Changes in adult neurogenesis after traumatic brain injury - the role of TGF-β cytokines and the transcription factor Runx1.

by

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ABSTRACT

Changes in adult neurogenesis after traumatic brain injury - the role of TGF-β cytokines and the transcription factor Runx1:

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Adult traumatic brain injury (TBI) is a common, acquired injury that often results in permanent loss of neurological function. There are currently no standard treatments aimed at preventing neuronal degeneration during the secondary injury phase or to facilitate regeneration of the central nervous system (CNS). Numerous studies indicate that traumatic brain injury (TBI) increases neurogenesis in the forebrain subventricular zone (SVZ) and the hippocampal dentate gyrus (DG). It is possible that these endogenous stem cell populations could be manipulated to provide replacement neurons to facilitate CNS repair and recovery after injury. Transforming growth factor-β (TGF-β) superfamily cytokines are important regulators of adult neurogenesis, but their involvement in regulation of neurogenesis after adult brain injury is unclear. We utilized a craniotomy and controlled cortical impact (CCI) model of TBI in the mouse to investigate the hypothesis that TBI would induce changes in the TGF-β superfamily signaling pathways within the adult neurogenic regions. Through analysis of qPCR array data generated from RNA isolated from the SVZ and DG of adult mouse at different time points after CCI, we identified major changes in expression of genes involved in the TGF-β, bone morphogenetic

(BMP), and activin signaling pathways, which could bear relevance to the endogenous neural stem and progenitor cell (NSPC) response to injury.

TBI induced expression of the transcription factor Runx1 in microglia and a subpopulation of NSPCs of the SVZ and DG. Runx1 expression was associated with microglial activation and proliferation after injury, suggesting it could regulate these processes. We then investigated the hypothesis that Runx1 induces proliferation and neuronal differentiation of adult NSPCs in primary adult mouse NSPC cultures. We provide evidence that Runx1 acts as a pro-mitotic factor and can also promote neuronal fate choice in adult NSPCs. Taken together, our data suggest that Runx1 is induced by injury and could drive post-injury neurogenesis from a specific sub-population of adult NSPCs. The Runx1 pathway may therefore be a valuable target for manipulation in order to facilitate endogenous neurogenesis following TBI.

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CHAPTER 1: Introduction

ABBREVIATIONS:

Basic fibroblast growth factor (bFGF), BMP and activin membrane-bound inhibitor homolog (BAMBI), β-3-tubulin (Tuj1), bone morphogenetic (BMP), central nervous system (CNS), controlled cortical impact (CCI), core-binding factor β (CBFβ), cyclin dependent kinase (CDK), days post-injury (dpi), dentate gyrus (DG), epidermal growth factor (EGF), forkhead box protein P3 (FOXP3), glial fibrillary acidic protein (GFAP), granulocyte colony stimulating factor (G-CSF), hematopoietic stem and progenitor cell (HSPC), hematopoietic stem cell (HSC), inducible nitric oxide synthase (iNOS), inhibitor of kappa light polypeptide gene enhancer in B-cells kinase (I□B-kinase), insulin-like growth factor-1 (IGF-1), interleukin-6 (IL-6), macrophage colony stimulating factor receptor (M-CSFR), Mrgpr class G-, protein coupled receptors (Mrgprs), neural stem and progenitor cell (NSPC), neural stem cell (NSC), nuclear factor kappa-light-chain-enhancer of activated B cells (NF-□B), retinoic acid (RA), S100 calcium binding protein B (S100β), subgranular zone (SGZ), subventricular zone (SVZ), T-cell aculte lymphocytic leukemia 1 (SCL), Tgf-β receptor 1 (TβRI), Tgf-β receptor 2 (TβRII), Transforming growth factor-β (TGF-β), traumatic brain injury (TBI), tumor-necrosis factor α (TNF-α), V-ets avian erthroblastosis virus E26 oncogene homolog 1 (ETS1).

TRAUMATIC BRAIN INJURY: A SIGNIFICANT PROBLEM IN NEED OF NOVEL TREATMENTS

Traumatic brain injury (TBI) is a major clinical problem in the United States, with an estimated 1.7 million people suffering a TBI each year, resulting in approximately 275,000 hospitalizations (47). TBI is significant concern to the US military, with the majority of all head injuries in the recent wars in Iraq and Afghanistan being closed-head injuries involving mild-moderate TBI (51). TBI frequently affects children and young men and women (37), and can

often result in a permanent loss of physical and mental abilities as well as behavioral and emotional changes, and a significant drop in the individual's quality of life (39; 125). Improved treatments could thus provide a long lasting benefit to patients who may otherwise be suffering permanent deficits from this acquired disorder. Currently, clinical treatment of TBI primarily focuses on stabilizing patients and managing intracranial pressure, brain oxygenation, and blood glycemic and osmotic levels, and treating secondary symptoms such as seizures. These practices are delivered in the immediate hours and days following injury, and there are no effective long-term treatments for TBI in clinical practice that are aimed at preventing neuronal degeneration of the central nervous system or facilitating its regeneration (65; 81).

TBI begins with a single event, but it initiates what can be thought of as a long-term neurodegenerative disorder, with clear pathological processes and changes occurring in the CNS for weeks to years after the injury, and a lifelong increase in the risk of co-morbidities (102; 125). TBI involves the actual injury event and physical trauma, known as the primary injury, as well as a phase of secondary injury. Cell death occurs from the moment of primary injury through the secondary injury phase for a period of several months after the insult (102; 199). The largest amount of cell death occurs during the secondary injury phase, in a time period when patients can be treated. Secondary injury involves cell death throughout the brain, with extremely high levels in the contusion site and pericontusional region often with resultant cavitation in the subsequent post-injury weeks (169). There is also cell death in sites away from the cortical injury site, such as the thalamus and hippocampus (199). Despite the large amount of apoptotic and necrotic cell death during the secondary injury phase, many potential anti-apoptotic and neuroprotective treatments that showed promise in animal models have failed at the level of human clinical trials (77; 124). It may not be enough for TBI treatments to only prevent cell

death during the secondary injury, rather, researchers must seek new avenues to regenerate the lost tissue and cells. It is on this hope that stem cell therapies, both through the transplantation of exogenous cell sources to the injured region and through the manipulation of endogenous proliferative stem and progenitor cell populations, are being investigated as a potential means to regenerate CNS tissue and recover lost functions following CNS injury.

ADULT NEUROGENESIS OCCURS IN THE ADULT BRAIN, IS ELEVATED BY TBI, AND MAY CONTRIBUTE TO POST-INJURY RECOVERY.

There are two major sites in the adult mammalian brain where neurogenesis occurs. These are: the subventricular zone (SVZ), underlying ependymal cell layer surrounding the lateral ventricles of the forebrain, and the subgranular zone (SGZ), in the dentate gyrus (DG) of the hippocampus. Neural stem and progenitor cells (NSPCs) in these regions actively divide throughout life, renewing the stem cell population, and generating new neurons and glia (76; 123; 129). Immature neuroblasts generated in the SVZ migrate over a long distance ventrally through the brain in the rostral migratory stream to reach the olfactory bulb where they differentiate into mature neurons, while new cells generated in the SGZ migrate only a very short distance and generate new neurons in the higher levels of the granule cell layer of the DG (129). Following various types of brain injury, including ischemic, excitotoxic, and traumatic injury, NSPCs of the two neurogenic regions proliferate at an increased rate. Many neuroblasts generated in these regions also alter their usual migratory routes and instead move to the site of the lesion, where they can differentiate into neurons and glia, although the majority of these cells die (55; 126; 154; 168; 170; 200; 204; 208).

Understanding the signals regulating the post-injury NSPC proliferative response may contribute to the development of therapeutic interventions that manipulate NSPCs in order to

regenerate CNS cells and tissue that were lost due to injury. Rodent studies using treatments that increase endogenous neurogenesis have improved post-TBI recovery in rodents, demonstrating the proof-of-concept that enhancing endogenous neurogenesis could improve cognitive recovery in patients with brain injury (67; 96; 206). Conversely, a few studies have shown that ablating the endogenous neurogenic response impedes cognitive recovery after injury (11; 206), suggesting that the normal endogenous response is adaptive and facilitates recovery.

THE TGF-B SUPERFAMILY OF PROTEINS REGULATE ADULT NEUROGENESIS.

The TGF-β superfamily of proteins is a group of structurally and evolutionarily related proteins, and includes bone morphogenetic proteins (BMPs), activin proteins, and the so-named TGF-β proteins and receptors. Signaling by these proteins critically regulates adult neurogenesis in both the SVZ and DG. The best characterized of these regulatory pathways in the neurogenic niche is the BMP pathway. BMP signaling serves as an important, continually active suppressor of proliferation and differentiation for the adult NSPC population. BMP receptors are expressed by primary stem cells, as well as intermediate progenitors and immature neuroblasts derived from both SVZ and DG. Exogenous treatment of NSPCs with BMP proteins potently inhibits NSPC division and neurogenesis *in vivo* and in culture (113; 130). Active signaling by pSmad1, an intracellular transducer of BMP signaling is seen in quiescent primary NSCs and neuroblasts, but not in those cells when they are dividing (130). The BMP ligands BMP2, -4,- 6, and -7 are expressed in the SVZ and DG (29; 130), as is the extracellular BMP antagonist protein Noggin, which binds to BMP cytokines and prevents them from binding their target transmembrane receptors. The balance between BMP and Noggin is critical to maintaining normal levels of neurogenesis, and altering this balance can impede or stimulate NSPC proliferation and neurogenesis. Increasing BMP levels causes a profound inhibition of NSPC division and

neurogenesis, while increasing Noggin levels increases NSPC proliferation and neurogenesis (113; 130). However, the facilitation of neurogenesis by Noggin treatment is transient (130). After long term treatment, the NSPC pool becomes depleted due to higher than normal levels of cell differentiation from the primary, self-renewing stem cell population, eventually causing an overall drop in the available pool of intermediate neuronal precursor cells and a resultant drop in the levels of neurogenesis (130).

Similar to the BMP proteins, TGF-β proteins are also potent inhibitors of adult neurogenesis. Expression of the receptors TβRI and TβRII have been demonstrated on early, nestin-expressing NSPCs of the adult SVZ (128; 185; 187). TGF-β1 is expressed in both the SVZ and DG (185; 195), and exogenous administration of TGF-β1 inhibits NSPC proliferation in cells of either region and inhibits neurogenesis both *in vivo* and through a direct effect on NSPCs *in vitro* (19; 86; 187). Endogenous TGF-β1 expression increases in the DG as mice age, and it is possible that this increase may be a source of age-dependent decreases in neurogenesis (4; 195).

Yet, the overall effect of TGF-β signaling in the neurogenic regions may be different in the injured brain than in the healthy brain. Based on the strong anti-inflammatory actions of TGF-β1, Ma and colleagues administered intranasal aerosolized TGF-β1 as a treatment for mice that were subjected to middle cerebral artery occlusion. They demonstrated that this treatment improved post-ischemic cognitive functions and facilitated neurogenesis (122). A similar result was shown in adrenalectomized rats, which showed a marked increase in hippocampal NSPC proliferation and neuron generation after adrenalectomy that was dependent upon an increase in TGF-β1 expression within the DG (6). While it has been clearly demonstrated that TGF-β suppresses the proliferation of cultured NSPCs (19; 187), TGF-β treatment can also increase the

fraction of NSPCs that generate neurons when they are exposed to differentiating culture conditions (6).

Activin receptors are also expressed in the DG (16; 109). Contrary to TGF-β and BMP signaling, activin signaling is necessary to promote and maintain adult neurogenesis, and is critical for neurogenesis to occur in the adult DG. Overexpression of the activin inhibitor protein, follistatin, causes an almost complete ablation of adult neurogenesis due to the death of intermediate, doublecortin (DCX)-expressing neuronal progenitor cells, though the survival and proliferation of primary neural stem cells remains unchanged (2).

Despite their well-known effects as regulators of neurogenesis, few published studies have investigated changes to TGF-β superfamily signaling in the neurogenic niche after TBI. After TBI, there is a chronic up-regulation in expression of proteins from each of the BMP, TGF-β, and activin families at the actual injury site (49; 66; 117). If long-lasting changes in the expression of these proteins occur in the neurogenic regions as they do in and around the lesion site, then those changes could have major effects on neurogenesis. Indeed, it has been found that elevated noggin expression underlies the mechanism of exercise-induced neurogenesis (60), and elevated activin expression following excitotoxic injury to the DG is required for the elevated post-injury neurogenic response to occur (1). Thus, changes to the TGF-β superfamily pathways can be part of the endogenous mechanisms for controlling post-injury neurogenesis. If such changes occur after TBI, they could be regulating the endogenous neurogenic response, and would also need to be taken into account when designing potential therapeutic interventions aimed at manipulating post-TBI neurogenesis. Our experiments sought to investigate changes in these pathways in both the SVZ and DG after a traumatic brain injury.

THE TRANSCRIPTION FACTOR RUNX1 INTERACTS WITH THE TGF-B SIGNALING PATHWAYS, AND INDEPENDENTLY OF THEM.

Runx1 protein, also called Aml1, exists in three major isoforms in humans, called Runx1a, Runx1b, and Runx1c. Runx1b and Runx1c expression are driven by two different promoters, but generate a similar protein, while Runx1a generates a truncated protein that contains the DNA-binding region but lacks the trans-regulatory domains. Runx1a has been demonstrated to have antagonistic effects to the Runx1b and c isoforms and is generally considered to act as a dominant-negative competitive inhibitor of the other two isoforms (182). For the purposes of this work, 'Runx1' indicates the full-length protein isoforms Runx1b or Runx1c, unless specifically indicated otherwise.

Runx1 can interact directly with the receptor-associated Smad proteins, the intracellular transcription factors that transduce signals mediated by TGF-β superfamily transmembrane receptor proteins (68; 111; 146; 203). A diagram of the canonical TGF-β superfamily signaling pathway is shown in Fig 1 (185). The interaction between Runx1 and the rSmads is dependent upon the prior activation of the TGF-β transmembrane receptor, indicating that the association is part of the TGF-β signal transduction pathway (146). Through its interaction with rSmads, Runx1 can synergistically activate or repress TGF-β target genes (68; 131; 207). Runx1 can induce Smad protein expression and facilitate Smad mediated gene transactivation, and Smads can reciprocally regulate levels of Runx1 expression (68; 97).

RUNX1 CO-ACTAVATORS AND CO-REPRESSORS.

