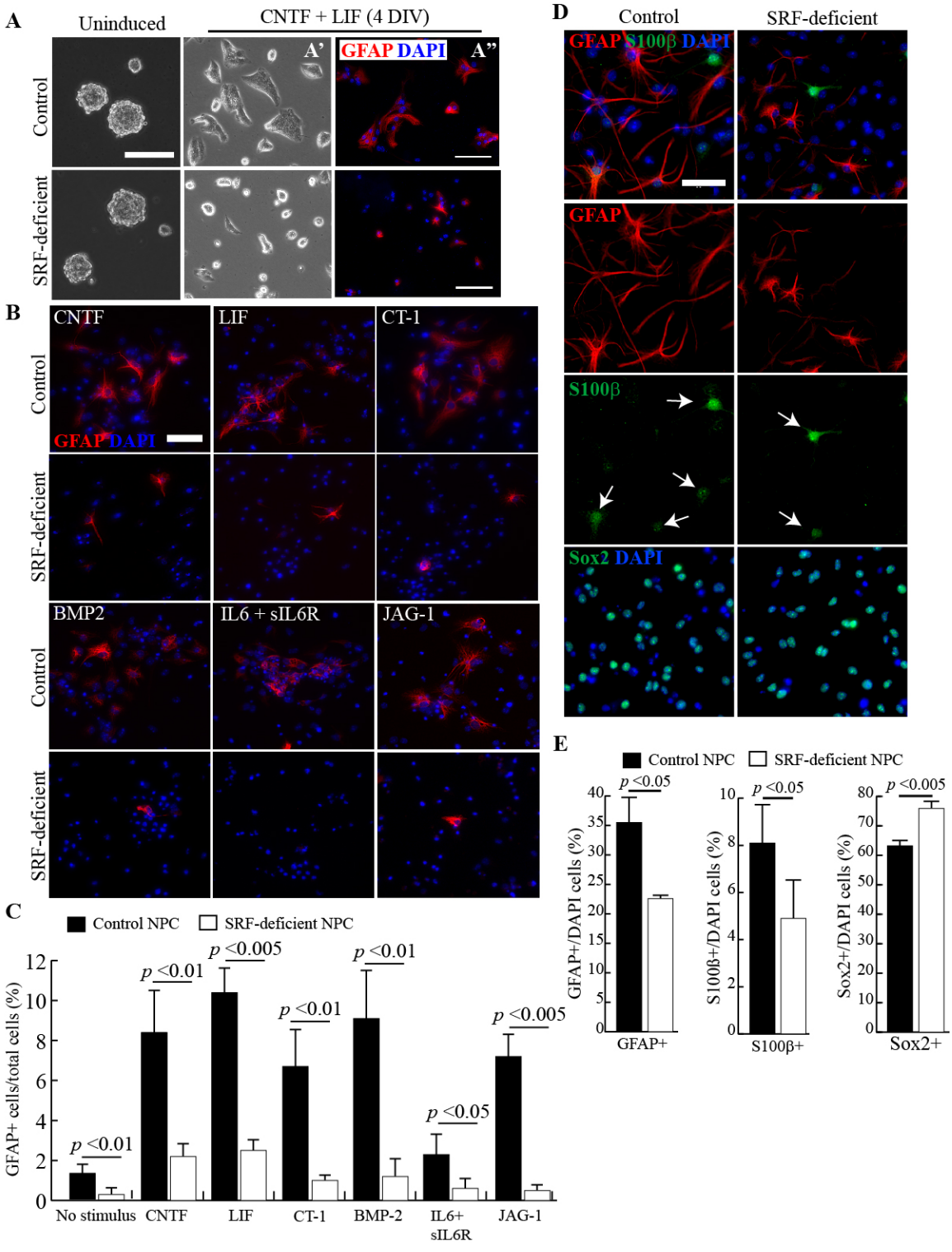

Figure 3. SRF-deficient NPCs fail to differentiate in response to pro-astrocyte stimuli. **(A)** NPCs from control and *Srf*-Nestin-cKO mice were cultured as neurospheres for 4 DIV. SRF-deficient neurospheres do not appear morphologically different to control neurospheres. The neurospheres were trypsinized and induced for astrocyte differentiation with CNTF (100ng/ml) and LIF (40ng/ml) (A', A"). SRF-deficient NPCs generated fewer astrocytes compared to wild type cells as visualized by GFAP immunostaining (A"). The SRF-deficient astrocytes also appeared smaller and less stellar (A'). A' and A" represent independent experiments. Scale bars indicate 100 μm in A and 50 μm in A' and A". **(B)** NPCs from control and *Srf*-Nestin-cKO mice were induced into astrocytes by several pro-astrocytic ligands. At 4 days post-induction, SRF-deficient NPCs generated significantly fewer astrocytes compared to NPCs from control littermates as seen by GFAP immunolabeling. Scale bar, 25 μm. **(C)** Quantification of GFAP+ astrocytes in (B) shows that SRF is broadly required for astrocyte specification (n=3 experiments). **(D)** Control and SRF-deficient NPCs were cultured in the presence of all ligands shown in (B) and cells were immunostained at 4 DIV for astrocytes (GFAP and S100β) and NPCs (Sox2). Arrows point to S100β+ cells. Scale bar, 25 μm. **(E)** Cell count analyses for (D) show that loss of SRF impairs commitment to astrocytes even in the presence of several pro-astrocyte signals and that more SRF-deficient NPCs remain as Sox2+ precursors in culture.

SRF is required cell-autonomously for astrocyte differentiation *in vivo*

Recent observations have shown that newborn neurons and committed NPCs promote astrocyte specification by secreting Notch ligands, including Jag-1, activating Notch signaling in neighboring uncommitted NPCs (Namihira et al., 2009). Since, SRF is also deleted in neurons in *Srf*-Nestin-cKO mice, the deficits in astrocyte specification could be a result of the influence of SRF-deficient neurons. To address whether SRF-deficient neurons contributes to the astrocyte specification impairment observed in *Srf*-Nestin-cKO brains, and hence understand whether SRF regulates astrocyte differentiation cell-autonomously or in a paracrine way, we analyzed astrocytes in *Srf*-NEX-cKO mice, in which SRF deletion was restricted to glutamatergic neurons of the neocortex and hippocampus starting around E11.5 (Lu and Ramanan, 2011a). Immunostaining of P0.5 brains from *Srf*-NEX-cKO mice and control littermates using anti-GFAP and anti-Aldh1L1 antibodies showed no differences in the number of astrocytes localized in *Srf*-NEX-cKO mice neocortex and hippocampus compared to control littermates (Fig. 4*A,B* and data not shown). Unlike the Srf-Nestin-cKO mutants, the NEX-Cre driven SRF mutants do not experience neonatal lethality; similarly, no deficits in astrocytic numbers was also observed in the brains of 3-month old *Srf*-NEX-cKO mice compared to control littermates (Fig. 4*C,C'*).

In sum, these observations indicate that SRF plays a cell-autonomous role within NPCs to promote astrocyte specification both *in vitro* and *in vivo*, and that SRF deletion in neurons does not have any discernible effect on astrocyte specification and maintenance.