In addition to interacting with the TGF-β signaling pathway, Runx1 has complex interactions with many different transcriptional cofactors and can have diverse effects on cell proliferation, migration, and differentiation (52; 91). Runx1 is a highly versatile transcription factor which can transactivate or repress many target genes in a highly cell-context dependent

manner. This is largely due to the many transcriptional co-activators and co-repressors it can interact with, allowing Runx1 to have unique effects in different cells at different times, as the host of available cofactors changes. The most significant and well-characterized co-factor of Runx1 is CBFβ. CBFβ does not itself bind DNA, nor does it affect the direction of Runx1 transcriptional activation or repression. Rather it interacts with the DNA binding domain of the Runx1 protein, stabilizing Runx1and protecting it from proteasomal degradation (78). CBFβ binding also enhances the affinity of Runx1 for DNA by approximately 10-fold, thereby strongly increasing the transcriptional regulatory effects of the Runx1 protein (36; 175; 176). Although Runx1 is capable of regulating transcription in its absence, inhibiting this interaction strongly impedes Runx1 activity (36), and *CBFβ* genetic knockouts phenocopy Runx1 knockouts (190).

CBFβ interacts with Runx1 in the DNA-binding Runt domain, near the N-terminus, while the Smad interaction site has been mapped to a different region near the C-terminus (42; 146). No published studies have directly described the relationship between CBFβ and its ability to affect Runx1-rSmad binding, although one study demonstrated that at the very least, increasing CBFβ concentrations does not compete with or inhibit the formation of the Runx1-rSmad complex (146).

V-ets avian erthroblastosis virus E26 oncogene homolog 1 (ETS1) is another cofactor for Runx1. ETS1 and Runx1 can interact directly, and the complex formed by these two proteins binds a unique DNA sequence that neither protein normally recognizes on their own (72). ETS1 also facilitates the formation of the Runx1-CBFβ complex, implicating it as a general activator of Runx1 transcriptional regulation (94). Runx1 also directly interacts with the transcriptional co-activators p300 and CREB-binding protein, and this interaction is involved in the stimulation of hematopoietic stem cell (HSC) differentiation by Runx1 (95).

The Groucho and TLE family of transcription factors, as well as the transcription factor forkhead box protein P3 (FOXP3), are other important transcriptional co-regulators that directly interact with Runx1. These protein interactions can suppress the transactivation ability of Runx1 while increasing its transcriptional repression of target genes (108; 143; 159).

RUNX1 UPSTREAM REGULATORS

Only a few molecules are established as inducers of Runx1 expression, with one of the most prominent being retinoic acid (RA). RA induces Runx1 expression in various myeloid cancer cell lines (145; 166; 172), murine embryonic pluripotent stem cells (90), and in HSCs (22). Like Runx1, retinoic acid is a critical factor for the development of HSCs and for the process of hematopoeisis (22). Retinoic acid has been reported to increase in the CNS in the acute phase following an injury, which could in turn induce Runx1 expression in CNS cells (92).

Notch signaling is also upstream of Runx1, and, like Runx1, Notch has important regulatory roles in hematopoiesis (21; 56). Genetic ablation of Notch1 prevents the formation of Runx1+ HSCs (107), and signaling by the Notch intracellular domain induces Runx1 expression in adult mouse bone marrow stem cells and endothelial cells (21; 56). Although, this induction of Runx1 appears to be indirect, as there are no binding sites for specific notch family transcription factors in the *Runx1* promoter. Rather, Notch signaling appears to induce *Runx1* induction through its activation of Gata2. Gata2 is a direct target of Notch signaling and can directly induce expression of the *Runx1* gene (139).

It has also been reported that basic fibroblast growth factor (bFGF, FGF2) can induce expression of Runx1 and the nerve growth factor receptor *TrkA* in human olfactory neuroblastoma cells (138), suggesting that the bFGF pathway could potentially stimulate Runx1 expression in neural cells.

Lastly, Runx1 binding sites exist in its own promoter region, demonstrating the potential for autoregulation of the protein (145).

RUNX1 DOWNSTREAM TARGETS

Runx1 contains both transactivation and transcriptional repression domains (43; 87), allowing it to either promote or repress expression of its target genes (164). Runx1 can even have opposite effects on the expression of a single gene within the same cell population as time progresses during development, and the intracellular cohort of co-activators and co-repressors changes (164).

To obtain a broad view of the pathways which could be regulated by Runx1, I examined Chip-seq data available online in the BloodChiP database (http://www.med.unsw.edu.au/CRCWeb.nsf/page/BloodChIP), coupled with pathway analysis using the GATHER (Gene Annotation tool to Help Explain Relationships) software available online through Duke University (http://gather.genome.duke.edu/). The results indicated that Runx1 binds to the promoter regions of roughly 8000 unique genes in the human myeloblastic leukemia cell line SKNO-1. Pathway analysis indicated that in the SKNO-1 cell type, Runx1 target genes were most significantly grouped into genes regulating cellular physiological processes, and also significantly grouped as regulators of cell proliferation, cell cycle regulators, and regulators of apoptosis. This is unsurprising, as Runx1 is a well-known oncogene and can regulate the survival and proliferation of cancerous cells. Genes related to G-protein coupled receptor signaling and sensory perception, particularly olfactory perception, were also bound by Runx1 with great frequency. Runx1 is expressed in developing olfactory neuronal precursor cells (179), and is known to regulate the expression of various receptors in differentiating neuronal precursor cells, including Mrg class G-protein-coupled receptors (115). In CD34-expressing

HSCs, Runx1 bound to approximately 9600 unique genes. Similar to the groupings found in the SKNO-1 cell line, the most significant and largest groupings of genes that were bound by Runx1 were those regulating cell physiological processes, G-protein coupled receptor protein signaling, and olfactory sensory perception. Runx1 also bound several hundred genes regulating cell proliferation and apoptosis, indicating that regulating these functions is a common action of Runx1 across cell types.

Runx1 directly binds to and transactivates the *Cxcr4* promoter in mouse HSCs (189). In HSCs, signaling by the SDF1 ligand to the CXCR4 receptor attracts HSCs to migrate into the bone marrow (88). CXCR4 also regulates the migration of transplanted neural stem cells to an injury site in the CNS (25), and regulates the migration of endogenous SVZ NSPCs towards endothelial cells of blood vessels via up-regulating their expression of integrin alpha6 (100). Through regulation of this pathway, it is possible Runx1 could affect cell migration.

Runx1 can also regulate the expression of genes of the Wnt pathway. Runx1 knockout in epeithlial cells produces a drop in expression of the Wnt ligands Wnt11 and Wnt2, as well as a drop in expression of the Wnt receptor frizzled-4 (144). Through its interaction with the cofactor TLE1, Runx1 directly represses expression of *wnt6* and *wnt8* in sea urchin blastula (155), and can also inhibit β-catenin mediated promoter transactivation (108). Runx1 may also inhibit β-catenin signaling independently of TLE1. It was shown that the DNA-binding domain of Runx1 fused to a GFP tag is sufficient to inhibit β-catenin mediated transactivation in a luciferase reporter assay (144).

RUNX1 REGULATES THE CELL CYCLE.

In addition to the above mentioned cofactors and targets of Runx1 signaling, Runx1 also interacts with and regulates the expression of many proteins directly related to cell cycle

progression, including cyclins, cyclin dependent kinases (CDKs), CDK inhibitors, histone deacetylases, and histone methylases. Through these interactions Runx1 is a potent regulator of proliferation in many different cell types, sometimes promoting and sometimes inhibiting cell proliferation. In murine HSCs, Runx1 protein represses cell division, with over-expression of Runx1 increasing cell cycle arrest at G_0 and inducing expression of the *Notch1* receptor and its target gene *Hes1* (27). In agreement, over-expression of the truncated RUNX1a protein increases murine HSC proliferation (182). Conversely, Runx1 over-expression promotes proliferation in the 32D.3 myeloid progenitor cell line and in cultured murine hair follicle stem cells (71; 105; 165).

Runx1 directly binds and activates the cyclin D1 promoter in murine HSCs and sea urchin blastula cells, respectively (134; 155). The *cyclin D3* promoter can also be directly transactivated by Runx1 binding in myeloid and lymphoid cell lines as part of the mechanism by which Runx1 facilitates S phase entry (9). In addition to being downstream targets of Runx1, the cyclins can reciprocally influence Runx1 activity. Cyclin D1, D2, and D3 all directly bind Runx1 in its Runt DNA binding domain, and cyclin D3 physically disrupts the binding of Runx1 to its required cofactor CBF-β, functionally inhibiting Runx1 activity (147). Cdk6 is also able to inhibit Runx1 transcriptional activation by disrupting its DNA binding ability, and can alleviate the Runx1 mediated inhibition of myeloid progenitor cell proliferation (53).

Runx1 also interacts with various CDK inhibitors. Runx1 directly binds to the promoter of the CDK inhibitor *p21* (179). Runx1 represses *p21* expression to facilitate cell proliferation in embryonic cortical progenitor cell cultures and in NIH3T3 cells (121; 179), whereas Runx1 activates the *p21* promoter in K562 cells (121). Runx1 also directly binds the promoter of the CDK inhibitor *p14* and induces its expression in the HeLa and Kasumi-1 myeoblast cell lines

(114). Expression of the fusion protein Runx1-ETO, which is comprised of the Runx1 DNA-binding domain followed by the ETO transcriptional repressor domain, also represses expression of *CDK4* and the CDK inhibitor *p19* and in myeloid progenitor cells and cell lines (20; 98; 114).

Runx1 can directly recruit the DNA-modifying histone deacetylaces HDAC1 and HDAC3 as co-repressor to suppress its target genes (63). This interaction can be disrupted by Runx1 phosphorylation from the protein complex of Cdk1 and cyclinB, which shifts the balance of Runx1 transcriptional regulation from repression towards activation (63).

RUNX1 REGULATES PROLIFERATION OF CERTAIN NEURAL CELLS DURING DEVELOPMENT.

Within the nervous system, Runx1 has been identified to regulate proliferation and neuronal differentiation in a few selected cell progenitor populations primarily during development, but prior to our work, has not been identified as active in the adult neurogenic regions or in adult NSPCs. Runx1 is active in embryonic and early post-natal olfactory ensheathing cells, which are a particular class of glia, where it suppresses their proliferation (135). Runx1 also regulates the neuronal-subclass differentiation/specification in certain populations of neuronal-restricted post-mitotic progenitor cells. It can drive cells of the spinal cord to become motor neurons as opposed to interneurons (163), and cells of the dorsal root ganglia to become Ret-expressing as opposed to TrkA-expressing peptidergic nociceptors (30). Runx1 is active in proliferating embryonic Mash1+ olfactory bulb neuronal progenitor cells and NeuroD+ olfactory neuronal precursor cells that are near the end of their proliferative phase (179). In these cells, Runx1 expression is pro-mitotic and extends the period of time over which these cells proliferate, while simultaneously driving them to express the neuronal marker NeuroD1(179). Additionally, adenoviral over-expression of Runx1 causes increased proliferation

and NeuroD1 expression in cultures of embryonic olfactory bulb NSPCs or embryonic cortical NSPCs, mediated, in part, through a direct inhibition of p21 expression (179).

ADULT NSPCS ARE AFFECTED BY LOCAL MICROGLIA

In addition to their intrinsic responses to the environmental changes caused by injury, adult NSPCs of the SVZ and DG are potently regulated by local microglia. Most studies have indicated an overall inhibitory effect of activated microglia on NSPC proliferation and neurogenesis, particularly in the acute phase of inflammatory microglial activation (45). Systemic inflammation over the course of one week activates microglia in the adult brain, and also represses cell proliferation and DCX+ cell generation in the DG, while anti-inflammatory treatments relieve this effect and restore normal levels of neurogenesis (44; 133). In culture, microglia activated by treatment with lipopolysaccharide (LPS) directly repress neuron generation from cultured adult rat hippocampal progenitor cells, while treating the progenitor cells directly with LPS in isolation from microglia has no effect (133). This action of microglia is likely to be due to their secretion of inflammatory cytokines such as interleukin-6 (IL-6), or tumor-necrosis factor α (TNF-α), which can directly repress neurogenesis *in vivo* and in culture (133; 183).

Yet, the action of microglia on neurogenesis is not clear-cut, and several studies have demonstrated supportive roles of microglia for neurogenesis. In addition to pro-inflammatory cytokines IL-6 and tumor necrosis factor-alpha TNF-α, inducible nitric oxide synthase (iNOS), is expressed by activated microglia following brain injury (69; 142), and is expressed in the DG following ischemia (209). Yet, the response of elevated neurogenesis following ischemia is ablated in iNOS null animals (120; 209), and iNOS inhibitor treatment also ablates seizure induced DG neurogenesis (84), indicating that iNOS is an essential component of the post-injury

neurogenic response. Additionally, microglia express insulin-like growth factor-1 (IGF-1) after seizures. IGF-1 supports neurogenesis, and infusion of IGF-1 antibodies suppresses this post-SE neurogenesis (34; 140; 181). Though there seems to be a clear net negative effect of acute inflammatory microglial activation following injury on the neurogenic response (44; 133), there are factors produced by microglia that contribute to post-injury neurogenesis, and a finer understanding of these regulatory pathways is necessary before the regulatory role of microglia on post-injury neurogenesis is clear.

RUNX1 REGULATES THE EXPRESSION OF CYTOKINES IN BLOOD CELLS THAT CAN REGULATE MICROGLIAL BEHAVIOR.

Runx1 activates the expression of interferon-γ, myeloperoxidase, and interleukins 2 and 3, in T-cells and HSCs (36; 121; 143; 205). These factors are all expressed by pro-inflammatory microglia after injury to the nervous system, suggesting that Runx1 could regulate these genes following injury (12; 73; 161). Runx1 also activates the macrophage-colony stimulating factor receptor (M-CSFR) in T-cells and HSCs (148; 205). M-CSFR is expressed by microglia after brain injury, and the cytokine macrophage-colony stimulating factor (M-CSF, CSF1) promotes microglial proliferation and phagocytosis after injury (161), representing another potential pro-inflammatory pathway that could be regulated by Runx1 in CNS microglia following injury.

RUNX1 REGULATES THE DEVELOPMENTAL PROLIFERATION AND MORPHOLOGY OF MIROGLIA.

In early-postnatal microglia, Runx1 expression appears throughout the microglial population of the brain at the same time as microglial stop their period of developmental proliferation. At this time, Runx1 functions to halt their proliferation and drive their progression from an amoeboid progenitor state to the small, ramified, quiescent state of microglia in the healthy postnatal brain (210). Heterozygous knockout animals (only possessing one functional

Runx1 allele instead of two), show a large increase in the number of proliferative microglia during the normal time period of developmental Runx1 expression (210). Runx1 over-expression represses proliferation and also represses expression of iNOS in cultures of embryonic microglia (210). Runx1 expression is absent in microglia in the normal adult brain, but has been shown to re-appear in activated microglia of the spinal cord following sciatic nerve injury, although the function of this re-expression was not investigated (210).

OUR INVESTIGATIONS

In the first part of my thesis, described in chapter 2, we utilized a craniotomy and controlled cortical impact (CCI) model of brain injury to investigate the hypothesis that TBI would induce changes in the TGF-β superfamily signaling pathways within the adult neurogenic regions. CCI induces persistent elevations in DG and SVZ NSPC proliferation and neurogenesis (11; 31; 38; 151; 200). We identified major changes in expression of genes involved in the TGF-β, BMP, and activin signaling pathways, which affect post-injury NSPC proliferation or differentiation. These results provide novel information on the changes in the signaling environment of the neurogenic niche that could inform the design of potential interventions aimed at manipulating the post-injury neurogenic response.

CCI injury also induced Runx1 expression in microglia in the injured cortex and in the neurogenic regions. Our investigations reveal a novel correlation of Runx1 expression with post-injury microglial activation and proliferation, and suggest that Runx1 may be induced in microglia after CNS injury in the adult to regulate these functions.

We also identified a novel population of Runx1-expressing putative NSPCs in the SVZ and DG after TBI injury, and chapter 3 describes our studies investigating the hypothesis that Runx1 could regulate the proliferation or neuronal differentiation of adult NSPCs. We provide

evidence that Runx1 acts as a pro-mitotic factor and can also promote neuronal fate choice in adult NSPCs, suggesting that it is activated endogenously following injury as a driving factor for post-injury neurogenesis in a specific sub-population of adult NSPCs, and that the Runx1 pathway may be a valuable target for manipulation in order to facilitate endogenous neurogenesis following brain injury.

Chapter 2: TGF-β superfamily gene expression and induction of the Runx1 transcription factor in adult neurogenic regions after brain injury.

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ABSTRACT

Traumatic brain injury (TBI) increases neurogenesis in the forebrain subventricular zone (SVZ) and the hippocampal dentate gyrus (DG). Transforming growth factor-β (TGF-β) superfamily cytokines are important regulators of adult neurogenesis, but their involvement in the regulation of this process after brain injury is unclear. We subjected adult mice to controlled cortical impact (CCI) injury, and isolated RNA from the SVZ and DG at different post-injury time points. qPCR array analysis showed that cortical injury caused significant alterations in the mRNA expression of components and targets of the TGF-β, BMP, and activin signaling pathways in the SVZ and DG after injury, suggesting that these pathways could regulate post-injury neurogenesis. In both neurogenic regions, the injury also induced expression of Runt-related transcription factor-1 (Runx1), which can interact with intracellular TGF-β Smad signaling pathways. CCI injury strongly induced Runx1 expression in activated and proliferating microglial cells throughout the neurogenic regions. Runx1 protein was also expressed in a subset of Nestin- and GFAP-expressing putative neural stem or progenitor cells in the DG and SVZ after injury. In the DG only, these Runx1+ progenitors proliferated. Our data suggest potential roles for Runx1 in the processes of microglial cell activation and proliferation and in neural stem cell proliferation after TBI.

INTRODUCTION

Adult traumatic brain injury (TBI) is a prevalent injury that often results in permanent loss of neurological function. In cases of severe TBI, clinical treatment focuses primarily on stabilizing the patients, performing intubation and ventilation if necessary, and monitoring and managing intracranial pressure, blood pressure, oxygenation, and glycemic levels (65). Secondary to stabilization, specific symptoms such as seizures are treated (81), but currently there are no standard clinical avenues available to facilitate repair, regeneration, or to enhance neuronal survival (37; 65). In the days following cortical TBI, massive amounts of cell death occur in the lesion core, pericontusional region, and in distal regions, such as the hippocampus (102). Treatments which help regenerate neurons could be beneficial, and significant recent research has focused on the possibility that the endogenous neural stem cell (NSC) population could be harnessed to stimulate regeneration and recovery of the central nervous system (CNS) following injury (93; 198). Widespread inflammation occurs concomitantly with cell death after injury, with microglia and astrocytes becoming activated and blood borne immune cells entering the lesion. This post-injury inflammation has broad impact on processes in both the lesion area and the neurogenic regions (45; 61; 156).

In the adult mammalian brain, NSCs and/or neural progenitor cells (NPCs) are maintained in two neurogenic niches: the forebrain subventricular zone (SVZ) around the lateral ventricles, and the subgranular zone (SGZ) of the dentate gyrus (DG) (129; 177). TBI increases the rates of NSC proliferation and neurogenesis in the adult mammalian SVZ and DG (55; 154; 200). This injury-induced neurogenesis may contribute to the limited spontaneous recovery and post-injury maintenance of cognitive abilities seen in rodents (11), as well as to the repopulation of neurons in damaged areas (5; 162; 200). Indeed, treatments that increase endogenous neurogenesis have also improved post-TBI recovery in adult rodents (67; 96; 206). Thus, post-

TBI neurogenesis represents a potential avenue for endogenous repair of tissue and recovery of cognitive functions following injury. Defining how the normal regulatory pathways of adult neurogenesis are altered by TBI is an essential step in attempting to manipulate post-TBI neurogenesis for therapeutic benefit.

Members of the transforming growth factor-β (TGF-β) superfamily of cytokines, including the bone morphogenetic proteins (BMPs), activins, and TGF-βs regulate many processes after TBI, including cell survival, gliosis, inflammation, and cell proliferation (7; 28; 32; 49). These cytokines also regulate adult NSC division and neurogenesis in uninjured animals (1; 60; 130; 187), although the involvement of TGF-β superfamily members in regulating post-TBI neurogenesis has not been demonstrated. Basal BMP signaling inhibits adult NSC proliferation and keeps the majority of adult primary NSCs in a slowly dividing, quiescent state (130). TGF-β1, 2, and 3 proteins inhibit NSC division and favor neuronal differentiation of NSCs in uninjured animals, but can increase NSC division rates in different injury contexts (6; 19; 127; 187). Activin-A is a crucial survival factor for immature neurons in the DG (2). Most importantly, experimentally increasing or decreasing the levels of TGF-β, BMP, or activin signaling in the neurogenic regions can have drastic effects on adult NSC division and neurogenesis (2; 130; 187). Therefore, we investigated how CCI injury alters expression of these cytokines and their related signaling molecules in the neurogenic regions.

Runx1 is a transcription factor that plays important roles in hematopoiesis (141; 171), olfactory neurogenesis (179), and neuronal development (99; 202). Runx1 physically interacts with the intracellular Smad transcription factors (the canonical intracellular transducers of TGF-β, BMP, and activin signaling) (68), although not exclusively, and can act as a transcriptional cofactor to either promote or repress the expression of TGF-β superfamily target genes (82; 131).

Developmentally, in the mouse CNS and in the dorsal root ganglia, Runx1 is expressed in certain populations of postmitotic motor and sensory neurons, where it functions to stimulate and maintain their differentiation into particular neuronal subclasses (30; 163; 202). It is also an important proliferative factor for Mash1-expressing neuronal progenitor cells in the embryonic olfactory bulb (179), and is expressed in adult NSC neurosphere cultures (15). Interestingly, adult microglia derive from Runx1-expressing precursors, and Runx1 regulates microglial proliferation during development (58; 210). Despite the fact that multiple populations of proliferative progenitor cells express Runx1, its expression has not previously been documented in the neurogenic regions of the adult brain.

In this paper we show that TBI causes major changes in the mRNA expression of many components of the TGF-β, BMP, and activin signaling pathways in both the SVZ and DG. We find that Runx1 is a novel injury-induced transcription factor expressed in NSCs of the adult SVZ and DG and is also expressed in proliferative and activated microglia in the neurogenic regions after TBI.

MATERIALS AND METHODS

Animals and controlled cortical impact (CCI) injury

All animal studies were approved by the USUHS Institutional Animal Care and Use Committee and were conducted in accordance with the NRC guide to the Care and Use of Laboratory Animals. Eight week old male C57BL/6 mice (NCI), weighing 22-28 g, were kept under 12:12 light and dark cycle with access to food and water ad libitum. Surgery was done after one week of recovery from transportation-related stress. Mice were anaesthetized with isoflurane (3% induction: 1.5% maintained) and affixed in a stereotaxic frame. Body temperature was kept constant using an isothermal heating pad (Stoelting). A craniotomy was performed

above the left parietal cortex, and a mild-moderate controlled cortical impact (CCI) injury was performed centered over the somatosensory cortex (coordinates; 2 mm lateral, -2 mm posterior to Bregma) to an impact depth of 1 mm, with a 2 mm diameter round impact tip (speed 3.6 m/s, dwell time 100 msec) and angle of 12º from dura, using an electromagnetically driven CCI injury device (Impact One™ stereotaxic impactor CCI, Leica Microsystems Gmbh, Wetzlar, Germany). The removed bone was replaced but not sealed and the skin sutured. The mice were allowed to recover fully from anesthesia before transfer back to their cages. The control group for all comparisons described herein was comprised of age-matched uninjured male mice.

Tissue isolation and RNA purification

For mRNA analysis, animals were sacrificed by CO_2 inhalation and the brains were immediately removed and frozen in cold isopentane on dry ice, then stored at -80º C. Serial brain sections (300μm thick) were cut by cryostat. Tissue-punches from the sections were taken using a 0.75 mm diameter circular tissue sampling tool (Harris Uni-Core, Sigma-Aldrich) to dissect out the SVZ and the DG only from the hemisphere ipsilateral to the injury. SVZ tissue was collected around the supralateral corner of the ventricle, beginning approximately 0.98 mm anterior and ending -0.34 mm posterior to bregma (50). DG tissue was taken from the anterior intersection of the superior and inferior blades of the DG, at approximately 1.46 mm to 2.30 mm posterior to bregma (50). Tissue punches from 6 animals were combined into each independent sample of either SVZ or DG to obtain sufficient amounts of RNA for PCR array analysis. Dissected tissue punches were immediately placed in Trizol reagent (Invitrogen) and stored at -80ºC. Total RNA was isolated using RNeasy Micro RNA purification kits and genomic DNA removed with RNase-Free DNase (Qiagen). RNA integrity was verified by Experion gel electrophoresis analysis (Bio-Rad) and only samples with a QI of 7 or greater were utilized.

cDNA synthesis, qPCR, and qPCR array analysis

300ng of RNA was used to generate cDNA with the RT2 first strand synthesis kit (SA Biosciences). Quantitative-PCR reactions were then carried out using the TGF-β/BMP signaling pathway PCR array (SABiosciences, PAMM-035), RT2 qPCR SYBR® Green master mixes (SABiosciences) and a Bio-Rad CFX96 real time system (Bio-Rad), following the manufacturer's instructions. Expression of 85 genes related to TGF-β superfamily extracellular and intracellular signaling, were compared between the SVZ or DG of injured and control mice. mRNA expression levels were quantified using the ΔΔCt method, with ΔCt values being normalized to the average Ct value of the five housekeeping genes: β-glucuronidase (Gusb), hypoxanthine guanine phosphoribosyl transferase (Hprt), heat shock protein 90kDa α, class B member 1 (Hsp90ab1), glyceraldehyde-3-phosphate dehydrogenase (Gapdh), and β-actin on the array. We compared gene expression values from 3 independent samples of SVZ or DG tissue from control mice to 3 samples taken from the same area in injured mice at each time point. For individual qPCR analysis of Runx1, two sets of primers were used, corresponding to mRNA isoforms driven by either the P1 promoter or by both the P1 and P2 promoter. The primer sequences were as follows: Runx1 P1 forward primer: 5'TTTCGCAGAGCGGTGAAAGA3' reverse: 5'GCACTGTGGATATGAAGGAA3'; Runx1 P1-P2 forward primer: 5'TGGCACTCTGGTCACCGTCAT3', reverse: 5'GAAGCTCTTGCCTCTACCGC3'.

Bromodeoxyuridine (BrdU) treatment

5-bromo-2´-deoxyuridine (BrdU, Sigma-Aldrich) was dissolved in 0.9% NaCl at a concentration of 10 mg/ml. In order to label all the dividing cells at each post-injury time point, on the day of sacrifice mice received four i.p. injections of the mitotic marker BrdU (100 mg/kg)

delivered once every 2.5 hours over a total of 7.5 hours. Animals were euthanized 30 minutes after last BrdU injection at 1, 3, 7, 14, 30, and 60 days post-injury (dpi) (n=5 /time point).

Double and triple immunofluorescent labeling

For immunohistochemical analysis, animals were sacrificed by transcardial perfusion with 4% paraformaldehyde (PFA) in phosphate buffer. Brains were removed and placed in 4% PFA overnight, then transferred to 30% sucrose solution stored at 4ºC. 30μm thick sections were cut by cryostat and stored in a solution of 30% glucose, 30% ethylene glycol, and 1% polyvinylpyrrolidone in 0.01M phosphate buffer at −80°C until use. Free-floating parallel sections were washed with 0.1% Triton-X100 in 0.1M phosphate buffered saline (PBST) and blocked in PBST/10% normal goat serum (NGS) for 1 hour, then incubated overnight at 4oC in PBST/1% NGS containing Runx1 antisera (dilution 1:2500; kind gift of Dr. Thomas Jessell,) together with one of the cell-specific marker antisera as described in Table 1. Sections were then washed in PBST three times and incubated with the corresponding secondary antibodies (Table 1) for 2 hours at room temperature. Sections were rinsed once with PBST followed by a distilled water rinse before coverslipping with ProLong Gold antifade reagent (Invitrogen). Negative control slides were blocked and then incubated only in secondary antibody solution. No cell labeling was observed in negative controls.