Lu and **Ramanan, Figure 4**

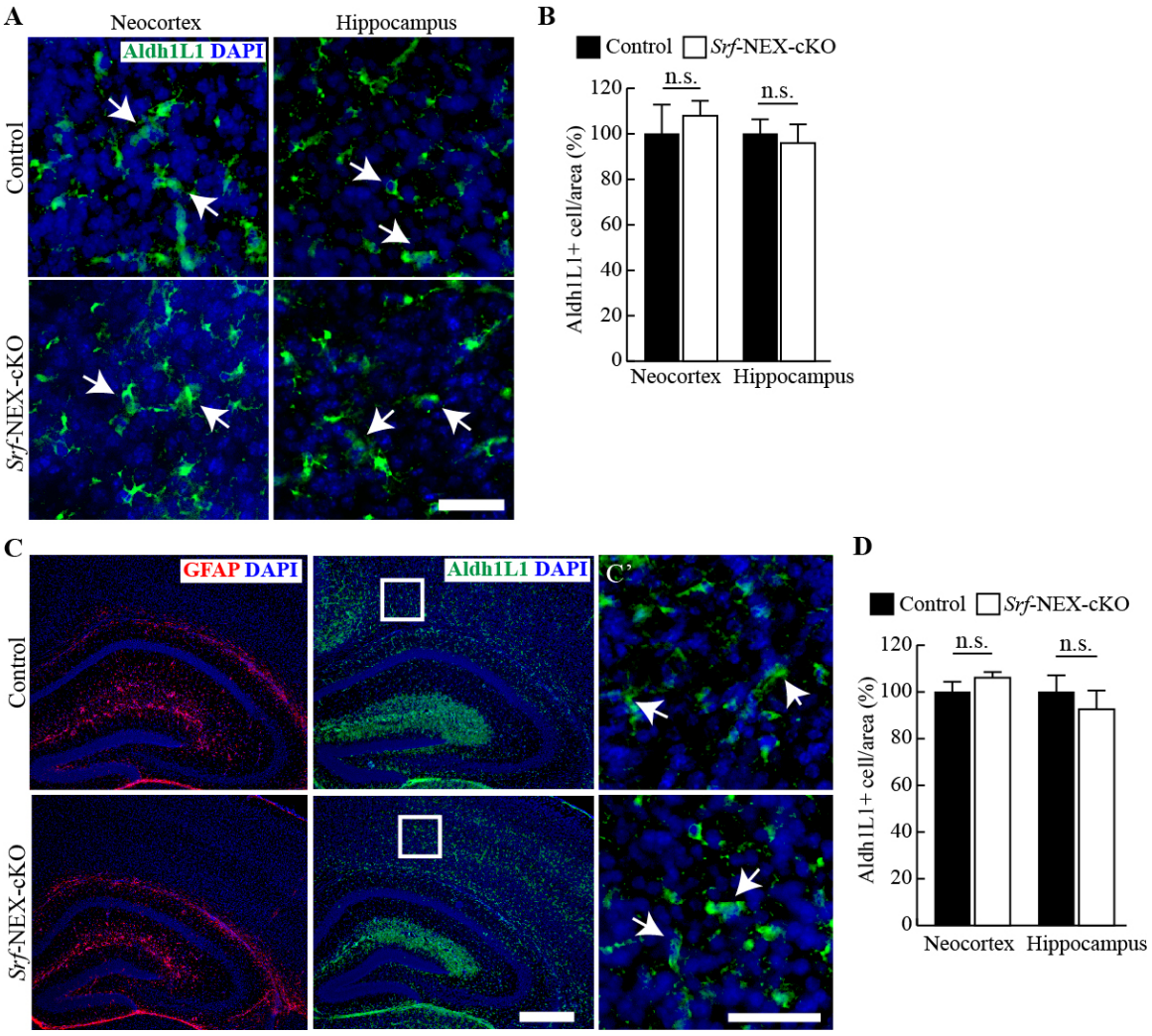

Figure 4. SRF deletion in neurons does not affect astrocyte differentiation. (**A)** Brain sections from P0.5 control and *Srf*-NEX-cKO mice were immunostained using anti-Aldh1L1 antibody to label all astrocytes (arrows). Neocortex and hippocampus are shown. Scale bar, 20 µm. **(B)** Quantification of Aldh1L1+ astrocytes from (A) shows no significant difference in astrocyte populations in *Srf*-NEX-cKO mice compared to control littermates (n=3 mice). **(C)** Immunofluorescence staining for GFAP and Aldh1L1 labeling astrocytes in 3-month old control and *Srf*-NEX-cKO mice. No GFAP+ astrocytes were seen in neocortex since GFAP expression in adult brain is restricted to astrocytes in the white matter and hippocampus but not in the neocortex. In contrast, Aldh1L1 labels all astrocytes including those in neocortex of both groups of mice. Scale bar, 200 µm. ***C'*** represents magnified view of the boxed region. Scale bar, 20 µm. **(D)** Quantification of Aldh1L1+ astrocytes in (C) shows no significant difference in the neocortex and the hippocampus between *Srf*-NEX-cKO and control littermate adult mice (n=3 mice).

Srf-Nestin-cKO mutants also exhibit a reduction in oligodendrocyte precursors

Although it has been shown that SRF modulates oligodendrocyte maturation via the secretion of connective tissue growth factor (CTGF) from neurons (Stritt et al., 2009), and we too observed a decrease in the number of differentiated oligodendrocytes *in vitro*, from neurosphere assays upon mitogens withdrawal.

However, what remains unknown is whether SRF-dependent transcription regulates oligodendrocyte specification in NPCs. Contrary to the observation that loss of neuronal SRF leads to more oligodendrocyte precursors (OPC) at around 2 weeks of age as maturation halts (Stritt et al., 2009), we observed significantly fewer OPCs, identified by Olig2 and Olig1 immunoreactivity (Lu et al., 2000; Zhou et al., 2000), in multiple regions of P0.5 *Srf*-Nestin-cKO brains compared to wild type littermate control brains (Fig. 5*A,B,C,D*). These findings suggest that in addition to controlling oligodendrocyte maturation, SRF could also be required for oligodendrocyte specification.

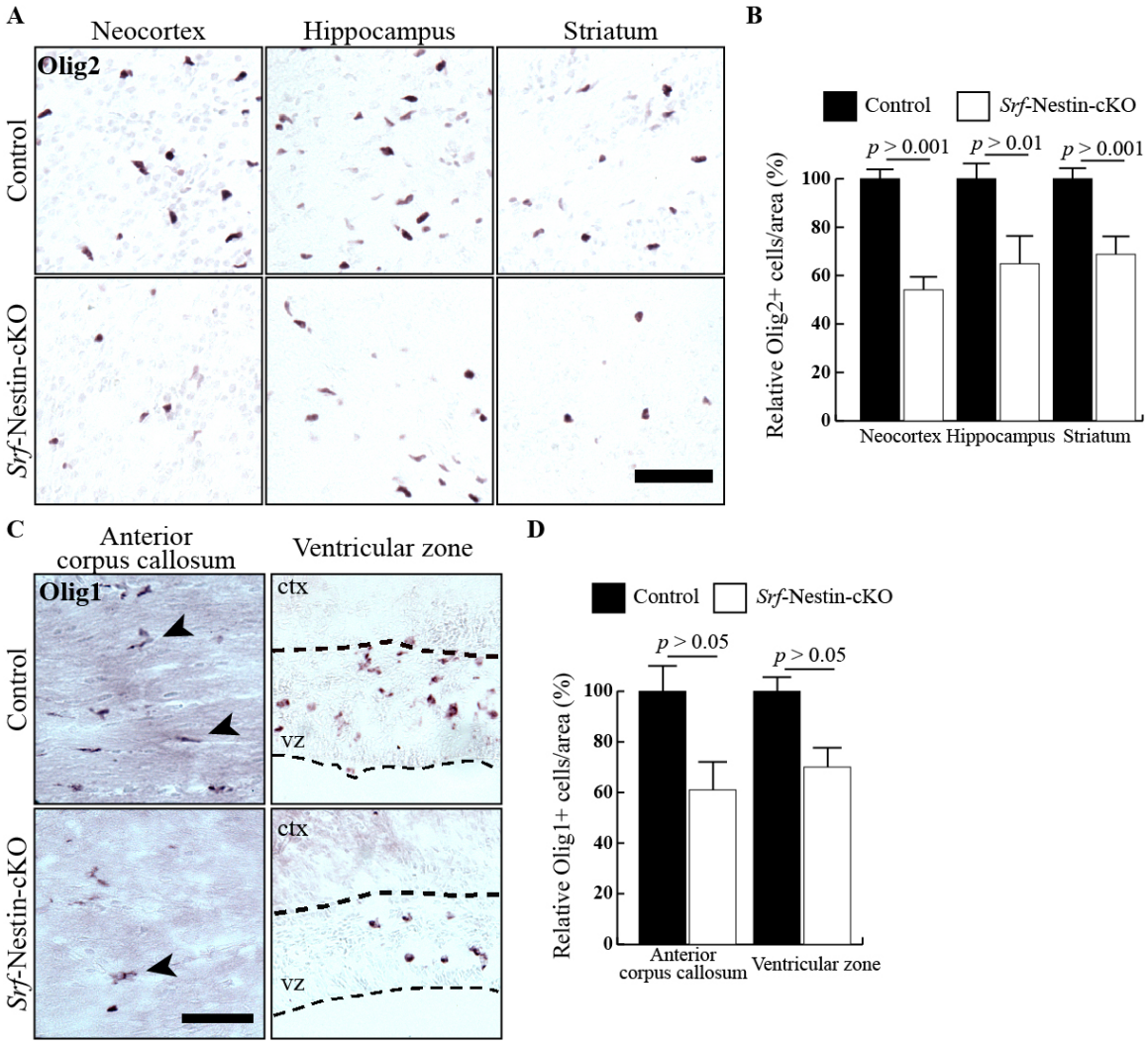

Figure 5. SRF is important for oligodendrocyte specification. **(A)** Oligodendrocyte precursor cells (OPCs) in the brain were identified through immunostaining for Olig2 expression in *Srf*-Nestin-cKO and control mice at P0.5, showing neocortex, hippocampus, and the striatum. Scale bar, 50 µm. **(B)** Quantification of Olig2+ cells per area in (A) shows that the number of Olig2+ OPCs is substantially reduced in *Srf*-Nestin-cKO mutants. **(C)** Olig1+ OPCs were also analyzed at P0.5 in control and *Srf*-Nestin-cKO brains, showing regions of the anterior corpus callosum and ventricular zone. Scale bar, 50 µm. **(D)**

Quantification of Olig1+ cells per area in (C) also shows a significant reduction of OPC population in the mutant brain.

SRF-deficient NPCs show impairment in oligodendrocytes specification

Besides neurons, astrocytes were found to promote OPC survival *in vitro* by the secretion of platelet derived growth factor (PDGF) (Gard et al., 1995). To understand whether the oligodendrocyte lineage commitment deficit observed in *Srf*-Nestin-cKO brains is cell-autonomous or is a result of reduced number of astrocytes and/or other neuronal defects, both occur developmentally prior to oligodendrocyte genesis, we cultured control and SRF-deficient NPCs from neonatal wild type and *Srf*-Nestin-cKO neocortex as neurospheres. PDGF, which promotes OPC proliferation (McKinnon et al., 1990; Robinson and Miller, 1996), and triiodothyronine (T3), which drives oligodendrocyte differentiation (Almazan et al., 1985), were supplemented to induce and enrich for differentiated oligodendrocytes. At 4 days post-induction, we found less than 0.5% of neurons and astrocytes under this pro-oligodendrocyte condition (data not shown), and SRF-deficient NPCs generated significantly fewer differentiated oligodendrocytes, as identified by the cells that co-express APC and O4, than control NPCs did (Fig. 6*C, D*). Moreover, when analyzed at 2 days post-induction, we also observed fewer OPCs generated by SRF-deficient NPCs as indicated by the percentage of PDGFRα+ and Olig2+ cells compared to control NPCs (Fig. 6*A, B*).

These findings recapitulate the *in vivo* observations made from *Srf*-Nestin-cKO brains and demonstrate that, in addition to being functionally crucial for maturation, SRF is necessary for oligodendrocyte specification.

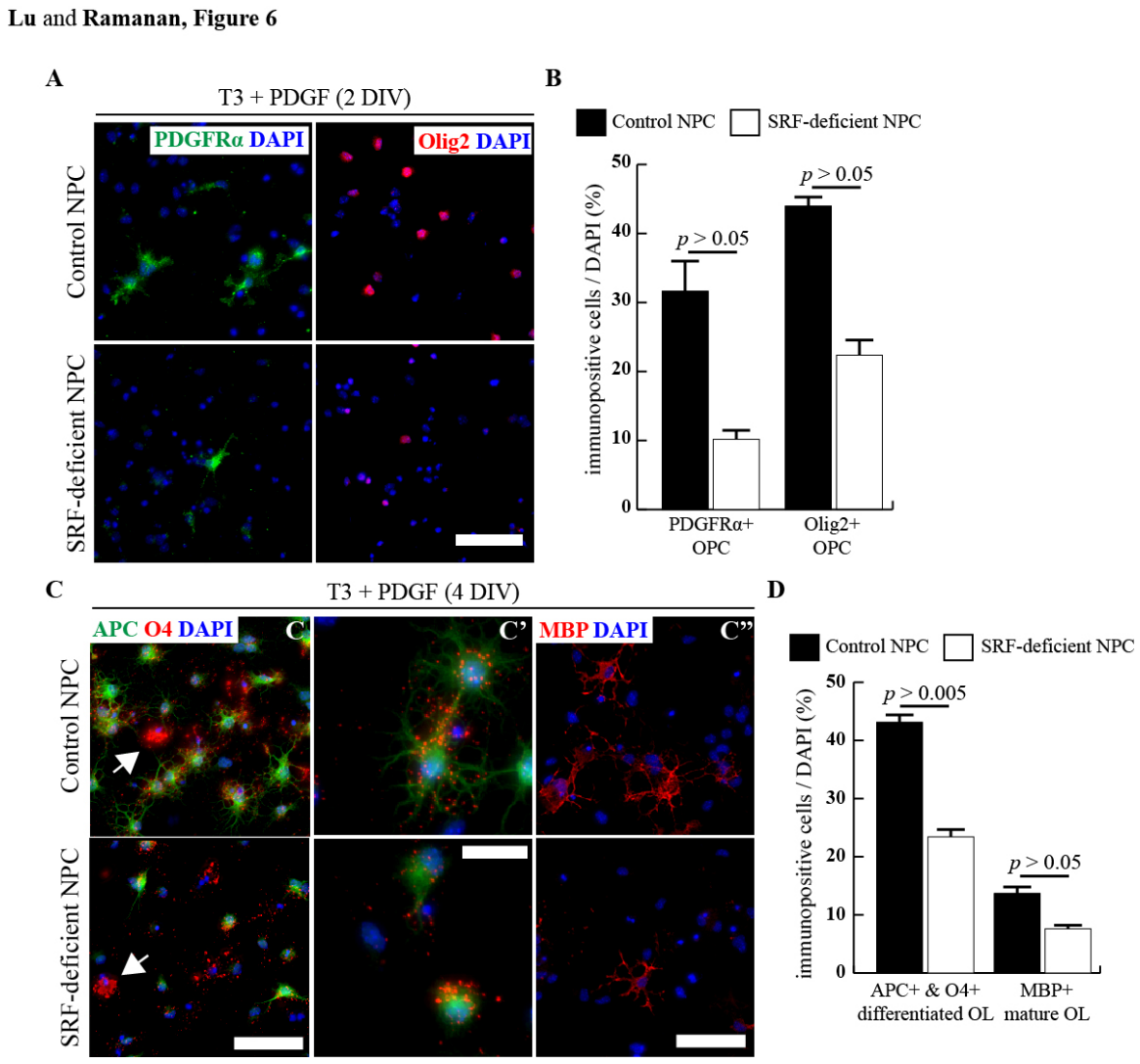

Figure 6. SRF-deficient NPCs show impairment in oligodendrocyte specification. **(A)** Control and SRF-deficient NPCs were induced for oligodendrocyte lineage commitment using T3 (10 ng/ml) and PDGF (2.5 ng/ml). At 2 DIV, OPC were

identified by Olig2 or PDGFRα positive immunoreactivity. Scale bar, 25 μm. **(B)** Quantification of PDGFRα+ or Olig2+ cells show that SRF-deficient NPCs exhibit a reduced capacity for giving rise to OPCs. **(C)** At 4 DIV, differentiated oligodendrocytes were identified from APC and O4 co-expression; magnified images of co-localization of APC and O4 staining are shown in (C'). Arrows point to only O4+ positive late-OPCs. Myelinating mature oligodendrocytes were identified from MBP expression (C"). Scale bar, 25 μm in (C) and (C") and 10 μm in (C'). **(D)** Cell count analysis demonstrates that at 4 DIV the number of differentiated oligodendrocytes is also significantly reduced in SRF-deficient NPC cultures, thus recapitulating the *in vivo* phenotype of *Srf*-Nestin-cKO mutants.

Ectopic expression of SRF rescues both astrocyte and oligodendrocyte specification in SRF-deficient NPCs

We questioned whether the astrocyte and the oligodendrocyte specification defects could be rescued by reintroduction of functional, wildtype SRF or are the cells fundamentally impaired in the absence of SRF. We cloned the SRF gene into a murine stem cell virus vector tagged with GFP (MSCV-SRFwt). By analyzing for GFP expression of infected cultures of control or SRF-deficient NPCs, we find an expression efficiency of 98 – 99% for both MSCV alone and, as a control, for MSCV-SRFwt (data not shown). The addition of MSCV-SRFwt did not appear to influence either control or SRF-deficient NPCs' proliferation rate, and we find that the expression of MSCV-SRFwt in SRF-

deficient NPCs is sufficient to restore the potential for astrocyte and oligodendrocyte differentiation, induced by CNTF and LIF and by PDGF and T3 addition respectively, to the levels comparable to wild type NPCs (Fig. 7*A, B, C, D*).

Lu and **Ramanan, Figure 7**

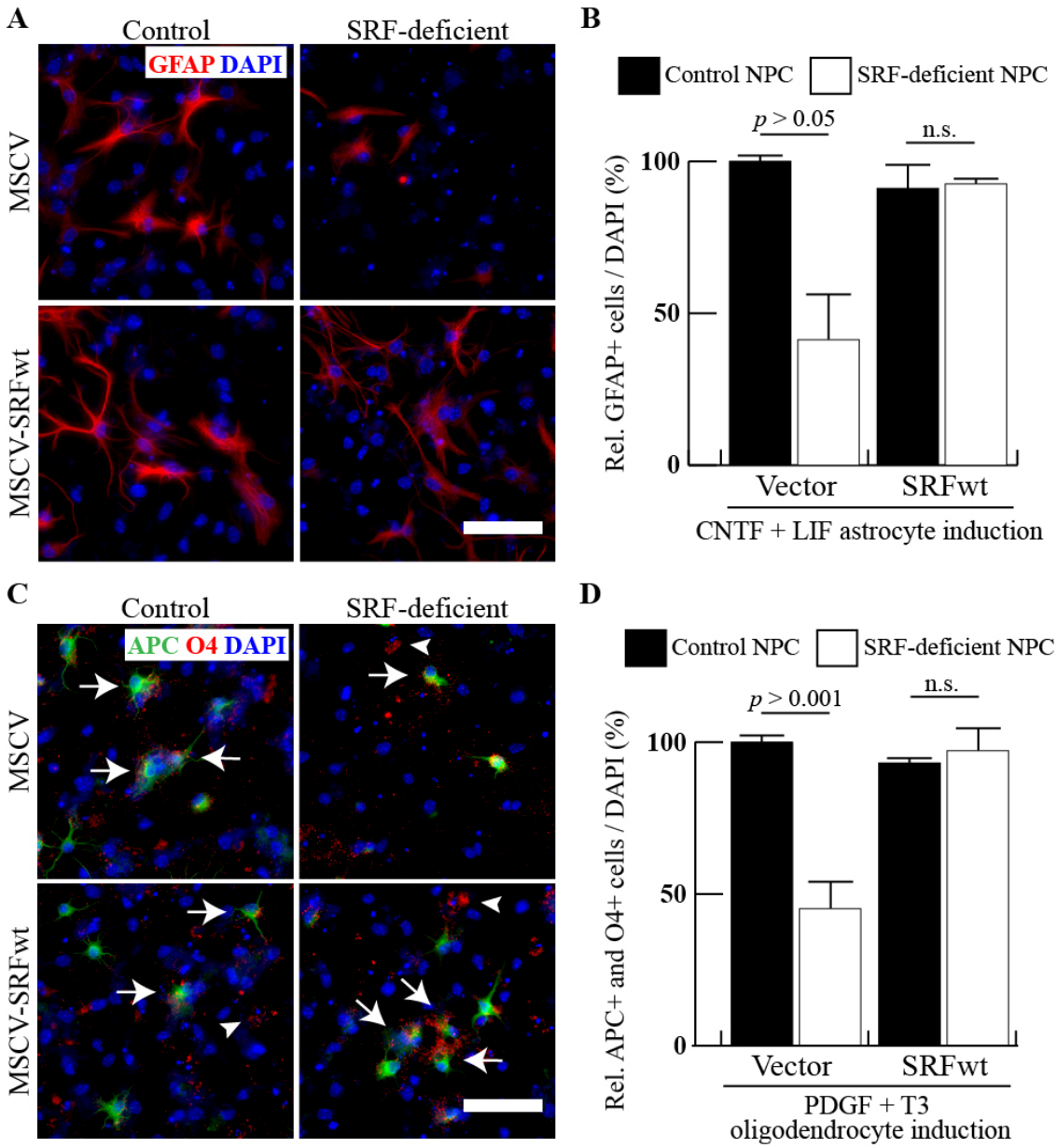

Figure 7. Re-introduction of wildtype SRF rescues glial specification defects. **(A)** Control and SRF-deficient NPCs were harvested from P0.5 brains and were infected with MSCV-SRFwt or with the vector alone as a control. NPCs were induced for astrocyte differentiation using CNTF and LIF and immunostained for GFAP expression at 4 DIV. Scale bar, 25 μm. **(B)** Analysis of the percentage of astrocyte generated shows that the mere presence of MSC-SRFwt does not augment astrocyte specification but restores SRF-deficient NPCs' capacity for astrocyte generation. **(C)** Similarly, control and SRF-deficient NPCs were infected with MSCV-SRFwt and induced for oligodendrocyte lineage commitment using PDGF and T3. Arrows indicate APC+ and O4+ differentiated oligodendrocytes; arrowheads point to O4+ late-OPCs (4 DIV). Scale bar, 25 μm. **(D)** Quantification shows that extra copies of SRFwt do not potentiate oligodendrocyte differentiation in control NPCs; however, MSCV-SRFwt rescues the oligodendrocyte differentiation deficits of SRF-deficient NPCs.

Constitutively active SRF augments the effect of astrocyte induction but is insufficient for inducing glial specification

Our results demonstrate that cell-intrinsic SRF is necessary for the proper cell-fate commitment to astrocyte and oligodendrocyte. We questioned whether activated SRF alone is sufficient to drive NPCs cells to astrocyte and/or oligodendrocyte lineage: we cloned the SRF-VP16 gene – a constitutively active variant of SRF (Johansen and Prywes, 1994; Schratt et al., 2002) – into the MSC vector tagged with GFP (MSCV-SRF-VP16).