Quantitative analysis of Runx1 and Iba1 positive cells

Quantifications of Runx1, Runx1+/BrdU+ double and Runx1+/BrdU+/Iba1+ triple-labeled cells were performed on a series of 5 randomly selected, parallel, coronal brain sections (30μm thick), along the ipsilateral SVZ (approx. 0.86 mm rostral to -0.34 mm caudal from bregma) or DG (-1.46 mm to -2.30 mm from bregma), using a stereological approach. The first rostral SVZ section was randomly selected for staining and analysis at approximately +0.86 mm

from bregma, and each subsequent section selected was 210 μm caudal to the previous section. From the DG, the first rostral section was randomly selected at approximately -1.46 mm from bregma and each subsequent section was 120 μm caudal to the previous. Five separate animals were analyzed at each timepoint after injury and for the control group. Images (20x) were acquired using an Olympus BX61 fluorescence microscope attached to a Retiga EXi Aqua CCD camera (Qimaging), using iVision software (BioVision Technologies). For the SVZ, images were centered over the supralateral corner of the SVZ. For the DG, the intersection of the superior and inferior blades of the DG was positioned at the medial edge of the visual field (as shown in Fig 3C). Cells counts were quantified by a blinded investigator using Image J software (NIH). Total Runx1 positive cells and total Iba1-positive cells, as well as Runx1-positive cells that were double or triple labeled with Iba1 and/or BrdU were counted in each image. Quantifications of Runx1 colocalization with other cell markers (listed in Table 1) were carried out on 3-5 randomly selected coronal sections from both the SVZ and DG, from 5 control animals and 5 animals from each post-injury group. Data were expressed as the average number of positive cells per square millimeter or percentage out of total Iba1 or Runx1-positive cells.

For colocalization studies, double and triple-stained cells were analyzed using a Zeiss confocal-laser scanning microscope (LSM 510) equipped with argon and He/Ne laser emitting at 488 nm and 568 nm. Images were taken at 20x, 40x and 63x magnification. Sequential Z sections were taken and overlay projections were acquired using Leica software and cropped and adjusted using Adobe Photoshop CS5.

Analysis of Iba1+ cell size and nuclear Runx1 staining intensity

Iba1+ immunoreactivity was thresholded and the average area of Iba1+ cell bodies was determined in each field (20x) using iVision software. The mean intensity of nuclear Runx1

fluorescence intensity in these same Iba1+ cells was also quantified by the iVision software. Data were expressed in gray scale values. All software settings (e.g., exposure time, area etc) were identical for all brain sections and animals.

Statistical analysis

Quantitative data for all figures and tables are expressed as mean ± s.e.m. All statistics were performed using Prism software (Graphpad). Statistical comparison of the qPCR array datasets were performed with a one-way, two-tailed ANOVA for each individual gene, comparing the expression in control animals as well as in each post-injury group, followed by Dunnet's post-hoc t-tests comparing each post-injury group to the control group. One-tailed student's t-tests were used to compare the expression of Runx1 between control and 1 dpi tissue in the DG and SVZ by individual qPCR for each set of primers. One-way, two-tailed ANOVA with subsequent Dunnet's post-hoc t- tests comparing post-injury counts to the control group were used to analyze changes in total Runx1+ and Runx1+/Iba1+ cell counts in the SVZ and DG, as well as to analyze changes in Iba1+ cell area. The mean area of Iba1+ cell bodies was correlated with the nuclear intensity of Runx1 staining using Pearson's correlation with a two-tailed P test.

RESULTS

Traumatic brain injury alters mRNA expression of components of the TGF-β, BMP, and activin signaling pathways in the adult neurogenic regions.

CCI caused significant changes in gene expression in tissue samples from the DG and SVZ, and altered mRNA expression of components of the TGF-β, activin, and BMP signaling pathways at all post-injury time points (Fig 2 and tables 2 and 3). Overall, CCI injury led to a more pronounced alteration in gene expression in the DG than in the SVZ (Fig 2). Gene

expression after injury reflected a general increase in TGF-β and BMP signaling. Expression of the cytokine TGF-β1 was increased in the DG and the SVZ at 1, 3, and 7 dpi (Fig 2). TGF-β2 expression was more variable, increasing in the SVZ at 3 dpi but decreasing in the DG at 7 dpi. TGF-β receptor expression was also generally increased by injury. TβRI (Tgfbr1) and TβRII (tgfbr2) were increased by 7 dpi in the DG. TβRII (tgfbr2) and TβR3 (tgfbr3) were increased in the SVZ at 7 dpi. Rapid stimulation of TGF-β signaling after injury was shown by the large increases in TGF-β1 inducible genes at 1 dpi. Serpine1, Tgfbi, and Runx1 were potently increased at 1 dpi in the DG and SVZ. At 3 and 7 dpi Tgfbi induction persisted in the SVZ. Runx1 induction was biphasic, diminishing at 3 dpi but returning to strong induction again at 7 dpi in both the SVZ and DG. Overall, these results indicate that traumatic injury increases TGF-β signaling in neurogenic regions.

BMP signaling was also increased in the neurogenic regions after injury. At both 3 and 7 dpi, BMP4 was increased in the DG and BMP6 was increased in the SVZ (Fig 2). Several other BMPs were increased after injury (shown in tables 2 and 3), while there was no significant change in expression of the BMP inhibitor Noggin (Fig 2).

CCI specifically induces expression of Runx1 isoforms driven by the proximal P2 promoter.

We were interested in proteins that could influence the injury-induced neurogenesis that occurs in both the DG and SVZ. We therefore focused on the expression of Runx1, a transcription factor that is known to be important for proliferation of hematopoietic stem cells and is expressed in mitotic neuronal precursors in development (171; 179). To verify the qPCR array data, we performed individual qPCR on independent samples to compare the expression of Runx1 mRNA between control tissue and tissue from animals at 1 dpi. Transcription of the

Runx1 gene is regulated by two alternative promoters, termed P1 (distal) and P2 (proximal). These promoters produce transcripts with different 5'UTRs and slightly different N-terminal coding sequences (54). To determine which promoter is active after injury, we generated two sets of primers: P1 and P1/P2. The P1 primer set amplifies a region of Runx1 spanning exons 1 and 2, which are only present in Runx1 isoforms driven by the P1 distal promoter. The P1/P2 primer set amplifies a region spanning exons 4 and 5, which are present in Runx1 isoforms driven by both promoters. The P1/P2 primers amplified transcripts were significantly increased in both the DG and SVZ at 1 dpi (Fig 2D and E), whereas P1 amplified transcripts were unchanged in the DG and significantly decreased in the SVZ at 1 dpi. Therefore, our data indicate that CCI injury induces a specific increase in P2 promoter activity, driving an increase in Runx1 expression in the neurogenic regions.

Runx1 protein is upregulated in the SVZ and DG after CCI injury.

To determine the spatial and temporal expression pattern of Runx1 protein, we stained brain sections taken from control mice or mice that were sacrificed at different time points after CCI injury. Cells with low-level Runx1 immunoreactivity were sparsely dispersed in regions including the DG (135±16 cells/mm2) and SVZ (45±3 cells/mm2) in control mice (Fig 3A, D). After injury, Runx1+ cells were prevalent throughout the ipsilateral hemisphere, in the SVZ and DG (Fig 3B, E), as well as the corpus callosum and prominently in the cortex surrounding the lesion (not shown). Quantitative evaluation showed that the total number of Runx1 expressing cells began increasing at 1 dpi in the DG, and statistically significant increases occurred at 3, 7, 14, and 30 dpi as compared to control mice (Fig 3C). In the SVZ, Runx1+ cell counts began increasing at 1 dpi, were significantly elevated at 7 dpi but returned to control levels by 60 dpi (Fig 3F).

Runx1 is expressed predominantly in microglial cells in neurogenic regions of the adult mouse brain.

After injury, the morphology of Runx1+ cells was suggestive of microglia. We therefore stained brain sections with the microglial marker, Iba1, together with Runx1. In control animals and at all post-injury time points, the majority of cells expressing Runx1 were Iba1+ microglia, in both the DG and SVZ (Fig 4A and C, and 4B and D, respectively). The total number of microglia expressing Runx1 increased following injury in a similar pattern to the number of total Runx1 expressing cells: Runx1+/Iba1+ cell counts were significantly increased over counts from control mice in the DG at 3, 7, and 14 dpi (Fig 4A) and in the SVZ at 7 dpi (Fig 4B). The percent of microglia that expressed Runx1 was significantly increased by injury, particularly at 1 dpi, increasing from ~47% to ~99% in the DG and from ~11% to ~64% in the SVZ (Table 2). There was a small number of Runx1+/Iba1- cells in both regions (Fig 4A and B) that varied over time. In the DG, the number of Runx1+/Iba1- cells was significantly increased at 30 dpi (Fig 4A). As will be discussed, Runx1 was also expressed by some neurons and neural progenitor cells of the DG after injury, and in a small percentage of astrocytes (data not shown). There was a particularly large population of Runx1+ neurons in the DG hilus region at 30 dpi, which likely comprised the majority of these Runx1+/Iba1- cells at this time point (Fig 4F). In the SVZ, these Runx1+/Iba- cells were also most likely neural progenitor cells, ependymal cells, or astrocytes.

Proliferating and reactive microglia cells in neurogenic areas express Runx1 after cortical injury.

Resting microglia in uninjured adult animals have a small nucleus and cell body and multiple branched processes extending outward. In response to injury, microglia become activated and hypertrophic, their processes thicken, branch, and retract, and their cell body increases in size, causing the cells to become amoeboid in shape (for a review see (116)). The

Iba1+ cells clearly exhibited these characteristics. The average size of Iba1+ cell bodies that expressed Runx1 was significantly increased in the DG at 1 and 3 dpi ($p<0.01$), indicating these microglia were activated. In accordance, the average intensity of Runx1 nuclear staining in Iba1+ microglia of the DG correlated significantly with changes in the average size of the microglial cell body from 1-30dpi (Fig 4E, Pearson $r=0.90$, $p=0.01$). The SVZ microglia, however, were less affected by the injury: the average cell body size of SVZ microglia was unchanged after CCI (Fig 4F). Our observations indicated that microglia in the SVZ were mildly activated after injury, although their activation was mostly to an intermediate phenotype whereby their processes thickened and branched without noticeable change in the size of their cell body (data not shown). In the SVZ there was no correlation between the intensity of microglial nuclear Runx1 staining and the size of the microglial cell bodies (Fig 4F).

To determine whether Runx1 expression was associated with cell proliferation, mice were injected with BrdU (100 mg/kg) in a saturation protocol to label all dividing cells at a single time point. On the day of sacrifice at each time point, we injected mice four separate times at 2.5 hour intervals, and sacrificed them 30 minutes after the last injection. Immunohistochemical triple labeling was then performed for Runx1, BrdU, and Iba1. No microglial proliferation was observed in the DG of control mice, but after injury a large population of Runx1+/Iba1+/BrdU+ cells were detected, particularly at 1 and 3 dpi, with a small but sustained population of these cells noticeable until 30 dpi (Fig 4A, C; Table 4). Microglial proliferation was also not observed in the control SVZ, but following CCI, a small number of Runx1+/Iba1+/BrdU+ cells were detected (Fig 4B, D; Table 4). This slight increase was sustained until 30 dpi. Strikingly, in both the DG and SVZ at all time points, virtually all of the microglia that incorporated BrdU after injury expressed Runx1 (Table 4).

Proliferative hippocampal neural progenitor cells and neurons express Runx1 after injury.

Examination of Runx1 staining in the SVZ and DG showed that a small number of Runx1+ cells had a different morphology from microglia, did not express Iba1, and were localized more specifically in the neurogenic niches. Therefore, to examine whether Runx1 was expressed by neural stem or progenitor cells, we performed immunohistochemical staining of the SVZ and DG with antibodies against Nestin, GFAP, Sox2, Mash1, DCX, NeuN, or S100β, together with Runx1. A subpopulation of cells expressing the neural stem and progenitor cell marker protein Nestin in the DG subgranular zone (SGZ) co-expressed Runx1 after injury (Fig 5A, C, D, Table 3). These cells bore the radial glia-like morphology of adult NSCs, with the soma in the SGZ and a radial process extending into the granule cell layer. Many of these Nestin+/Runx1+ cells co-expressed GFAP (Fig 5B, C). Runx1 was also expressed in a subpopulation of Sox2-positive radial cells in the SGZ after injury (Fig 4E). Many NSC marker proteins are also expressed in reactive astrocytes after injury. However, GFAP+/Runx1+ cells of the SGZ did not co-express the mature astrocyte marker S100β (Fig 6), indicating that they were in fact NSCs and not reactive astrocytes.

At 1, 3, and 7 dpi some of the Nestin and Runx1 co-expressing cells of the SGZ incorporated BrdU, indicating that Runx1 is expressed in a proliferative population of neural stem or progenitor cells after injury (Fig 5D; Table 2). We did not observe Runx1+ cells in the DG co-labeled with the immature neuroblast marker DCX+ or with the intermediate neural progenitor cell marker Mash1 at any time point studied.

In later stages, at 14, 30, and 60 dpi, Runx1 expression was colocalized with NeuN in neurons of the DG, mainly in the hippocampal hilus at 14, 30 and 60 dpi (Fig 5F, Table 5). Taken together, these findings suggest that Runx1 might regulate an early event in the response

of the NSC population to CCI injury, and in addition, Runx1 might have a later role during neuronal differentiation in the hilus. A summary of Runx1 colocalization with the NSC markers in the DG and SVZ is shown in Table 5.

Runx1 expression is induced in neural progenitor cells of the subventricular zone after injury.

Runx1 expression was observed in Nestin+ cells of the SVZ at 3, 7, 14, and 30 dpi (Fig 7A-C and Table 3). Interestingly, Runx1 expression was predominantly cytoplasmic in these SVZ cells, in contrast to its nuclear localization in the DG, and was usually observed as punctuate staining in the soma around the nucleus. Some, but not all of these Runx1+/Nestin+ double-labeled cells co-expressed GFAP (Fig 5B). Unlike Nestin+/Runx1+ cells in the DG, we did not detect BrdU incorporation in these SVZ cells at any time point. We also did not see Runx1 co-expressed with Mash1 or DCX in the SVZ, although Runx1 was often expressed in cells immediately adjacent to clusters of DCX+ cells, a characteristic of Type B NSCs (76) (Fig 7D). Our data indicate that in the SVZ, Type B neural stem or progenitor cells early in the NSC lineage express Runx1.

DISCUSSION

In this paper we demonstrate that CCI increases the expression of TGF-β and BMP cytokines in neurogenic regions in adult mice for up to a week after TBI. We also show that Runx1 is an early injury-induced transcription factor expressed in both microglia and NSCs in the adult neurogenic niches. Our data indicate that Runx1 expression is associated with microglial proliferation and activation, processes which themselves impact post-injury cell survival and neurogenesis. TBI also induced nuclear Runx1 expression in subpopulations of

proliferative NSCs in the DG and cytoplasmic Runx1 expression in non-proliferative NSCs of the SVZ, indicating that Runx1 expression could directly regulate to post-injury neurogenesis.