We find an infection and expression efficiency of ~ 98% for MSCV-SRF-VP16 in control and SRF-deficient NPCs (data not shown). Control and SRF-deficient NPCs expressing either MSCV or MSCV-SRF-VP16 were permitted to differentiate by replacing culture medium with those without mitogens; at 4 days post mitogen withdrawal we observed that SRF-VP16 not only did not was not sufficient to initiative differentiation but it is also slightly inhibitive for astrocyte differentiation (Fig. 8*A,B*). However, when NPCs were induced for astrocyte differentiation by the addition of CNTF and LIF, we find that SRF-VP16 substantially potentiated astrocyte differentiation in control NPCs and that it drives astrocyte differentiation from SRF-deficient NPCs to a comparable extent (Fig. 8*A,B*). As with oligodendrocyte specification, we also found that SRF-VP16 is not sufficient to induce differentiation in the absence of mitogens; unlike astrocyte specification, we observed that the expression of SRF-VP16 not only does not augment the effects of pro-oligodendrocyte factors T3 and PDGF but inhibits control NPC oligodendrocyte differentiation to levels comparable to SRF-deficient NPCs (Fig. 8*C, D*). Although the inhibitory effects of SRF-VP16 in oligodendrocyte differentiation conditions were counter-intuitive, similar negative effects were also found to be the case for oligodendrocyte maturation (Stritt et al., 2009) and could occur as SRF-VP16 is not subject to the natural regulatory machinery that modulates the activity of wildtype SRF and/or that SRF-VP16 maybe bound to sites/genes that interfered with the initiation of oligodendrocyte-specific set of genes. Given the difference influence SRF-VP16 confers on NPCs

between astrocyte and oligodendrocyte specification, it is likely that SRF acts through distinct pathways to mediate the differentiation signal transduction cascade for the two cell types. In sum, our results discovered SRF as a novel transcriptional regulator necessary but not sufficient for astrocyte and oligodendrocyte differentiation in the brain.

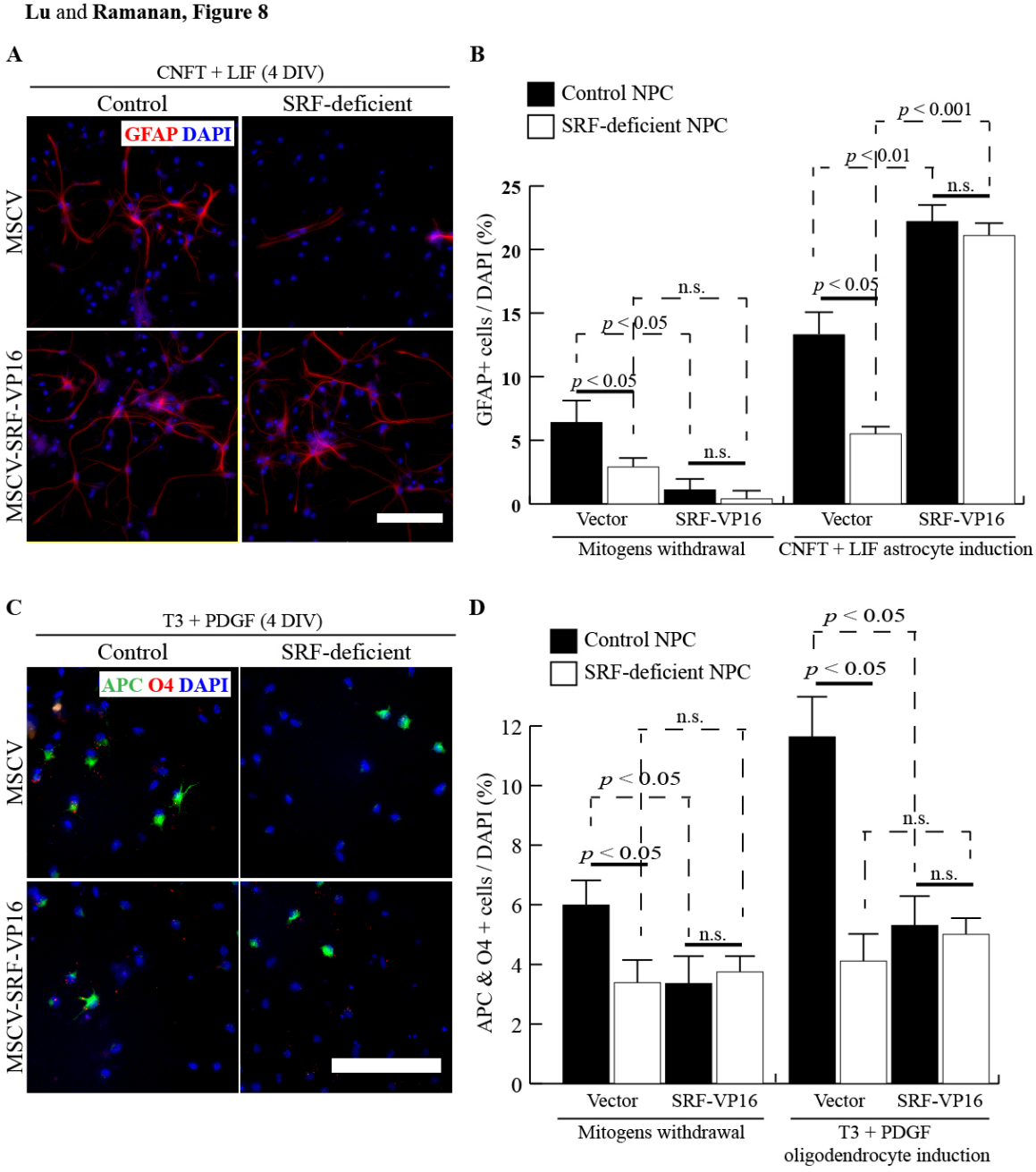

Figure 8. Constitutively active SRF is insufficient for glial specification but augments primed astrocyte differentiation. **(A)** Control and SRF-deficient NPCs were infected with either MSCV vector alone or MSCV-SRF-VP16. NPCs were induced for astrocyte differentiation with CNTF and LIF or had mitogens

withdrawal alone (representative images not shown) permitting differentiative states. Scale bar, 50 μm. **(B)** Quantification of GFAP+ astrocytes shows that by only withdrawing mitogens SRF-VP16 is unable to initiate astrocyte specification; however, when SRF-VP16 is expressed in conjunction with CNTF and LIF supplementation potentiates astrocyte differentiation of both control and SRF-deficient NPCs. **(C)** Control and SRF-deficient NPCs were infected with either MSCV vector alone or MSCV-SRF-VP16. NPCs were permitted for differentiation by mitogens withdrawal (representative images not shown) or were enriched for oligodendrocytes using T3 and PDGF. Differentiated oligodendrocytes were identified by positive immunoreactivity for both APC and O4. Scale bar, 50 μm. **(D)** Quantification for differentiated oligodendrocytes indicates that SRF-VP16 is not sufficient to induce oligodendrocyte specification in the absence of pro-oligodendrocyte factors and that it also does not potentiate oligodendrocyte differentiation in conjunction with T3 and PDGF.

DISCUSSION

Astrocytes is the most abundant cell type in the mammalian brain and perform critical roles in nearly every facet of brain function including synaptogenesis, transmitter homeostasis and synaptic plasticity (Barres, 2008; Allen and Barres, 2009). Oligodendrocytes are functionally indispensable for higher organism nervous system as they are responsible for the generation and maintenance of myelin that facilitates neuronal salutatory transmission as well as

for providing trophic support – for example, insulin growth factor-1 (IGF-1) and glial cell line-derived neurotrophic factor (GDNF) – to promote the survival of neurons and growth of axons (Wilkins et al., 2001; Wilkins et al., 2003). Disruptions to oligodendrocyte development or health contributes to disorders such as multiple sclerosis and leukodystrophies (Baumann and Pham-Dinh, 2001).

In this study, we found that mice with conditional deletion of SRF in NPCs (*Srf*-Nestin-cKO) exhibit deficits in astrocytes and oligodendrocyte specification in the brain. In contrast, SRF deletion in neurons (*Srf*-NEX-cKO) does not affect astrocyte development suggesting a cell-autonomous role for SRF in astrocyte specification. Because earlier studies had already extensively analyzed for the developmental effect of selective ablation of SRF in neurons and found SRF contributes in a paracrine fashion to modulate oligodendrocyte maturation (Stritt et al., 2009), we did not perform oligodendrocyte population studies in our *Srf*-NEX-cKO animals. Cultured SRF-deficient NPCs do not display any defects in proliferation or exhibit increased apoptosis. As observed *in vivo*, SRF-deficient NPCs were unable to properly differentiate into astrocytes and oligodendrocytes in response to pro-astrocytic and pro-oligodendrocyte stimuli, respectively. Thus, our study identifies a previously unknown cell-intrinsic role for SRF in glial cell-fate specification in the brain.

SRF deletion in *Srf*-Nestin-cKO resulted in loss of about 40% to 60% astrocytes and oligodendrocytes *in vivo*. SRF-deficient NPCs also exhibited a similar failure to generate glia when induced to differentiate *in vitro*.

This could reflect a likely delay in glia specification, which is difficult to assess in *Srf*-Nestin-cKO mice since they exhibit neonatal lethality. However, we found that differentiation of SRF-deficient NPCs for longer periods (until 12 days) in culture did not yield more astrocytes or oligodendrocytes suggesting that a delay in specification is unlikely to be the underlying cause of the glial deficits in *Srf*-Nestin-cKO mice (our unpublished observations). Another possibility is that SRF is required for specification of a particular glial precursor cell (GPC) subpopulations or within a particular subpopulation for glial differentiation. To date, the exact nature and identities of GPCs are poorly understood in the brain. Recent studies have shown that astrocytes can be generated from more than one precursor cell type (Liu et al., 2004; Lin and Goldman, 2009; Cai et al., 2011). Future studies will aim to identify these precursor subpopulations in the brains of *Srf*-Nestin-cKO mice and study their capacity to generate astrocyte and oligodendrocytes in the absence of SRF.

Recent observations have shown that SRF-deficient neurons affect maturation of oligodendrocyte precursors in a non-cell autonomous manner (Stritt et al., 2009). In the reported *Srf* mouse mice, widespread SRF loss in forebrain neurons caused deficits in tangential neuronal migration leading to cell accumulation in the subventricular zone (Alberti et al., 2005). This led to massive apoptosis and consequently triggered an increase in astrocytes postnatally due to reactive astrogliosis (Alberti et al., 2005; Stritt et al., 2009).