TGF-β superfamily cytokines are essential regulators of adult neurogenesis, and can also mediate changes in neurogenesis in response to injury (1; 60; 130). We have shown that expression of TGF-β1, TGF-β receptors, and TGF-β target genes are increased in the SVZ and DG after traumatic injury, with TGF-β responsive genes showing a dramatic induction as early as 1 dpi. The role of TGF-β in regulation of neurogenesis is complex. The majority of published work indicates that TGF- β signaling is inhibitory to adult neurogenesis, with several studies showing that chronic overexpression or infusion of TGF-β1 to uninjured rodents leads to drastically reduced hippocampal and SVZ neurogenesis (19; 86; 187). This is mediated in part through a direct antiproliferative effect of TGF-β1 on adult NSCs, which halts their cell cycle progression in the G0/1 phase (19; 187), and can prevent the differentiation and progression of Sox2+ NSCs through the neural progenitor lineage (86). Conversely, a few publications have indicated that TGF-β1 can increase NSC proliferation and neurogenesis when administered to animals after certain inflammatory or ischemic injuries (6; 62; 122). Additionally, Smad3 null mice, which have defective TGF-β signaling, show reduced DG and SVZ neurogenesis, though this effect may also be due to impaired activin signaling (193). Therefore, upregulation of TGF-β cytokines and receptors after injury has the potential to affect multiple cell types in the neurogenic niche. This injury–induced upregulation of TGF-β may have multiple opposing effects on neurogenesis. TGF-β1 could facilitate neurogenesis through its anti-inflammatory actions, while inhibiting it through its direct anti-mitotic effects on NSCs. Further, TGF-β1 has neuroprotective functions (103; 110; 122), and could enhance survival of newborn neurons.

Enhanced TGF-β signaling after CCI injury is demonstrated by the strong induction of two TGF-β target genes, Serpine1 and Tgfbi (41; 201) (Fig 1). Both genes are primarily expressed by astrocytes after CNS injury (41; 75; 201). Serpine1 (also called PAI-1) is an extracellular serine protease inhibitor whose major targets include urokinase type plasminogen activators (uPA) and tissue-type plasminogen activators (tPA) (reviewed in (180)). Serpine1 is expressed after CNS injury, can protect neurons against excitotoxic or ischemic lesions (41; 136), and may also modulate microglial activation and migration (83). Serpine1 is therefore part of the endogenous neuroprotective response to injury. However, it has recently been suggested that Serpine1 may exacerbate vascular disruption and worsen outcome in certain ischemic injury models (136; 157). Tgfbi is a secreted extracellular matrix (ECM) protein whose function is not completely clear. It can act as a ligand for several integrins and can regulate the migration/cell adhesion properties of cells (for a review see (178)). Thus, Tgfbi may influence the migratory response of CNS or immune cells after injury, or participate in the post-injury tissue remodeling process.

BMP signaling is a critical and highly regulated modulator of normal adult neurogenesis, and has well documented inhibitory effects on adult NSC proliferation and neurogenesis (14; 113; 130). Increased expression of the BMP inhibitor noggin mediates the elevation in neurogenesis that occurs after voluntary exercise (60), demonstrating that alterations to BMP signaling can underlie changes in neurogenesis. However, our results demonstrate increased expression of many BMP cytokines in the neurogenic regions, without any significant change in noggin levels. Post-TBI neurogenesis therefore proceeds despite increased local levels of BMP expression.

Increased NSC proliferation can begin as early as 4 hours after CCI injury, and persists at 7 dpi (55; 200). Since both TGF-β and BMP can act directly on NSCs to impede their proliferation (19; 130), the increase in expression of these cytokines from 1 to 7 dpi suggests that the environment of the neurogenic niche after injury is not optimal to maximally promote endogenous neurogenesis. Therefore, pharmacologic manipulations to inhibit signaling by TGF-β and BMP proteins in the neurogenic regions could be beneficial to further enhance post-injury neurogenesis and recovery. However, these cytokines regulate many processes after CNS injury, and can have beneficial neurotrophic and anti-inflammatory effects (122; 191), suggesting that combinatorial treatment of TGF-β or BMP signaling inhibitors along with anti-inflammatory and/or neurotrophic treatments may be necessary.

Our qPCR array data show only global changes from the whole of the DG or SVZ, which are obviously composed of many different cell types. They may therefore not reflect smaller more localized changes specifically in microenvironment of the NSCs within the DG or SVZ. The predominant cell type in the DG, the mature neurons of the granule cell layer, may contribute more to changes in RNA expression than the neural stem cells of the SGZ, and local microglia and astrocytes would also contribute. RNA isolated from whole SVZ would reflect changes in ependymal cells, astrocytes and microglia in addition to the NSCs.

CCI strongly induced Runx1 protein expression in cells of both neurogenic regions. Runx1 interacts directly with intracellular Smad proteins and can potentiate Smad activation of target genes (82; 131). It is therefore possible that Runx1 may affect the TGF-β/Activin or BMP Smad signaling pathways after injury. We found Runx1 expression to be a widespread, early component of the microglial response to TBI, primarily in the DG, which is near the injury site, and to a more limited degree in the more distal SVZ. Runx1 is often expressed in hematopoietic

cells, but its expression in microglia has only recently been appreciated. CNS microglia derive from a Runx1 expressing precursor population (58). Further, a recent publication showed that Runx1 regulates the proliferation and activation of early postnatal microglia in the developing CNS (210). In this paper, Zusso and colleagues show that Runx1 inhibits proliferation and activation of early postnatal microglia as they move from an amoeboid, proliferative state to the ramified, quiescent state characteristic of resting adult microglia (210).

In control animals, we found low-level Runx1 expression in roughly 45% and 10% of the total DG and SVZ microglial populations, respectively, and these cells were predominantly ramified. CCI induced dramatic increases in microglial Runx1 expression as early as 24 hours after injury. At this time, the fraction of microglia that expressed Runx1 increased to near 100% in the DG and to 65% in the SVZ. This occurred concomitantly with an increase in the intensity of their nuclear Runx1 expression, which correlated with an increase in microglial hypertrophy in the DG. At all post-injury time points and in both neurogenic regions microglial BrdU incorporation was strongly associated with Runx1 expression, with the percentage of BrdU-incorporating microglia that expressed Runx1 remaining at or near 100% at all post-injury time points, even out to 30 dpi, when the fraction of Iba1 cells expressing Runx1 returned to control levels. The thorough, robust, and early nature of Runx1 expression in microglia, together with its high expression in proliferating microglia after injury places it appropriately to act as a driver of post-injury microglial activation in adult mice. Runx1 is a developmental regulator of microglial activation and proliferation, and has also been found to be upregulated in adult spinal cord microglia following sciatic nerve injury, supporting the idea that it is expressed as part of the injury response (210).

Yet, given that Runx1 was expressed at low levels in ramified microglia of uninjured animals, and that its post-injury expression in the SVZ did not correlate with increased size of the microglial cell bodies, Runx1 expression alone is clearly not sufficient to induce microglial activation. However, in our injury paradigm, the DG was located much closer to the actual site of impact than the SVZ. SVZ microglia were less activated after injury, having no change in the size of their cell bodies. Additionally, a smaller fraction of the SVZ microglial population expressed Runx1, with a peak of 64% expressing it at 1 dpi and only 10-30% at other time points. The increase in the total number of Iba1+/Runx1+ cells after injury was also much smaller in the SVZ than in the DG. The fact that a correlation was observed in the DG and not in the SVZ may indicate that Runx1 is part of the microglial activation pathway, and perhaps necessary for the process, but requires other signals that are present in the vicinity of the lesion.

An alternative explanation for Runx1 function in microglia is that Runx1 may be part of a feedback inhibition response to activation. In such a scenario Runx1 would be expressed after injury to inhibit microglial activation, as it does during cortical development, to prevent an excessive microgliosis response (210), and low Runx1 expression in ramified adult microglia would help maintain their resting state. In other cell types, Runx1 has been shown to either promote or inhibit proliferation (9; 27; 52; 165). Thus, a third possibility is that Runx1 is a necessary transitional component for microglia to move in either direction between a quiescent, ramified state and a proliferative, activated state. Transgenic mouse experiments with temporal microglia-specific knockouts of Runx1 after injury will be necessary to elucidate the role of Runx1 and determine whether it is necessary for the activation or suppression of microglial reactivity and proliferation in adults.

We also found that Runx1 is co-expressed with several well-characterized markers of adult NSCs after CCI injury, indicating that Runx1 may have a direct role in regulating post-injury NSC behavior. The mechanisms driving post-injury neurogenesis are largely unknown, and it is important to define novel factors involved in the process. Runx1 is expressed by neural progenitor cells of the embryonic olfactory bulb to promote their proliferation, and exogenous Runx1 expression increases proliferation of embryonic neural progenitor cells derived from either the olfactory epithelium or cerebral cortex (179). In cell cultures of adult NSCs, high *Runx1* mRNA expression is associated with cell proliferation, and *Runx1* mRNA levels drop when the cells are stimulated to differentiate (15). However, to our knowledge Runx1 expression has not been previously documented in proliferative populations of adult neural progenitor cells.

After CCI, Runx1 is expressed in subpopulations of Nestin+/GFAP+ Nestin+/GFAP-, and Sox2+ cells, specifically in the SGZ of the adult DG. These cells have the distinct radial-glial like morphology of type 1 adult NSCs (129; 167; 194), and some of these cells incorporated BrdU. Within the population of adult SGZ neural progenitor cells, GFAP expression is characteristic of type 1 quiescent adult radial NSCs, while Nestin and Sox2 are expressed in both type 1 NSCs and in the more rapidly dividing type 2 progenitor cells (40; 129). However, Runx1 was not co-expressed with DCX or NeuN in the SGZ or granule cell layer of the DG, suggesting that Runx1 is expressed in early stages of NSCs and is switched off as they begin differentiating into immature migratory neuroblasts or neurons. It is currently unclear whether adult NSCs in vivo constitute a single homogenous population, or rather many different populations of more restricted NSCs and NPCs (129). Thus, Runx1 may be expressed in a specific subpopulation of NSCs to regulate their proliferation and/or differentiation after TBI.

At later time points after injury there was a consistent population of mature NeuN-labeled neurons in the hilus adjacent to the SGZ that expressed somatic and nuclear Runx1. It is possible that Runx1 expression is repressed as NSCs differentiate into neuroblasts, and re-expressed as these neuroblasts become mature neurons in this region. Alternatively, these hilar neurons may be a population of mature neurons unrelated to the NSCs that expressed Runx1 at earlier time points.

In the SVZ, Runx1 was also expressed by Nestin+/GFAP+ and Nestin+/GFAP- cells after injury, but not by Mash1+ cells or DCX+ migratory neuroblasts. The Nestin+/Runx1+ cells were often immediately adjacent to clusters of DCX+ neuroblasts, which suggests that these are type B NSCs (76). Therefore, cortical injury induces Runx1 expression early in the SVZ NSC lineage, and its expression is downregulated upon progression to type C, Mash1+ progenitors. In contrast to the DG, Runx1 expression in the SVZ was primarily cytosolic, and the Nestin+/Runx1+ cells did not incorporate BrdU, indicating they were not proliferative. Nuclear expression in the NSCs of the DG may promote proliferation, whereas cytoplasmic expression in the NSCs of the SVZ may serve a different purpose. Sequestration of Runx1 in the cytoplasm of myeloid and leukemic cell lines can inhibit cell proliferation (24). Additionally, cytoplasmic Runx1 in hematopoietic stem cells reduces NF-ĸB signaling, by inhibiting the activity of IĸB-kinase (137). As NF-ĸB signals may promote proliferation of NSCs (197), it is possible, that cytoplasmic Runx1 is sequestered in the cytoplasm in NSCs of the SVZ to reduce their proliferation.

The induction of neurogenesis after injury in the SVZ and DG may contribute to the ability of the brain to recover from the insult. Understanding the signaling environment of the neurogenic niche and the mechanisms by which neurogenesis is stimulated are paramount in the effort to enhance this endogenous recovery process. We show that CCI elevates potentially

inhibitory TGF-β and BMP cytokine expression in the neurogenic regions, suggesting that post-injury neurogenesis could be enhanced by manipulating these signaling pathways. We also demonstrate that the transcription factor Runx1 is induced in both NSCs and microglia in the neurogenic regions after injury. Our results suggest that Runx1 is involved with microglial activation following injury, and is expressed in a proliferative population of hippocampal NSCs. In the future, cell-specific Runx1 gain- and loss-of-function studies will be extremely interesting to further clarify the role of Runx1 in adult microglial activation, post-injury neurogenesis, and the relationship between the two processes. We expect that Runx1 and its target genes could present valuable targets for therapeutic manipulations in instances of pathologic microglial activation, and in therapies designed to stimulate NSC proliferation.

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Chapter 3: Runx1 promotes proliferation and neuronal differentiation in adult mouse neurosphere cultures

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ABSTRACT

Traumatic brain injury alters the signaling environment of the adult neurogenic niche, and may activate unique proliferative cell populations that contribute to the post-injury neurogenic response. Runx1 is not normally expressed by adult neural stem or progenitor cells (NSPCs) but is induced in a subpopulation of putative NSPCs after brain injury in adult mice. We therefore investigated the role of Runx1 in cultures of adult mouse subventricular zone NSPCs. We show that Runx1 is expressed in adult neurosphere cultures, and removal of the mitogen bFGF or addition of 1% FBS decreases Runx1 expression. Inhibition of Runx1 activity with Ro5-3335 inhibits NSPC proliferation without affecting differentiation. Lentiviral mediated over-expression of Runx1 in neurospheres caused a significant change in cell morphology without reducing proliferation. Lentivirally transduced cells changed from floating spheres to adherent colonies and individual unipolar or bipolar cells. Flow cytometry analysis indicated that Runx1 over-expression produced a significant increase in expression of the neuronal marker TuJ1 and a minor increase in S100 β . Thus, Runx1 expression drove adult NSPCs predominantly towards a neuronal lineage. These data suggest that Runx1 could be manipulated after injury to promote the neuronal differentiation required for repair of the CNS.

INTRODUCTION

Neural stem and progenitor cells (NSPCs) in the hippocampus and subventricular zone (SVZ) are a constant source of new neurons in the adult mammalian brain (129). Following various types of brain injuries, including traumatic brain injury, these NSPCs proliferate and generate new neurons at an increased rate (31; 33; 55; 154; 168; 200; 208). This injury-induced neurogenesis may contribute to post-injury maintenance and recovery of cognitive ability (11), and to the repopulation of neurons in damaged areas (5; 162; 200). However, the regulatory signals that control injury induced neurogenesis and the stem cell population on which they act are poorly understood.