In contrast, we found that SRF ablation in developing glutamatergic neurons of the neocortex and hippocampus in *Srf*-NEX-cKO mice did not cause cell death or affect astrocyte differentiation even at 3 months of age. However, SRF loss in NPCs alone caused a significant reduction in astrocytes both *in vivo* and *in vitro*. Together, these observations strongly suggest that SRF plays a cell-intrinsic role in regulating astrocyte specification in the brain. Unlike other less than a handful of known gliogenic transcription factors identified from the spinal cord, such as NFIA which is necessary and sufficient (Deneen et al., 2006), we find that SRF is necessary but insufficient for astrocyte and oligodendrocyte specification.

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Chapter 4: Conclusions and future directions

This body of work stems from our interests to better understand the genetic programs that guide various aspects of central nervous system development, particularly with respect to the functional role of SRF – a highly evolutionary conserved, brain enriched, and stimulus-dependent transcription factor. Our research began by generating a conditional mutant of SRF, in which deletion of the gene is driven by the Nestin promoter for NPC restriction (*Srf*-Nestin-cKO), and by analyzing anatomically and histologically for cellular populations, cell identity and architecture abnormalities. Because the *Srf*-Nestin-cKO mutants invariably die with the first day of birth, we know that SRF must play certain important functional roles. Our preliminary results show that SRF is important the cortical neuronal innervations *in vivo* as well as for glia, which include astrocytes and oligodendrocytes, cell-fate specification within the brain.

Several studies have shed light on the crucial requirement of SRF for neurite outgrowth in culture, partly by regulating actin and growth cone dynamics (Ishikawa et al.; Stern et al., 2009), but our anatomical analysis is the first to shed light on the major neuronal projections and tracts that are affected in the absence of SRF (Lu and Ramanan, 2011b). Astrocytes play multiple critical roles in brain development and functioning (Barres, 2008), and abnormalities in astrocyte development have been implicated in neurological disorders including epilepsy, neurodegenerative disorders and brain tumors (Lobsiger and Cleveland, 2007; Oberheim et al., 2008).

On the other hand, oligodendrocytes are essential for myelination (Hirano, 1968; Emery, 2010), and dysregulated myelination causes multiple sclerosis and leukodystrophy (Emery, 2010). Therefore, it is of critical importance to further elucidate the molecular and cellular mechanisms that underlie SRF-dependent transcription required for astrocyte and oligodendrocyte differentiation and development in the brain.

Hints and lessons learned from *Srf*-Nestin-cKO mutants

Although the mutant brains and animals are physically indistinguishable from control littermates, the first striking abnormality that we observe is cerebral hemorrhages frequently displayed on the surface of cerebral cortex and olfactory bulbs. We speculate that this could be the result of two scenarios: 1) compromised blood brain barrier, which consists of structural interactions between astrocytes and endothelial cells, and/or 2) leaky expression of the Cre recombinase in certain endothelial populations. Even though the particular Nestin transgenic line we utilized has been selected to have highly targeted expression of Cre mostly in ectodermal/neural cell lineages (Tronche et al., 1999), but slight leaky expression was reported in certain kidney and lung cells by the vendor (Jackson Labs). Non-targeted Cre-mediated deletion of SRF in a minor population of endothelial cells, although unverified, could have been possible.

Indeed, a study of SRF in endothelial cells found it to be important for angiogenesis and vascular integrity and the lack of SRF can result hemorrhagic phenotypes as well as embryonic lethality by E14.5 (Franco et al., 2008). Despite that we are not fully certain of the causes of cerebral hemorrhages observed from our *Srf*-Nestin-cKO mutants at P0.5, we begun our analysis of the gross structure and integrity of mutant brains coronally and sagittally using Nissl staining. We first noticed that mutant brains displayed enlarged ventricles as well as higher cellular densities at the subventricular zone (SVZ) and subgranular zone (SGZ), which are both regions that host precursor populations and are responsible for neurogenesis and gliogenesis. However these abnormalities alone are inconclusive in suggesting what SRF is required developmentally. Previous studies of SRF using a postnatal neuronal CaMKIIα Cre line also revealed increased cellular densities around the SVZ at 2 – 3 weeks of age in mutants, as a result of impaired tangential migration (Alberti et al., 2005). Using immunohistochemistry staining against NeuN, Aldh1L1, S100β, GFAP, Olig1, and Olig2 expressions (cell marker for neurons, astrocytes, and oligodendrocyte precursors respectively), we were able to get a preliminary analysis of whether SRF contributes to cell-type differentiation and generation. Quantifying for the number of cells per μm^2 of area from microscopy images, we learned that without SRF the number of astrocytes as well as OPCs are reduced on a magnitude of 40 – 60%, a result that would likely undermine the structural integrity of blood brain barrier as fewer astrocytes are available to reinforce

endothelial cells through astroglial end-feet interaction. This loss of glia result was consistent across several regions that we analyzed, which include the neocortex (analyses were restricted to the somatosensory and motor cortex regions), hippocampus, striatum, and the thalamus. A particularly interesting observation is that we did not observe a significant difference in neuronal numbers between mutant and control brains as measured by NeuN, an antibody that labels epitopes of mature neuronal nuclei. This was counter-intuitive as most transcriptional regulators of neurogenesis or gliogenesis were founded to promote one lineage at the expense of another, e.g. Ngn1 which is pro-neuronal and NFIA which is pro-glial.

Possible contributors of reduction in astrocyte and oligodendrocyte numbers

We hypothesized several potential causes of the decrease in glial numbers: 1) loss of SRF elevates cell death, possibly concentrated in glial precursor lineages, 2) absence of SRF impaired proliferation and/or self-renewal properties of a population of NPCs, 3) deletion of SRF disrupted the radial migration and hence the ability of glia to populate the regions we examined, and 4) SRF is important for the differentiation of astrocytes and oligodendrocytes. By employing TUNEL assay and activated-Caspase 3 staining, we analyzed for cell death activities at multiple stages of brain development (E14.5, E16.5, E18.5, and P0.5) in both control and mutants brains.

Although we see occasional TUNEL- or activated-Caspase 3- positive cells in control and mutant brain, a natural phenomenon of brain development, our quantification did not indicate a statistically significant different level of apoptotic activity in the mutant brains compared to wildtype littermates. This is interesting because previous studies in ES cell cultures found that SRF may control Bcl-2 expression, an anti-apoptotic gene, and hence promote survival during mesodermal differentiation (Schratt et al., 2004). Given the discrepancy in our observations, we believe that SRF likely plays different functions within embryonic stem cells and neural stem cells. Our results demonstrate that the deletion of SRF does not contribute to a higher level of cell death and that the reduction in glial cells is not the result of selective apoptotic activity in astrocytes and oligodendrocytes or their precursor lineages.

Next we analyzed for the abundance and integrity of radial glial cells, which are a type of precursor cells that also act as structural scaffolds for radial distribution of astrocytes, oligodendrocytes, as well as neurons (Gasser and Hatten, 1990; Hatten, 1990; Hatten and Mason, 1990; Jacobsen and Miller, 2003). By immunostaining for vimentin, an intermediate filament enriched in radial glia, in both P0.5 and in embryonic brains and using con-focal images to analyze for cyto-architectures, we did not observe a notable difference between control and mutant brain radial glia cells in either abundance or structural integrity.

Furthermore, because we did not see any defects in neuronal cortical layering as identified using Cux1 and Tbr2 immunostaining as well as several layer specific transcription factors *in situ* staining – including Cux2, Lmo4, and Lhx4, we believe that loss of SRF not only does not functionally impair radial glia mediated cell migration. Together, these analyses led us to we believe that the deficits in glial populations in *Srf*-Nestin-cKO brains is not because of a radial migration defect.

By culturing NPCs as neurospheres, we also explored what the effects, if any, of SRF-deficiency are for precursor cell proliferation and self-renewal. We cultured NPCs derived from mutants and from their control littermates at P0.5 and E14.5 telencephalon in the presence of EGF and FGF – mitogens that support NPC proliferation – for several days. Cell samples were trypsinized and quantified at 12 hour intervals for measurements of growth rates of control and SRF-deficient NPCs. We did not see a statistically significant difference between wildtype, heterozygous SRF-deficient, and homozygous SRF-deficient NPC cultures up to 84 hours in culture, suggesting SRF is not required for NPC proliferation. Additionally, we analyzed the ability of single control and SRF-deficient NPCs to generate secondary clonal neurospheres, a measure for stem cell self-renewal, and also found no difference between control and SRF-deficient NPCs. These *in vitro* findings suggest that the removal of SRF does not impede the proliferative and self-renewal properties of NPCs.

If SRF deletion reduced the growth and renewal capacity of NPC/neuroepitheial cells, we would also expect to observe a change in neuronal numbers between control and mutant brains. Given these findings, we do not think an impairment NPCs proliferation and/or self-renewal was the underlying cause for the reduction of astrocyte and oligodendrocyte numbers.

To better study differentiation, we again employed the neurosphere culture system, which enables us to induce and enrich for a particular cell-type. For example, supplying cytokines CNTF and LIF can potently induce astrocyte differentiation from NPCs and providing PDGF and T3 can induce oligodendrocyte precursor cells (OPCs) formation as well as promote oligodendrocyte maturation. Just like our cell death analyses *in vivo*, we do not see higher levels of cell death for SRF-deficient NPCs or differentiating SRF-deficient NPCs by using both activated-Caspase3 immunostaining and propidium iodide staining. On the other hand, at 2 days and 4 days post induction, we found statistically lower numbers of astrocytes and oligodendrocytes were generated from SRF-deficient NPCs, which recapitulated our *in vivo* findings, indicating that the deficits in glial numbers were the result of impaired differentiation in the absence of SRF. The same results were derived when we induced astrocyte or oligodendrocyte differentiation with other different known cytokines or growth factors. These observations suggest that SRF is necessary fundamentally for glial specification rather than mediating a specific signaling pathway.

Uncommitted SRF-deficient NPCs remain in the precursor state

We questioned what is the fate of SRF-deficient NPCs in mutants that were not becoming astrocytes or oligodendrocytes. As we do not see an increase in apoptosis or conversion into neurogenesis, we suspected the majority of SRF deficient NPCs may remain as uncommitted precursor cells. In culture, we tested this hypothesis by staining for Sox2, a marker of NPCs, in both control and SRF-deficient samples after astrocyte induction; we found that at 4–days post induction there were 10 – 15% more Sox2 positive cells (as a percentage of total DAPI cells) in SRF-deficient cultures than control cultures. Moreover, this roughly matched the decrease in the overall percentage of GFAP+ and S100β+ cells in mutant cultures compared to control, suggesting that cells that failed to acquire the astrocyte cell-fate likely just remained in the precursor state even in the presence of pro-astrocyte factors. We suspect the same is likely true under oligodendrocyte induction but have not yet performed an experiment to confirm this. Interestingly, when we followed the number of total cells from both SRF-deficient and control cultures over a period of 4 days, we did not see an significant increase of cell numbers in the mutant culture, indicating that the SRF-deficient NPCs are not actively proliferating under differentiative conditions and that the astrocytic percentage is not skewed by the expansion of NPCs populations in the mutant culture.

In order to understand whether the same findings are true *in vivo*, we analyzed the NPC population at E14.5 and E18.5 in the SVZ using immunostaining against Sox2 and proliferative markers Ki67 and p-Histone H3. Indeed, we also observed a substantial increase in the number of NPCs within the mutant brain, furthering indicating that many of the SRF-deficient NPCs are unable to acquire glial cell-fate and are stuck in the precursor state.

Cellular context of SRF's requirement for astrocyte specification

Having unrevealed the necessity of SRF for astrocyte and oligodendrocyte differentiation, we plan to study more extensively the role of SRF in astrocyte development first. After obtaining a more concrete idea that the failure to properly generate astrocytes in the brain is the cause of reduction in the number of astrocytes observed in *Srf*-Nestin-cKO mutants at birth, we sought to better understand the cellular context of which SRF is necessary for astrocyte differentiation. We speculated that two scenarios could take place: 1) SRF is required **cell-autonomously** such that the loss of SRF within NPCs leads to an impairment in astrocyte specification, and 2) SRF is required in a **paracrine manner** such that ablation of SRF in neurons causes astrocyte differentiation defects. Although the majority of transcription factors have been found to regulate differentiation by coordinating cell-specific gene expression within a cell-type and are hence required cell-autonomously, neurons have been known

to influence the development and differentiation of astrocytes by secreting ligands, such as Notch ligands (Namihira et al., 2009), and that neurogenesis also precedes astrogenesis so that it is possible SRF-deficient neurons could be partly the cause of the glial developmental deficits that were observed in *Srf*-Nestin-cKO mutants. Furthermore, because SRF was reported to control oligodendrocyte maturation in a paracrine manner, by modulating the expression of neuronal CTGF which are secreted to suppressing IGF-1 signaling that promotes maturation (Stritt et al., 2009), it is certainly possible that SRF could also regulate astrocyte development in a similar extrinsic manner through potentially different signaling pathways. Even though in neurosphere cultures we can enrich for particular cell types, we believe the best way to test the cellular requirement of SRF is *in vivo* using cell-type specific conditional ablation of SRF. We expanded our tool set by generating *Srf*-NEX-cKO animals, in which deletion of SRF is initiated only in newborn neurons in the neocortex and hippocampus starting at ~E11.5 driven by the basic-helix-loop-helix NEX pro-neuronal transcription factor promoter. By analyzing for astrocytic populations in the *Srf*-NEX-cKO animals we are able to obtain an accurate gauge of the contribution of neuronal paracrine effect on astrocyte differentiation. Unlike the *Srf*-Nestin-cKO mutants, *Srf*-NEX-cKO animals do not experience neonatal lethality. We examined the astrocytes in these mutants as well as their control littermates at birth and in adults, using multiple astrocyte markers including Aldh1L1, GFAP, and S100β.

Quantifying immunostaining microscopy results, we found no statistically significant differences between control and *Srf*-NEX-cKO astrocyte population in multiple regions including the neocortex and hippocampus. This result enabled us to eliminate any cell-extrinsic contribution of SRF-deficient neurons towards modulation of astrocyte differentiation; we conclude that SRF is necessary for astrocyte specification cell-autonomously.

Mechanisms of how SRF control's astrocyte differentiation

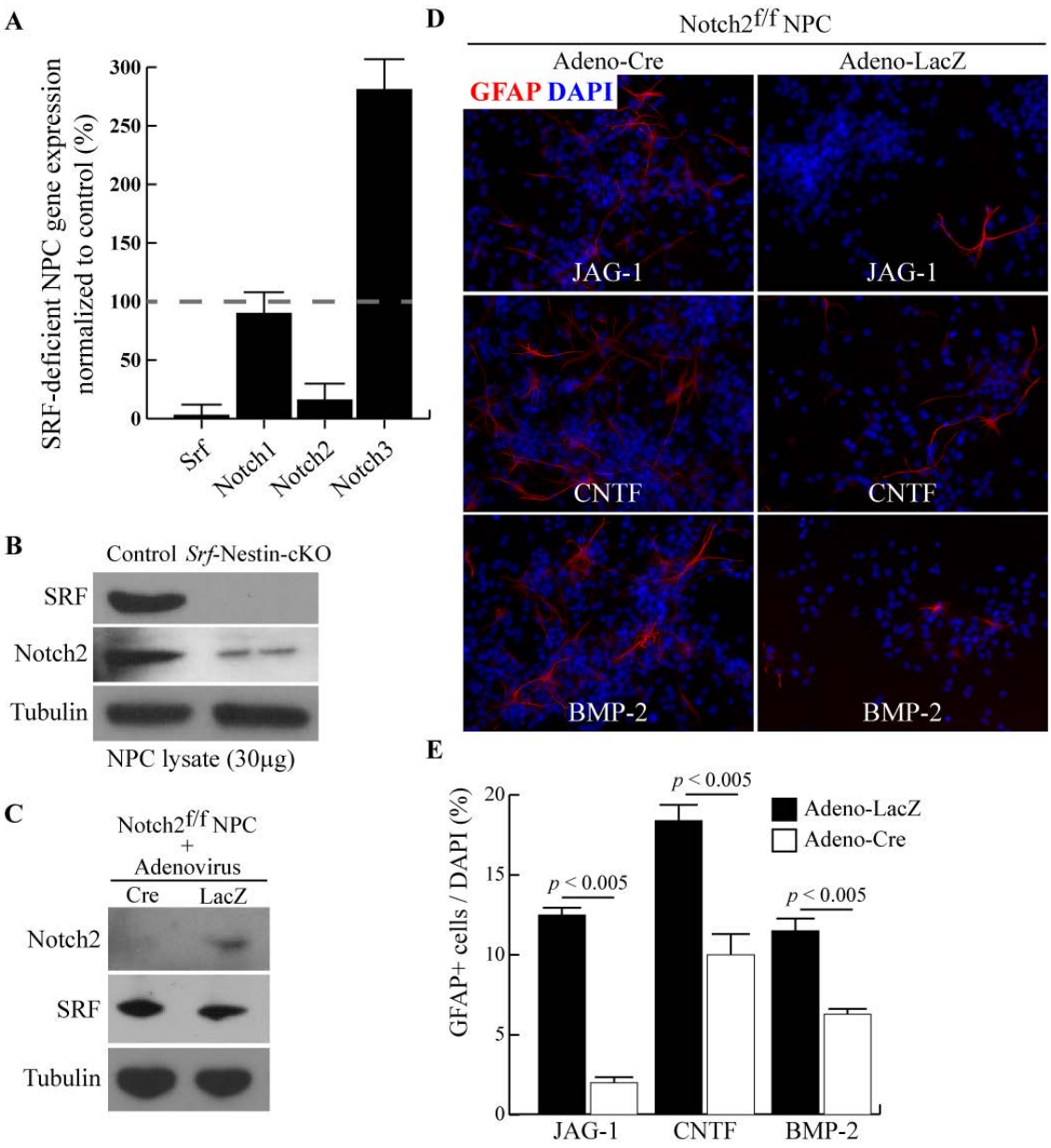
We know that with the aforementioned findings that we have uncovered novel functions of SRF within the brain, but do we know what are the underlying molecular mechanisms that guide these CNS developmental processes? Earlier studies demonstrated that SRF is important for controlling the expression of cytoskeletal genes and immediate early genes (IEGs), but no established findings implicate that SRF is crucial for activating the expression of genes important for astrocyte differentiation. To gain insights on pathways and target genes through which SRF regulates astrocyte differentiation, we conducted microarray analyses comparing differential gene expression between control and *Srf*-Nestin-cKO in hippocampi and neocortex. We observed that several components of the Notch signaling pathway were expressed in lower levels in the mutant brain – including Notch, Hes5, Hes7, Jag1, Jag2, Dll1, and RBP_{jk} – from our arrays.

We validated the microarray results by performing quantitative PCR for these genes on RNAs isolated from control and SRF-deficient NPCs as well as through western analysis of protein expression levels. We hypothesized that SRF is unlikely to be modulating Notch signaling at the ligand level, because the addition of excess Jag1 in culture was unable to rescue differentiation impairment from SRF-deficient NPCs. Using a public bioinformatics tool (Alibaba 2.1 Transcription Binding), we found that only Notch2 – but not Notch1, Notch3, and Notch4 – contains a perfect SRF target CArG domain within the 5kb sequence upstream of the transcription start site (TSS). We did find 1~3 near perfect SRF target CArG domains in the promoter region of Notch1, Notch3, and Notch4 as well as, however it is unclear whether these near perfect sites are functional and actual SRF targets. Studies have shown SRF can transactivate certain genes via binding to CArG domains that mismatches by 1 to 2 nucleotides (Sun et al., 2006); further confirmation can be best achieved through promoter luciferase assays and chromatin-immunoprecipitation (ChIP) experiments. Although, through mostly receptor overexpression and ligands supplementation studies, Notch signaling pathway was found important for astrocyte differentiation (Tanigaki et al., 2001; Morga et al., 2009), but the precise receptor contribution by the 4 different Notch receptors was not clear. We hypothesize that SRF specifically targets the receptor expression of Notch2 but not others to modulate astrocyte differentiation. We eliminated Notch3 as a contributing downstream gene of SRF for astrocyte specification because when we analyzed for astrocytic populations in null mutants

of Notch3 (generously provided by Kopan Lab at Washington University School of Medicine) no reduction in astrocyte numbers was detected at birth. We also do not think Notch1 is a candidate as conditional deletion studies have shown in the absence of Notch1 NPC proliferation and maintenance are severely compromised (Ables et al.; Imayoshi et al.; Yoon and Gaiano, 2005; Corbin et al., 2008), a phenotype not observed in our *Srf*-Nestin-cKO mutants or in neurosphere cultures of SRF-deficient NPCs.