Injury alters the signaling environment of the neurogenic niche (118; 185), and may also activate unique proliferative cell populations that contribute to the post-injury neurogenic response (119). We recently found that expression of the transcription factor Runx1 is induced in a subpopulation of proliferative putative adult NSPCs in the SVZ and dentate gyrus after traumatic brain injury in adult mouse brain, areas where Runx1 is not normally expressed (118). The function of Runx1 induction post-injury in the neurogenic niche is not known.

Runx1 was originally identified in cases of acute myeloid leukemia (AML), in which fusion of the Runx1 gene with the ETO gene produces an oncogenic mutant protein (80; 132). Subsequently, Runx1 was found to be essential for definitive hematopoiesis, regulating the transcription of several critical genes for hematopoietic development (52). In hematopoietic stem cells (HSCs), over-expression of full length Runx1 causes murine HSCs to exit the cell cycle and become quiescent, whereas over-expression of an endogenous truncated dominant negative Runx1 (Runx1a) leads to increased proliferation (27; 182). In numerous other cells types Runx1

regulates cell proliferation, sometimes promoting, and sometimes inhibiting, cell division (13; 52; 71; 158). Runx1 is primarily a sequence specific DNA binding protein. Runx1 stability and DNA binding is enhanced approximately ten-fold through complexing with the protein, CBFβ (17; 175; 176). Both proteins are necessary for normal Runx1 function in vivo, as demonstrated by the similar phenotype of mice that are null for either Runx1 or CBFβ (190).

Runx1 also controls the proliferation and neuronal differentiation of certain neural progenitor cell populations. In embryonic olfactory bulb progenitor cells *in vivo*, as well as in cultures of either embryonic olfactory progenitor cells or embryonic cortical NSPCs, Runx1 increases cell proliferation and also increases the expression of the neuronal marker protein NeuroD (179). However, Runx1 inhibits the proliferation of embryonic microglia (210) and olfactory ensheathing cells (135). Given its ability to regulate stem and progenitor cell proliferation and differentiation, induction of Runx1 in some NSPCs after injury (118) suggests that Runx1 could be regulating the injury induced proliferation or neuronal differentiation of a specific subpopulation of adult NSPCs.

We therefore investigated the expression and function of Runx1 in adult NSPCs, using neurosphere cultures of adult mouse NSPCs. We demonstrate that Runx1 is expressed in adult neurosphere cultures, and expression decreases in different culture conditions. We found that inhibiting Runx1 activity can inhibit adult NSPC proliferation, and that induction of Runx1 through lentiviral mediated over-expression pushes NSPCs predominantly towards a neuronal lineage. Thus, Runx1 may play an important role in the injury-induced neurogenesis seen in the adult mammalian brain after models of traumatic brain injury.

MATERIALS AND METHODS

Adult neurosphere cultures.

All animal care and procedures were approved by the Institutional Animal Care and Use Committee at Uniformed Services University and performed in accordance with their guidelines. Adult male C57/BL6 mice (NCI, Frederick, MD), approximately 9 weeks of age, were deeply anesthetized by isofluorane inhalation and euthanized by decapitation. Brains were excised, placed in ice-cold PBS, and the tissue adjacent to the anterior portion of the lateral ventricles containing the SVZ carefully dissected out. This tissue was homogenized (Miltenyl neural tissue dissociation kit, Miltenyi Biotec, MA), passed through a 70μM filter to obtain a single cell suspension, and plated in Neurocult complete culture media (Stem Cell Technologies, WA, dfe#05702) supplemented with 20ng/mL EGF, 20ng/mL bFGF, and 2μg/mL heparin ('growth media'). These primary cultures were grown initially for 7 days. Cells were then passaged every 5 days for subsequent passage numbers. Cells from passages 3-5 were used for all experiments. For treatment of neurosphere cultures with the Runx1 inhibitor Ro5-3335 (Calbiochem, MA, #219506), Ro5-3335 was resuspended at 250mM in DMSO, and further diluted in sterile PBS for treatments. The highest concentration of inhibitor resulted in a 0.1% DMSO concentration. Thus 0.1% DMSO was used as a control. For treatment with Ro5-3335, cells were plated at a density of 6 x 10^5 cells/100mm dish. For lentiviral transduction experiments, cells were plated in 48 well culture dishes at a density of 2,000 cells/well.

Lentivirus generation and treatment

Lentiviral plasmids pLenti-suCMV(Runx1)-Rsv(GFP-Bsd) (termed LV-Runx1) and LV-PL3 (LV-RSV(GFP-Bsd) (termed LV-GFP) were purchased from GenTarget inc (Gentarget, CA). Lentiviral particles were packaged in human embryonic kidney 293T cells, in serum-free media, by lipofectamine transfection with LV-Runx1 or LV-GFP plasmid together with VSVG and delta 8.2 packaging plasmids. Media was changed 12 hours after the transfection, and then

viral particles were harvested from the media of transfected cells 48 hours later. This media was centrifuged at low speed to clear large debris, filtered through a 0.45μM filter, then concentrated by ultracentrifugation at 25,000g for 2hr, and resuspended in sterile PBS. Viral titer was determined by analysis of GFP expression in HEK 293T transduced cells, 72 hours after viral transduction by flow cytometry. Neurosphere cultures were transduced by LV-Runx1 or LV-GFP at an MOI of 10.

Protein extraction and western blots

Neurospheres were grown in media containing the indicated growth factors for 24 hours before harvest. Both floating neurospheres and loosely adherent cells were collected, pelleted by low speed spin at 4ºC, washed with ice cold PBS, and resuspended in 1% RIPA lysis buffer containing protease and phosphatase inhibitors. Cells were lysed before centrifugation at 1600g, at 4ºC for 20 minutes. Protein concentration in the supernatant was determined by the BCA protein assay (Pierce, Rockford, IL, USA). 15 μg protein was combined with loading buffer, boiled for 5 min, before loading onto an SDS-polyacrylamide gel. Separated proteins were transferred onto nitrocellulose membranes, and probed with rabbit anti-Runx1 antisera (1:1000, Novus, #NBP1-89105), or rabbit anti-β-actin (1:8000, Sigma, #A1978-200), followed by incubation with goat anti-rabbit IgG HRP-linked secondary antibody (Cell Signaling, MA). Blots were developed with supersignal enhanced chemiluminescence reagent (Thermo Scientific) and detected using a Fuji LAS-3000 image acquisition system (Fuji, Stamford, CT, USA) equipped with a cooled CCD camera. For each experiment, blots were first probed with the Runx1 antiserum, before being stripped and reprobed for β-actin. Runx1 expression was normalized to the expression level of β-actin.

RNA isolation and qPCR

Cells were harvested, washed with ice cold PBS and resuspended in TRIzol reagent (Invitrogen, CA). RNA was isolated by adding 0.2 volumes of chloroform, centrifuging at 16000g for 15min, and extracting the aqueous layer. Further purification proceeded with the RNeasy mini kit (Qiagen, MD). Isolated RNA was then incubated with RNase-Free DNase according to the manufacturer's instructions and quantified with a Nanodrop spectrophotometer (Thermo Scientific, DE). RNA integrity was checked by agarose gel electrophoresis. qPCR was performed with SYBR Green qPCR MasterMix (Qiagen, CA) using the BioRad CFX96 instrument and the following gene-specific primers: Runx1; forward 5'TGGCACTCTGGTCACCGTCAT3', reverse 5'GAAGCTCTTGCCTCTACCGC3', cyclin D1; forward 5'AGAGGGCTGTCGGCGCAGTA3', reverse 5'GGCTGTGGTCTCGGTTGGGC3', p21; forward 5' TCCAGGAGGCCCGAGAACGG3', reverse 5'CTCCGAACGCGCTCCCAGAC3'. GAPDH; forward 5'ACCATCTTCCAGGAGCGAGA3', reverse 5'GGCGGAGATGATGACCCT3'. Reactions consisted of 10 minutes incubation at 95ºC, then 40 cycles of denaturation at 95ºC for 15 seconds and annealing/elongation at 58ºC for 10 seconds. Expression levels of all genes of interest were normalized to GAPDH, and relative changes in gene expression were calculated using the ΔΔCt threshold cycle method (169).

Cell culture imaging and quantifications

Neurospheres were treated 24 hours following passage with either different concentrations of Ro5-3335 or lentiviral particles. Cells were analyzed 5 days after treatment. Each dish was divided into four quadrants by marking the cap, and an experimenter blind to the treatment conditions randomly captured one image from each quadrant and one from the center of the plate. The image fields captured covered roughly a 3.75mm^2 area, and images were

analyzed with ImageJ software (NIH) to quantify the number of neurospheres, adherent colonies, and morphologically differentiated cells in each image, as well as to measure the average diameter of the neurospheres. In each experiment at least three culture dishes were treated for each condition, and measurements from each of the dishes were averaged. Each experiment was replicated 3 times.

Cell harvest and processing for flow cytometry and cell survival analyses.

Cells were treated as described and on the fifth treatment day, cells were harvested and washed in ice cold PBS, pelleted again, and incubated with 0.025% trypsin with EDTA in PBS at 37ºC for 10 minutes. 4 mL of growth media was added and cells were triturated to obtain a single cell suspension. Cells were then pelleted and resuspended in 2 mL PBS, and passed through a 70μM filter to obtain single cell suspension prior to further processing.

Analysis of proliferation

Cells were treated as indicated for five days. On the fifth day, EdU was added (final concentration 100μM), and 7 hours later the cells were harvested. Cells were then fixed and processed using the Click-iT flow cytometry assay kit (Invitrogen, CA, #C10424) with EdU covalently bound to an Alexa Fluor 647-coupled fluorescent molecule. EdU incorporation was visualized using a BD Accuri C6 flow cytometer and CFlow plus software (BD biosciences, CA). Events were gated for cells based on forward and side scatter parameters to exclude cell debris or clusters. A minimum of 10,000 gated cells were quantified for each condition.

Cell survival analysis

Cells were treated in duplicate with the indicated concentrations of Ro5-3335. On the fifth day, cells were harvested as described, and for each sample, a 10μL aliquot of cell solution

in PBS was removed and combined with equal volume of trypan blue solution (0.4%, BioRad, CA). The percentages of cells excluding the dye in each sample were determined using a Biorad TC10 automated cell counter (BioRad, CA).

Flow cytometry for differentiation markers

Cells were harvested, fixed and permeabilized for intracellular antigen staining using the Fox3P/Transcription Factor Staining Buffer set (eBioscience, CA, #00-5524), following manufacturer's protocols. Cells were immunostained with the primary antibodies: mouse anti-Tuj1 (1:12.5, Promega, WI, #G712A), mouse anti-GFAP (1:50, Millipore, MA, #MAB360), mouse anti-S100β (1:50 Sigma Aldrich, MO, #S2532), or guinea pig anti-doublecortin, (1:100 Milllipore, #AB2253) followed by secondary antibody staining with goat anti-mouse Alexafluor 405 (1:1000 Invitrogen, CA, #A31553) or goat anti-guinea pig Alexaflour 647 (1:1000 Invitrogen, #A21450). Cell counts were quantified using a BD LSR II flow cytometer (BD biosciences, CA). Events were gated for cells based on forward and side scatter parameters to exclude cell debris or clusters. A minimum of 6,000 cell events were quantified for each measurement. Population analyses were then performed using FlowJo software (TreeStar inc, OR).

RESULTS

Adult neurospheres express Runx1 protein at highest levels in growth-promoting conditions.

Runx1 was expressed in adult mouse neurospheres cultured in typical growth media containing epidermal growth factor (EGF), a required trophic and mitogenic factor (153), and basic fibroblast growth factor (bFGF, FGF2), also a potent mitogen (57; 104; 184; 188) (Fig 7A). Removing bFGF from the media reduced Runx1 expression, and addition of 1% fetal bovine serum (FBS) reduced Runx1 expression further (Fig 8A,C). Removal of bFGF resulted in smaller neurospheres without any change in morphology (Fig. 8D). As previously described, addition of 1% FBS resulted in NSPC differentiation primarily into astrocytes (Fig 8D) (15). We did not, however, detect any significant change in *Runx1* mRNA expression in these different culture conditions suggesting that Runx1 regulation was post-transcriptional (Fig 8B).

Inhibition of Runx1 transcriptional activity inhibits neurosphere growth.

The compound Ro5-3335 is a potent inhibitor of the interaction between Runx1 and its partner protein CBFβ (36), and inhibits Runx1 mediated transcriptional regulation although Runx1 remains able to bind DNA (36). To examine the function of Runx1 mediated transcription in neurospheres, we treated neurospheres cultured in growth media (containing EGF and bFGF) with increasing concentrations of Ro5-3335 for five days. The size of the neurospheres formed decreased significantly when cultured in 5 or 25 μM Ro5-3335 without a decrease in the total number of neurospheres (Fig 9A, B). 5 μM and 25 μM Ro5-3335 was reported to reduce Runx1 driven luciferase reporter activity by approximately 10 and 50%, respectively (36). Thus inhibition of Runx1 activity led to a decrease in the overall proliferation rate or proliferative fraction of the NSPCs within the neurosphere.

To more directly determine whether inhibition of Runx1 activity led to a decrease in the rate of proliferation, we determined the amount of incorporation of EdU (a thymidine analog) into DNA by neurosphere cells after a five day treatment with different concentrations of Ro5-

3335 (Fig 10A, B). After a seven hour pulse of EdU, cells were fixed, processed and analyzed by flow cytometry. Ro5-3335 inhibitor treatment reduced the percentage of cells within neurospheres that incorporated EdU from approximately 40% in control media to 30% after treatment with 25 μM Ro5-3335 (Fig 10A). This corresponds to an approximately 25% decrease in the percentage of cells which incorporated EdU. The amount of EdU incorporated into cells also dropped, with a 50% decrease in the mean fluorescence intensity of the EdU signal, in cells treated with 25 μM Ro5-3335 compared to control cells (Fig 10B). Cell survival was not altered by treatment with any concentration of Ro5-3335 for 5 days (Fig 10C). Thus, inhibition of Runx1 activity with Ro5-3335 led to a reduction in the overall proliferation of neurosphere cultures.

Treatment with 25 μM Ro5-3335 induced expression of mRNA encoding the cell cycle regulators *p21* (CDKN1A, $p=0.0505$) and *cyclin D1* (CCND1 $p<0.05$) approximately 2-fold after a 5 day treatment (Fig 10D, 10E). Both of these genes are direct targets of Runx1 gene transcriptional regulation (155; 179) and thus their induction acts as a positive control for inhibition of Runx1 transcriptional activity by Ro5-3335.

Runx1 over-expression induces morphological differentiation of neurosphere cells.