To further understand the interaction between SRF and the different Notch receptors, we should perform luciferase promoter analysis and chromatin-IP approach, in which we analyze for SRF protein binding to putative Notch CArG domains when induced for astrocytes in the neurosphere system. These are experiments that we had planned for and are in the process of conducting. Despite the lack of precise transcription factor-promoter interaction insights, we were able to obtain Notch2$^{f/f}$ P0.5 animals (generously provided by Kopan Lab at Washington University School of Medicine) and culture their NPCs as neurospheres. Using adenovirus mediated Cre recombinase knockout of Notch2 in NPCs and inducing both control – where adenovirus containing the LacZ gene instead of Cre was delivered – and Notch2-deficient NPCs for astrocytes, we found that NPCs that lack Notch2 expression – successful knockout was confirmed via western blotting – showed substantial deficits for generating astrocytes, thus recapitulating the impairment observed in SRF-deficient NPCs.

These results show that SRF targets Notch2 expression and that the absence of Notch2 gives rise to phenotypes similar to the absence of SRF. We believe it is very likely that one of the major mechanisms by which SRF regulates astrocyte differentiation is through the modulating of Notch2 receptor expression, and we have generated Nestin promoter driven conditional mutants of Notch2 (Notch2-Nestion-cKO) to help us further understand the interactions between SRF and Notch2 and how they are required for astrocyte differentiation.



Supplementary Fig 1. SRF regulates astrocyte differentiation through Notch2 receptor expression. **(A)** Using quantitative PCR, we verified that the expression of Notch2 transcripts is indeed substantially decreased, while Notch3 is expressed 2 – 3 fold higher in SRF-deficient NPCs. We analyzed Notch3 null-mutant brains at both P0.5 and at 10 months of age and found no changes in the astrocyte numbers (data not shown). **(B)** We confirmed via western blotting that Notch2

protein expression is indeed down-regulated in SRF-deficient NPCs. We hypothesize that SRF regulates astrocyte differentiation through the expression of Notch2 receptors. **(C)** To test this, we cultured neurospheres from control and Notch2$^{f/f}$ brains at P0.5, and used an adenovirus with Cre recombinase (Adeno-Cre) to knockout Notch2 expression and an adenovirus with lacZ (Adeno-lacZ) as control. Western blotting was employed to show that deletion of Notch2 was effective and that there was no consequential effect on SRF protein expression, suggesting SRF is upstream to Notch2. **(D)** By inducing for astrocytes, we found that Notch2-deficient NPCs also failed to respond to pro-astrocyte factors, phenocopying the differentiation impairment exhibited by the ablation of SRF.

SRF is necessary but not sufficient for glial differentiation

Our finding is the first to demonstrate that SRF is a transcriptional factor necessary within the brain for glia differentiation and that re-introduction of wildtype SRF gene (SRFwt) via viral infection rescues the differentiation defects observed from the neurosphere culture assay; however, what we have not addressed is whether SRF-dependent transcription is sufficient for the initiation of astrocyte and/or oligodendrocyte differentiation. For example, the transcription factor Klf15, identified from functional assays in a genome-wide screen for regulators of gliogenesis, was found to be sufficient for astrocyte differentiation within the spinal cord, activating GFAP expression (Fu et al., 2009).

To study whether SRF is sufficient for either astrocyte or oligodendrocyte specification, we made viral constructs in which the constitutively active variant of SRF – SRF-VP16 – was cloned into the MSCV vector (Johansen and Prywes, 1994; Schratt et al., 2002).

By infecting control and SRF-deficient NPCs and following the induction protocols that we have adopted, we discovered that in the presence of pro-astrocyte factors SRF-VP16 augments astrocyte differentiation by giving rise to about 1.5 times as many GFAP-positive cells. However, in the absence of any other factors that induces astrocyte differentiation, the introduction of SRF-VP16 into either control or SRF-deficient NPCs was **not sufficient** to confer more astrocyte fate commitment than the default level observed in the absence of mitogens EGF and FGF. Interestingly, the effect of SRF-VP16 is different in oligodendrocyte specification: in the presence of pro-oligodendrocyte factors we did not observe an augmented oligodendrocyte differentiation with the supplementation of SRF-VP16; furthermore, in the absence of mitogens and pro-oligodendrocyte factors, SRF-VP16 was slightly inhibitive to oligodendrocyte cell-fate acquisition than the default level. The inhibitive effect is indeed unexpected, nevertheless one that was also reported by another study (Stritt et al., 2009). A possible explanation for this outcome is that SRF-VP16 lacks the physiological trans-activation domain and is not under any normal regulatory control of SRF activity.

SRF-VP16 could be preoccupying a range of target genes – perhaps cytoskeletal, immediate early genes, or astrocyte-specific genes – such that when the epigenetic landscape is permissible for oligodendrocyte specification other genes promoted SRF-VP16 could be overriding the set of genes required to produce oligodendrocyte specific transcripts or nullifying their intended effects.

This is a very interesting phenomenon and a question that we as well as other researchers of SF have little insights to. Future studies using gene profiling tools to accurately capture the differential gene expression in control NPCs infected with SRF-VP16 or with empty vector at various time points before and after oligodendrocyte induction could shed more light on the underlying molecular interactions.

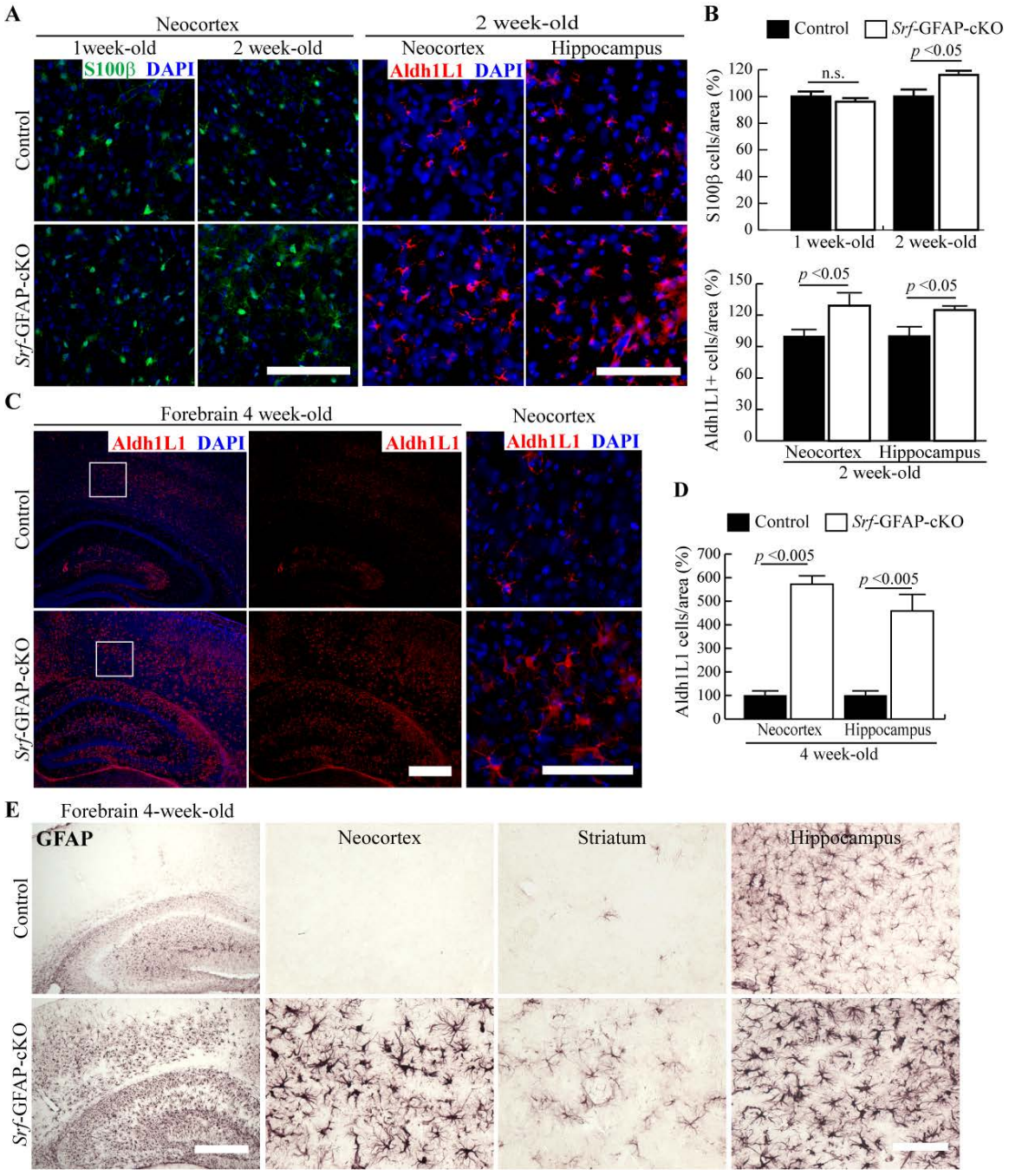
Loss of SRF in adult astrocytes leads to hypertrophy and gliosis-like phenotype.

Lastly, we wanted to determine whether the astrocyte differentiation impairment represents a block of astrocyte commitment or a temporal delay of differentiation. We generated an additional conditional knockout of SRF, within which SRF is deleted specifically in the astrocyte lineage (*Srf*-GFAP-cKO) (Bajenaru et al., 2002). At P7, we found that *Srf*-GFAP-cKO animals shown normal numbers of astrocytes as determined by Aldh1L1 and GFAP immunostaining for astrocytes. Surprisingly, by 4-week of age we observed a

4 – 6 fold increase in the number of astrocytes, as determined by both Aldh1L1 and GFAP cell count analyses. We found not only that there are more astrocytes in the adult *Srf*-GFAP-cKO brain, but those astrocytes are also hypertrophic. These observations are not the result of increased apoptosis in the mutant brain as we did not find elevated cell death activity measured by TUNEL staining and activated-caspase3 immunostaining in the same samples.

Electron microscopy of control and *Srf*-GFAP-cKO brains at 4-week revealed that SRF-deficient astrocytes contain an greater abundance of glial intermediate filaments, which are up-regulated in reactive astrocytes, and are substantially enlarged in cell size (data not shown), resembling a reactive gliosis phenotype (Pekny and Nilsson, 2005).