To determine the function of Runx1 in neurospheres we developed a lentiviral overexpression system for Runx1. Human Runx1b was expressed under the control of a modified high activity CMV promoter (suCMV), in the same plasmid as RSV-GFP, in order to visualize transduced cells (LV-Runx1). Human Runx1b is most homologous to murine Runx1 isoform 3 (Fig 11), an isoform which was actively expressed in our neurosphere cultures (Fig 11). The control lentiviral vector expressed only GFP under the identical RSV promoter in the same plasmid backbone (LV-GFP).

Neurospheres transduced with lentiviral particles containing LV-Runx1 showed a dramatic change in morphology (Fig 12). Treatment with LV-Runx1 induced the majority of cells to form colonies of adherent, irregularly shaped cell aggregates or to dissociate to individual adherent cells (Fig 12 a^{iii}, a^{iv} C, D). These adherent cells typically bore a small cell body with one or two long thin processes, often terminating in slightly bulbed end feet, reminiscent of the leading process of migratory neuroblasts. These cells could be seen growing alone and also within the cell clusters (Fig 12 a^{iii}, a^{iv}, a^{v}). Transduction with the control lentivirus, LV-GFP, had no effect on neurosphere morphology as the control transduced neurospheres grew similar to PBS-treated neurospheres, with spherical, floating colonies of small spherical cells with almost no adherent cells detected (Fig 12 a^{i}, a^{ii}, C, D). Quantification of neurospheres and adherent cells revealed an almost complete mutual exclusion between the number of floating neurospheres and the number of adherent aggregates (Fig 12C, D), with the total number of these cell aggregates remaining constant between treatment groups (Fig 12F). Thus, LV-Runx1 transduction resulted in a dramatic decrease in the density of floating neurospheres and a significant increase in the number of adherent aggregate cell formations counted (Fig. 12C, D).

The similarity in the total number of floating and adherent aggregates between LV-Runx1 and LV-GFP treatments suggested that there may be no change in the proliferation rate under these different conditions, despite dramatically different morphologies. We therefore determined the amount of EdU incorporated into lentiviral transduced neurosphere cultures. There was no difference in the percentage of cells incorporating EdU, either in all cells in the culture (Fig 12G) or specifically in lentivirally transduced GFP+ cells between cells transduced with LV-Runx1 or

LV-GFP (Fig 12H). Thus, overexpression of Runx1 induced a clear morphological change in many of the cells, but did not alter their proliferative capacity.

Runx1 over-expression induces the expression of primarily neuronal lineage markers.

To determine whether the morphological change after Runx1 overexpression was a result of a change in the differentiation of the NSPCs we examined expression of cell-specific markers for neurons and astrocytes. As LV-GFP transduced cells consistently exhibited no morphological differences from untreated control cells, these free-floating neurospheres were impossible to quantitate thoroughly by standard immunostaining techniques in our hands. We therefore employed flow cytometry analysis to quantify the percentages of cells transduced by either LV-GFP or LV-Runx1 that expressed various markers of differentiated cells (Fig 13). Overexpression of Runx1 in LV-Runx1 transduced cells increased the percent of cells expressing doublecortin (DCX), a marker of immature neuroblasts from 20.3% in the control LV-GFP transduced cells to 27.3% (Fig 13A). Runx1 overexpression also resulted in a striking increase in the population of cells expressing high levels of Tuj1, (also called ß-III-tubulin), a cytoskeletal protein that is a well-established marker of differentiated neurons within the central nervous system (89). Transduction by LV-Runx1 particles increased the percentage of Tuj1 + cells from 2.2% of cells in control LV-GFP cultures to 32.9% (Fig 13B). These data show that overexpression of Runx1 in neurosphere cultures promoted the differentiation of neuronal precursor cells and differentiated neurons in a significant percentage of NSPCs.

To determine whether there was any change in astrocytic differentiation we also examined expression of S100B, an astrocytic marker that is not expressed in adult NSPCs (8; 152). LV-GFP transduced cultures exhibited significant expression of S100B, with 24.4% of cells expressing this marker, showing that a quarter of the cells within the neurospheres have

already differentiated along the astrocyte lineage under our culture conditions. Overexpression of Runx1 through LV-Runx1 transduction increased the number of cells expressing S100β by 11%, to 35. 3% of the total cell population (Fig 13D). Thus, Runx1 overexpression resulted in an overall differentiation of the NSPCs, along both astrocytic and neuronal lineages, but with a more significant promotion of neuronal differentiation. Interestingly, this differentiation was not accompanied by decreased proliferation.

DISCUSSION

The induction of Runx1 in a subpopulation of putative adult NSPCs in the SVZ and hippocampus following traumatic brain injury provided the rationale for examining the function of Runx1in NSPCs in neurosphere culture (118). The data we present here indicate that Runx1 could be regulating the post-injury proliferative or neuronal differentiation response in these cells. We demonstrate that endogenous Runx1 expression is highest in proliferating NSPCs *in vitro*, that it drops on mitogen removal, and that NSPC proliferation is reduced upon Runx1 inhibition. Runx1 expression is lowest when bFGF is removed and neurospheres are stimulated to undergo astrocytic differentiation by 1% FBS treatment. Runx1 over-expression produced a dramatic morphological change in the neurosphere cultures, causing many cells to acquire a shape reminiscent of immature neurons or neuroblasts. This morphological change was accompanied by a large increase in the expression of the neuronal proteins DCX and Tuj1, with a much smaller increase in the expression of the astrocytic protein S100β. Overall, our results suggest that Runx1 is necessary for NSPC proliferation in cultured adult neurospheres, and promoting a pro-neuronal fate choice on cell differentiation. This model is summarized in figure 14.

bFGF is a pro-mitotic factor for cultured NSPCs, and can stimulate the proliferation of pro-neuronal progenitors (104; 184; 188). Here, we demonstrate removing bFGF from the media causes a significant drop in Runx1 protein expression (Fig 1). Thus, bFGF may directly stimulate Runx1 expression and NSPC proliferation, providing a link between Runx1 levels and neurosphere proliferation. Runx1 expression was further decreased upon addition of 1% FBS, a treatment that induces nearly exclusive astrocytic differentiation (15). Together these data indicate that Runx1 is expressed highest in neurosphere cells that are in an undifferentiated, highly proliferative state. As NSPCs are stimulated to undergo astrocytic differentiation, there is a concomitant decrease of Runx1 expression, possibly as part of an overall down-regulation of pro-neuronal differentiation pathways. These findings are in agreement with Bonnert and colleagues (15), who found the highest *Runx1* mRNA expression in neurospheres in growth media. *Runx1* mRNA expression dropped upon stimulation of neurospheres to differentiate by either removal of EGF and bFGF, or by treatment with 5% FBS. They also found that *Runx1* mRNA expression was enriched in FACS sorted cells isolated directly from the SVZ that were selected for their high expression of proliferative markers (15).

Inhibition of endogenous Runx1 activity with Ro5-3335 led to a decrease in neurosphere size as well as the percentage of cells incorporating EdU (Fig 2). Thus, inhibition of Runx1 activity results in decreased NSPC proliferation, suggesting that endogenous Runx1 protein expressed in neurosphere cultures is necessary to maintain normal levels of cell division in growth media. Although Ro5-3335 has not been demonstrated to affect any other pathways known to regulate NSPC proliferation, we cannot exclude the possibility of potential non-specific effects of the compound that are unrelated to Runx1 signaling. Inhibition of Runx1 activity with Ro5-3335 also increased the expression of *p21* and *cyclin D1* mRNA. Both of these

genes are immediate downstream targets of Runx1 regulation (155; 179). p21 is directly repressed by Runx1 in mouse embryonic cortical progenitor cells (179), and when induced is able to inhibit proliferation of many cells (26). The cyclin D1 gene (CCND1) is also a direct transcriptional target of Runx1, but inhibition of Runx1 can either induce or inhibit CCND1 expression (134; 186). Transcriptional regulation by Runx1 is highly context dependent: Runx1 can either activate or repress gene transcription in different cellular contexts (164). Cyclin D1 induction is typically associated with increased cell proliferation as it usually acts as a promoter of mitosis. However, this is not universal and cyclin D1 is sometimes increased in instances of growth inhibition (149). Indeed, in neurospheres cultured from adult mice, TGF-ß1 induced expression of both cyclin D1 and p21 proteins, concurrent with a potent inhibition of neurosphere proliferation (149). Thus, co-induction of p21 and cyclin D1 may be a common mechanism of inhibition of neurosphere proliferation.

Overexpression of Runx1 did not lead to any alteration in the proliferation of neurosphere cells, despite a dramatic increase in differentiation and morphology usually associated with post-mitotic cell populations in vivo. Theriault and colleagues showed that Runx1 over-expression in culture elevated expression of the proliferative marker Ki67, together with NeuroD1, a typical marker of mature post-mitotic neurons. They also found Runx1 to be expressed in NeuroD1-positive cells that were proliferative in vivo (179). Inhibition of Runx1 with Ro5-3335 inhibited NSPC proliferation while overexpression of Runx1 did not alter proliferation; this suggests that a threshold of Runx1 activity is necessary to support NSPC proliferation in neurospheres in normal growth media. However, Runx1 does not appear to be a rate limiting proliferative factor for neurospheres in normal growth media, as further increasing Runx1 expression does not further elevate proliferation.

Neurosphere cultures contain a heterogeneous mixture of cells, including self-renewing, tripotent stem cells, rapidly dividing intermediate progenitors, which can undergo a limited number of divisions before differentiating into the neuronal or glial lineages, more restricted glial or neuronal progenitor cells, as well as post-mitotic, differentiated cells. These cultures additionally contain dead cells, which exist in the core of the neurosphere colonies and have died due to the restricted flow of nutrients (57; 150; 153). In our proliferative neurospheres we detected a population of DCX+ neuronal progenitor cells (roughly 20%) as expected. Runx1 over-expression induced a small increase in the percentage of cells expressing DCX, suggesting that Runx1 can push stem cells to develop into these DCX+ neuronal-restricted progenitors. Runx1 overexpression also induced a sizable population of cells expressing high levels of Tuj1 (about a third of the total cell population), whereas this cell population was virtually absent in lentiviral transduced controls. Expression of Tuji1 corresponded with a distinct neuronal-like morphological change. As we treated with LV-Runx1 for 5 days it is possible that many of the stem cells progressed through the DCX+ stage to the more differentiated Tuj1+, DCX- phenotype. Thus, a shorter treatment may have resulted in a greater DCX+ transition. Our data show that Runx1 can act as a pro-neuronal differentiation factor in adult NSPCs. There was also a slight increase in the expression levels of S100β protein after LV-Runx1 treatment (Fig 5). As S100β protein is unique to differentiated astrocytes and not expressed in NSPCs (8; 152), our results indicate that there is a basal level of astrocytic differentiation in neurosphere cultures, and that Runx1 over-expression slightly increased this occurrence.

Given the heterogeneous nature of the neurosphere cultures, the divergent differentiation effects could be due to the actions of Runx1 over-expression in different cell populations within the neurosphere cultures. Unfortunately, since the protein markers we were investigating are all

intracellular, the cells had to be fixed and porated in order to perform the immunostaining necessary for flow cytometry quantification, and this led to a near complete loss of the GFP protein and signal produced by the lentiviral treatment. As such, we were unable to quantify the percentages of cells expressing the differentiation markers specifically within cells definitively transduced by our lentiviral treatment, and were limited to quantifying changes within the entire cell population. Thus, we cannot distinguish which effects were cell intrinsic and which may be downstream effects of cell-cell signaling within the culture. Intercellular communication has a significant impact on the behavior and phenotype of adult neurosphere cultures, and differentiating cell populations can induce other cells within the culture to differentiate as well.

Our experiments suggest that Runx1 may be a useful target to manipulate in order to direct the endogenous NSPC proliferative response towards maximal neurogenesis following traumatic brain injury. Runx1 over-expression has been demonstrated to facilitate neuronal differentiation and long-range axonal growth in cells derived from embryonic human spinal cord and transplanted into adult rat dorsal root ganglia (101). Our studies suggest the potential for a similar effect when using neural stem cells isolated or induced from adult tissue. Stimulation of Runx1 expression in neural stem cells may enable these cells to maintain their neuronal potential when transplanted and hence enhance the repair and repopulation of the damaged CNS.

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Chapter 4: Discussion

TBI involves widespread cell death and neuronal loss, but also produces a robust proliferative response from adult stem and progenitor cells in the SVZ and DG. Treatments that facilitate NSPC proliferation can improve outcome following brain injury (67; 96; 206), while treatments that inhibit neurogenesis are can impair measures of behavioral and cognitive recovery(11; 206). By facilitating and appropriately directing the proliferative response it may be possible to improve recovery from TBI. However, the signals that regulate NSPC proliferation and neurogenesis after injury are not fully understood, nor is the full complexity of the heterogeneous adult stem and progenitor cell population itself.

We examined the expression of signaling molecules within the neurogenic niches from several major signaling pathways that are affected by CNS trauma at the site of a lesion, and found significant changes occurring in these pathways in the neurogenic regions as well. This work provides valuable insights into changes related to the TGF-β, BMP, and activin signaling pathways, all of which are powerful regulators of adult NSPC division and neurogenesis.

We identified a robust expression of Runx1 protein in post-injury microglia that correlated with increases in microglial cell size in the DG, and describe an interesting association of BrdU incorporation with Runx1 expression, suggesting that Runx1's regulatory roles in microglial development may be in some way recapitulated to regulate their division or activation following CNS trauma.

We also describe the novel expression of Runx1 protein in adult NSPCs after injury, though it is absent from these cells in the uninjured brain. We provide evidence that Runx1 is a

regulator of adult NSPC proliferation and can stimulate their predominantly neuronal lineage differentiation.

TBI alters expression of TGF-β superfamily genes in both neurogenic regions.

To obtain a broad overview of changes that may be occurring in the major branches of the TGF-β superfamily we utilized qPCR arrays, comparing mRNA levels in the SVZ or DG from control uninjured mice to their expression levels at 1, 3, or 7 days following CCI injury. We found significant changes in many genes, but of the greatest interest were the changes related to the BMP, TGFβ1, and activin-A signaling, described below.