In summary, despite the pivotal roles that astrocytes play in the brain, the mechanisms that regulate their differentiation and development remain poorly studied. Our findings uncovered not only that SRF is a novel cell-autonomous transcriptional regulator of astrocyte differentiation, but also that it plays a distinct functional role in modulating the development of mature astrocytes.



Supplementary Fig 2. SRF loss in mature astrocytes causes hypertrophy and reactive-gliosis like phenotype. **(A)** Immunostaining for astrocytes using S100β and Aldh1L1 staining show that *Srf*-GFAP-cKO mutants show normal astrocyte numbers at 1-week after birth, however they increase significantly compared to control littermates at 2-week of age. **(B)** Quantification of cell counts at 1-week

and 2-week old brains of control and *Srf*-GFAP-cKO mice. **(C)** Mature astrocytes lacking SRF are hypertrophic and substantially more numerous at 4 weeks after birth. **(D)** Cell count analyses show that astrocytes increase by a magnitude of 4 – 6 time in *Srf*-GFAP-cKO brains. **(E)** GFAP immunostaining for astrocytes in multiple regions of the brain also confirms that loss of SRF in adult astrocytes results enlarged cell bodies and an increase in numbers, resembling gliosis-like phenotypes.

Neuronal projections require SRF *in vivo*

Despite mounting evidence and mechanistic insights on how SRF-dependent transcription controls axonal outgrowth and dendritic complexity *in vitro* utilizing both cortical and hippocampus neuronal culture, little evidence shows what these results actually translate to *in vivo*, physiological influences. Having the *Srf*-Nestin-cKO and *Srf*-NEX-cKO conditional mutants in our tool kit, we are uniquely positioned to ask 1) is SRF really necessary for neuronal tracts and innervations in the brain, as it is possible that neurite defects of SRF are compensated others in the much more complex physiological environment, and 2) if SRF is important, what tracts and innervations truly require SRF *in vivo*.

In order address the above questions, we analyzed for anatomical abnormalities in the *Srf*-Nestin-cKO and *Srf*-NEX-cKO mutants using a combination of Nissl staining – to examine gross architecture differences, immunohistochemistry staining – to specifically map various neuronal innervations and tracts in the brain, and DiI staining – a lipophilic dye that traces

the processes in entirety through diffusion over time. From coronal Nissl staining we first observed deficits, either absent or severely reduced, in the mutant brain in posterior corpus callosum, internal capsule, stria medullaris, and mammilothalamic tract. This finding is interesting because it shows that SRF is broadly required for neuronal innervations, but it is not necessary for all because the anterior corpus callosum, which is innervated by different areas of cortical neurons compared to posterior corpus callosum (for example, somatosensory projections constitute a part of the anterior corpus callosum whereas visual cortical neuron projections constitute a part of the posterior corpus callosum), is still intact. Another contributing factor for this difference could be that SRF-deficiency results varying degree of neurite defect in different populations of neurons. Future studies that compare the neurite length and number from different regions of neurons – for instance, surveying cortical neurons, hippocampal neurons, striatal neurons, and thalamic neurons – from *Srf*-Nestin-cKO brains in culture could provide more insights.

Loss of SRF leads to impairments of major axon tracts

Using immunostaining for the anti-neurofilament antibody 2H3 on coronal and sagittal brain slices of *Srf*-Nestin-cKO mutants and control littermates, we discovered further striking deficits in axonal innervations.

We observed a near absence of the entorhinal-hippocampal tract in the mutant hippocampus, substantial reduction in the cortical striatal projections, which relay between the neocortex and the striatum, and in the internal capsule innervations, and highly diminished anterior commissure as well as the fasciculus retroflexis in the mutant brains.

Because relying on immunostaining to assess the neuronal processes in the brain dependents on protein expressions, such as the levels of neurofilament or tubulin, as a proxy. It could be possible that the loss of SRF affects neurofilament or other cytoskeleton protein expressions. Thus, we applied the lipophilic neuronal process tracer dye DiI to visualize the entirety of the mutant neuronal projections compared to control brain neuronal projections. A tiny amount of DiI crystals were applied on the somatosensory cortex, and after 2 – 4 weeks due to diffusion DiI dye could fully label the processes that extend from the region of crystal placement. We observed substantial corticostriatal and corticothalamic innervation deficits in the *Srf*-Nestin-cKO brains.

Using ImageJ software we could accurately analyze for the projection length in pixels and then in micrometers; we found that these projections are shorter by as much as 2 – 6 folds in the mutant brains. DiI labeling also enabled us to confirm again that the posterior corpus callosum and anterior commissures are largely absent in the mutant brain and that the thalamocortical innervations are highly diminished, too. The observation that thalamocortical innervations are also affected in *Srf*-Nestin-cKO brains demonstrates that SRF is necessary for proper establishment of axonal tracts not only from cortical neurons but also from other regional neurons including thalamic neurons.

These results are the first to precisely pinpoint the requirement of SRF for several neuronal innervations and axonal tracts physiologically. Furthermore, our unpublished data also show that local dendritic projections are impaired too as MAP2 immunostaining reveals significantly reduced apical dendritic projections in the mutant brain.

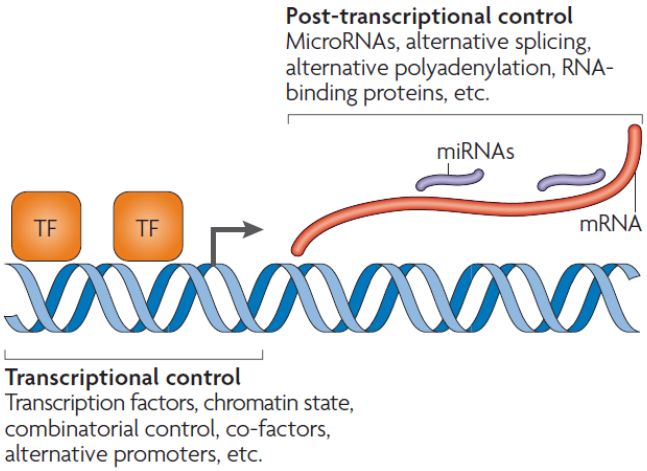
Srf-NEX-cKO brains indicate that SRF is necessary cell-autonomously for several neuronal tracts establishment

Although it is almost intuitive to attribute the neuronal projection deficits to a cell-autonomous requirement for SRF, especially given that several neuronal culture studies find SRF-dependent transcription to be necessary for normal neurite genesis and outgrowth; however, to ascertain this and to study whether

SRF-deficient glial cells could also contribute to the physiological impairments we turned to analyzing the *Srf*-NEX-cKO brains at P0.5. By employing the same techniques as performed on Srf-Nestin-cKO analyses, we found that *Srf*-NEX-cKO brains, which conditionally ablates SRF only in newborn neurons at around E11.5, also exhibit many of the same innervation and axonal tract defects. This demonstrates that SRF is indeed crucial cell-autonomously for many neuronal projections in the brain.

Could SRF control glial cell-fate specification through regulation of miRNAs?

Mounting evidence indicate that MicroRNAs (miRNAs) are another set of highly important regulator molecular entities in addition to transcription factors. miRNAs are capable of regulating gene expression by suppressing the translation of target mRNAs. The observation that the induction of numerous, lineage-specific miRNAs expression during neural differentiation had initiated the speculation that miRNAs may participate in the regulation of differentiation from embryonic stem (ES) cells. Although recent studies have demonstrated important roles of miRNAs in neural specification, but whether miRNAs are essential regulators that are sufficient and/or necessary for neural differentiation or fine-tuning modulators induced to ensure proper development is still highly debatable.



Transcriptional regulation and miRNA post-transcriptional modulation can work in concert to orchestrate cell-fate specific gene expressions (Chen and Rajewsky, 2007).

Studies on the most widely studied brain- and neuron-specific miR-124 show that it promotes neuronal differentiation by directly suppressing the action of anti-neuronal small c-terminal domain phosphatase 1 (SCP-1) (Visvanathan et al., 2007) and activating alternative pre-mRNA splicing that favors neurogenesis via inhibition of global alternative splicing repressor, polypyrymidine tract-binding protein 1 (PTBP1) (Makeyev et al., 2007; Makeyev and Maniatis, 2008). Another study showed potent influences of miR-124 with miR-9, which is also brain-enriched, reduced glial differentiation by decreasing the level of p-STAT3 (Krichevsky et al., 2006). Several other brain-specific or brain-enriched miRNAs such as miR125b and miR-134 are also known to have profound roles in promoting neural differentiation by maintaining the proliferation of differentiated cells (Lee et al., 2005) and suppressing Nanog, which is a transcription factor required for ES cell self-renewal (Tay et al., 2008).

Research on the cardiac and smooth muscle cells have identified that SRF can directly regulate the expression of several miRNAs, including miRNA-1 (Lewis et al., 2012), miRNA-21 and miRNA-143 (Horita et al., 2011), miRNA-143/145 in vascular smooth muscle cells differentiation (Boucher et al., 2011), and miRNA-1, miRNA-133, and miRNA-21 in cardiac hypertrophy and cardiogenesis (Zhao et al., 2005; Zhang et al., 2011). Additionally, SRF activity could also be a target of miRNA regulation, such as targeting by miRNA-483-5p in controlling the process of angiogenesis (Qiao et al., 2011).

miRNAs are non-coding transcripts, ~22 nucleotides in length, that provide crucial control to gene expression by translational inhibition and destabilizing their target mRNAs. They are initially transcribed as longer primary miRNAs (pri-miRNAs) and processed into about 70-100 nucleotides pre-miRNAs by Drosha and Pasha. pre-miRNAs are translocated into the cytoplasm and cleaved further by Dicer, a RNase III enzyme, and loquacious, to yield mature miRNAs (Kloosterman and Plasterk, 2006). miRNAs recognize their complementary target sequences imperfectly by binding to their 3' untranslated regions (UTR) and lead them to RNA-induced silencing complex (RISC) that prevents translation and occasionally degrades mRNAs (Kosik, 2006). Currently, more than 500 human miRNA sequences are predicted to exist (Xie et al., 2005; Berezikov et al., 2006) and each could influence the expression of tens, if not hundreds, of mRNAs (Lim et al., 2005).

A potential future direction following up the results of this thesis could involve discovering and studying the interactions between SRF and its target miRNAs in CNS, in order to understand lineage specific differentiation control of SRF even though this transcription factor is expressed almost ubiquitously, and to study whether SRF modulates the process of cell-fate acquisition by repressing the expression other non-glial lineage latent transcripts through miRNAs.

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