TBI increases expression of BMP cytokines and receptors with concomitant decreases in expression of BMP inhibitors

In the SVZ there was an upregulation of *BMP 2* and *4* mRNA expression out to one week after injury, while in the DG, *BMP 2, 6,* and *7* mRNA were increased. There was no increase in expression of the BMP inhibitor Noggin, and in fact a large decrease in DG expression of BAMBI (BMP and activin membrane-bound inhibitor homolog), another BMP signaling inhibitor. This indicates that increased BMP inhibitor activity is not part of the mechanism of post-TBI neurogenesis, as it is in exercise-induced neurogenesis (60). Rather, there is an increase in BMP signaling in the neurogenic niches following TBI injury. These BMP cytokines have been clearly demonstrated to inhibit NSPC division and neurogenesis in uninjured adult animals and in adult NSPC cultures (113; 130). BMP signaling can also push differentiating NSPCs towards an astroglial rather than neuronal fate (23; 160; 192). There is a great deal of astroglial differentiation from NSPCs following injury. Thus, the elevated BMP expression we observed may be a contributing factor to astroglial differentiation. The drop in BMP inhibitor expression with injury suggests that exogenous administration of the noggin inhibitor or inhibitors of BMP

transmembrane receptors could have a significant effect on promoting NSPC differentiation. Thus, therapeutic administration of BMP inhibitors may be able to negate the elevated post injury BMP signaling, and facilitate NSPC proliferation and subsequent neuron generation.

TBI increases expression of TGF-β cytokines, receptors and downstream target genes.

TGF-β1 expression was consistently induced in both regions at all time points observed, and there was a drastic increase in expression of TGF-β target gene *Serpine1*, particularly at 1 dpi, and persisting out to one week in the DG. *Tgfbr1* and *Tgfbr2* TGF-β receptor mRNA were also increased out to one week after injury in both regions. TGF-β1 has been clearly demonstrated to have a direct negative effect on the proliferation of NSPCs, suggesting that TGF-β receptor inhibitors, particularly directed at Tgfbr1 and Tgfbr2 receptors could facilitate post injury NSPC proliferation and thereby stimulate increased neurogenesis.

The net effects of elevated TGF-β and BMP expression in the post-injury environment may differ from their effects in non-injured animals.

TGF-β and BMP cytokines have clear, direct, repressive effects on NSPC proliferation, yet our results indicate that post-TBI neurogenesis occurs in an environment where their expression is upregulated. This is likely due to the fact that TGF-β and BMP proteins are important endogenous anti-inflammatory molecules. Inflammation is itself a potent repressor of adult neurogenesis (44; 133), and repressing TGF-β or BMP signaling may in fact exacerbate the inflammation-mediated repression of neurogenesis. Indeed, TGF-β and BMP treatments have been shown to actually facilitate neurogenesis in some post injury situations (6; 35; 122), likely due to their anti-inflammatory actions. This would need to be taken into account when designing neurogenic interventions. However, simply co-administering a general anti-inflammatory treatment along with BMP or TGF-β inhibitors, could potentially control inflammation and still

prevent these pathways from imposing their direct anti-neurogenic effects on the NSPC population. Indeed, administration of general anti-inflammatory treatments are capable of elevating post-injury neurogenic responses after brain injury (1; 70).

Additionally, caution would have to be taken to ensure that any treatment did not produce a repression of neurogenesis or depletion of the NSPC population in the chronic stage at late time points after treatment, as was demonstrated in uninjured animals after long term noggin treatment (130).

TBI decreases expression of activin inhibitors.

We found a drop in BAMBI expression, which binds and inhibits BMPs and activin-A. There was also a very large drop in expression of the activin inhibitor follistatin in both the SVZ and DG. Mice that overexpress follistatin in hippocampal neurons have normal rates of NSPC proliferation in the DG but impaired survival (2). Continuous follistatin over-expression leads to the death of almost all of the newly formed neurons within weeks, resulting in essentially no adult neurogenesis (2). These experiments strongly implicate endogenous activin as a crucial survival factor for immature neurons or neuronal precursor cells. Activin is critical for neurogenesis to occur following direct excitotoxic injury to the DG, as a requisite endogenous anti-inflammatory agent (1). The post-injury decrease in activin inhibitor expression may serve to facilitate neurotrophic activin-A signaling on newborn DG neurons and also repress inflammation. The decreased expression of activin inhibitors after injury also indicates that the neurogenic regions would be a permissive environment for activin signaling. Thus, exogenous activin protein could be a potential pro-neurogenic and neuroprotective/neurotrophic treatment for newly generated DG neurons.

TBI induces robust microglial expression of Runx1

Runx1 protein expression was strongly induced in microglia of both neurogenic regions following TBI. This effect was more pronounced in the DG than the SVZ, due to its closer proximity to the lesion site. Expression was highest in the acute stage, and Runx1 microglial expression returned to control levels in both regions by 7 dpi. In the DG, the increases in Runx1 microglial nuclear staining intensity correlated with increases in microglial hypertrophy. Hypertrophy is an indicator of microglial activation and this suggests Runx1 expression is associated with microglial activation near the lesion site. The immediate expression of Runx1 throughout the microglial population after CCI, particularly in and around the DG neurogenic niche, suggests that Runx1 could be involved in promoting or activating the acute inflammatory response of microglia to injury. The acute microglial response to injury can impede neurogenesis (196). Therefore, Runx1 and its downstream signaling targets in microglia may represent useful therapeutic drug targets for controlling microglial activation in the neurogenic niche, and limiting their ability to inhibit post-injury neurogenesis.

However, the lack of a correlation between these two factors in the SVZ dictates that Runx1 expression alone is not sufficient to induce microglial hypertrophy, and also suggests the alternate explanation that Runx1 may in fact be activated as part of feedback inhibition loop to prevent an excessive microglial response after injury. This possibility is supported by recent work demonstrating that embryonic expression of Runx1 inhibits activation in cultures of murine embryonic microglia, and stimulates the transition of hypertrophic, amoeboid embryonic microglia to the ramified resting state seen in uninjured juveniles and adults (210).

Runx1 expression also inhibits proliferation of cultured embryonic microglial cells (210). However, it is difficult to reconcile this function with the Runx1 expression pattern in the post-injury microglial population of the adult. Virtually all proliferative microglia expressed Runx1

after injury, beginning immediately at 1dpi. This extended out to our latest observed time point at 30 dpi, when the overall levels of Runx1+ microglia had dropped back to the control levels in the DG and SVZ, and only around 15% of the total SVZ microglial population was Runx1+. Taken together with the well-established roles of Runx1 as a regulator of cell proliferation, our data strongly suggests that Runx1 is regulating proliferation of activated microglia. If Runx1 were to inhibit proliferation, it follows that the immediate and thorough expression of Runx1 throughout the microglial population at 1 dpi would subsequently prevent any further microglial proliferation. Yet there is a clear increase in the proliferative microglial population from 1 to 3 days, and mitotic microglia were detected all the way out to 30 dpi. Therefore, it seems more likely that Runx1 is expressed after injury in proliferating microglia as a pro-mitotic factor, rather than as an anti-mitotic factor as it is embryonically (210).

Runx1 is capable of inducing the expression of receptors for both granulocyte colony stimulating factor (G-CSF) and the M-CSFR in blood cells (112);(145). Both of these receptors promote microglial accumulation at a wound site after injury (64);(161). M-CSFR promotes microglial proliferation and phagocytosis, and it was recently found that M-CSFR activity is required for the normal survival of adult microglia (46). Inhibiting M-CSFR causes almost total death of microglia in the brain, which then repopulate following cessation of M-CSF inhibitor treatment, suggesting that the activation of this receptor may also be involved in their adult proliferation. Both Runx1 and M-CSFR are required for the embryonic and early post-natal generation of brain of microglia (210);(58), and Runx1 is an activator of M-CSFR expression (205);(36). Thus, it is possible that the widespread induction of Runx1 expression in microglia after injury may serve to induce the expression of M-CSFR, and it would be very interesting to

investigate whether or not M-CSF is regulated by Runx1 to control post-injury microglial proliferation.

Such a regulatory switch does, in fact, occur in the transcriptional control of Mrgpr class of G-protein coupled receptors (Mrgprs) by Runx1. Embryonically, Runx1 activates the expression of many Mrgpr genes in neuronal nociceptor precursors of the trigeminal and dorsal root ganglia (115). However, if Runx1 expression persists in these cells in the postnatal period, Runx1 switches from being an activator to a repressor of Mrgpr gene expression (115). It is then indeed possible that Runx1 may repress microglial proliferation and hypertrophy during development, but then promote microglial proliferation upon post-injury induction of Runx1 expression in the adult.

To more definitively discern the function of Runx1 in adult microglia following injury, inducible Runx1 knockout animals or effective Runx1 siRNA treatments would be required, such that the normal microglial development would not be altered by the absence of Runx1, but so that its expression could be ablated at or just prior to the time of injury.

TBI induces Runx1 expression in a sub-population of adult NSPCs.

Runx1 expression has not previously been described in adult populations of NSPCs in the SVZ or DG in vivo in adult animals, nor did we observe its expression in these cells in uninjured animals. Our observations indicate that Runx1 was only expressed in a subset of adult NSPCs, specifically after injury, and this may represent a unique adult NSPC population. After CCI injury Runx1 protein was expressed in cells of the SVZ and DG that also expressed the early stem and progenitor cell markers Nestin and/or GFAP. In both regions, these cells were appropriately located within the neurogenic niche to be stem cells, and in the DG they bore the characteristic upward projection extending through the granule cell layer. Runx1 was not

expressed by GFAP+/S100B+ cells, indicating that the Runx1+/GFAP+ and Runx1+/Nestin+ cells were in fact NSPCs and not astrocytes. Some of these cells were proliferative, as demonstrated by BrdU incorporation. Runx1 did not co-localize with the markers of intermediate progenitor cells or immature neuroblasts, DCX or Mash1, in either region, indicating that its expression was repressed as these cells progressed further down the stem cell lineage, and limited to early-lineage NSPCs.

Runx1 supports proliferation and is a pro-neuronal differentiation factor for cultured NSPCs.

We provide multiple lines of support for the possibility that Runx1 is a pro-mitotic factor in cultured NSPCs, suggesting that it could also be promoting proliferation *in vivo* after injury. Runx1 protein expression was induced in cultured neurospheres by bFGF, which is a potent mitogen that facilitates the proliferation of neuronal specific-progenitors (104; 184; 188). The induction of Runx1 by bFGF in cultured adult mouse neurospheres therefore suggests Runx1 may be induced as part of the pro-mitotic pathway in adult neuronal progenitor cells. EGF is another potent trophic factor and mitogen for neurosphere cultures, and though some groups have cultured adult NSPCs in the absence of EGF (15), we found EGF to be indispensable for the survival of the cells, and thus could not evaluate its effects on Runx1 expression. Treating neurospheres with 1% FBS caused virtually complete astrocytic differentiation of the neurosphere cultures, and caused a drop in Runx1 protein levels. This suggests that Runx1 is inhibited as astroglial differentiation occurs, as it may be part of a competing pro-neuronal pathway. This idea is also supported by our observations *in vivo* after TBI, in which Runx1 expression was excluded from S100β+ astrocytes of the SVZ and DG after injury. Additionally, it has been reported that overexpression of Runx1a induces glial differentiation in cultured neurospheres that are derived from embryonic murine neural crest stem cells (85). Runx1a is the

truncated form of Runx1 that contains the Runt-DNA binding domain, but lacks the transcriptional regulation domains, and it acts as a dominant inhibitor of the full length Runx1b and Runx1c proteins (182), providing further evidence that full length Runx1 may suppress glial differentiation pathways.

We found that Runx1 protein levels were decreased on bFGF removal or 1% FBS treatment, but did not find any significant changes in the *Runx1* mRNA in different culture conditions. Thus, Runx1 protein levels must be regulated at the post transcriptional level to cause this decrease. Runx1 undergoes proteolytic degradation at an increased rate in the absence of its cofactor CBFβ (78), so any endogenous modification of the interaction with these two proteins could potentially evoke an increase in the degradation rates of Runx1, thereby lowering the overall levels of the protein in the cells. Extracellular signal-related kinase (ERK) can phosphorylate Runx1 at multiple sites, and this phosphorylation enhances its transactivation ability (173). However, ERK phosphorylation of Runx1 also disrupts the interaction between Runx1 and its corepressor protein mSin3A, and this disruption facilitates the proteolytic degradation of Runx1 (79). MircroRNA-27 has also been demonstrated to reduce Runx1 expression levels by interacting with the 3' untranslated region of the *Runx1* mRNA transcript and post-transcriptionally downregulating its expression (48). Additionally, the Cdk4/cyclin D1 complex can phosphorylate Runx1 and target it for ubiquitination and degradation (10).

Runx1 inhibition reduces neurosphere proliferation.

Inhibiting Runx1 transcriptional regulation with Ro5-3335 decreased neurosphere size and EdU incorporation, and also caused increased expression of the Runx1 target genes p21 and cyclin D1. It has been shown that Runx1 directly binds to the *p21* promoter and represses its expression in embryonic cortical and olfactory NSPCs (179), and Runx1 also directly binds the

cyclin D1 promoter (155), demonstrating a disinhibition of these two Runx1 targets by the inhibitor treatment. There were no changes in cell survival by any dose of the inhibitor treatment, indicating the decrease in proliferative cells was not due to toxicity of the treatment. The Ro5-3335 inhibitor was only recently identified as an inhibitor of Runx1 transcriptional regulation and has only been used in this manner in a few publications (18; 36). As such, there is limited information available as to its specificity. However, there is no published information demonstrating its interaction with any other physiological pathway in normal human or rodent cells. Chemically, Runx1 is a benzodiazepine, although when compared to the benzodiazepine drug diazepam, Ro5-3335 has less than 1% of the affinity of diazepam for benzodiazepine receptors in rat cerebral cortex membranes (74). There are several cell lines that express mutated Runx1 proteins resulting from gene fusions, and in these cell lines the activity of the Runx1 mutant protein is essential for cell survival (36). In cell survival assays, the Ro5-3335 inhibitor is highly toxic to these cells expressing Runx1 mutant proteins, with IC_{50} values ranging from approximately 1 through 20 μM. Conversely, in cell lines bearing no Runx1 mutant protein, such as the Kasumi-6, the IC_{50} is greater than 200 μM (36). This suggests that at concentrations below 200 μM, the Ro5-3335 inhibitor is specific enough in its inhibition of the Runx1 pathway that it does not disturb other cell signaling pathways to the extent that it alters cell viability. This is also supported by our results, which demonstrated no effect on neurosphere cell survival after Ro5-3335 inhibitor treatment. Additionally, treatment of zebrafish embryos with the Ro5-3335 inhibitor phenotypically mimics Runx1 genetic knockout, indicating the overall specificity of the inhibitor for the Runx1 pathway (36).

The inhibition of cell proliferation produced by the Runx1 inhibitor Ro5-3335, along with the high Runx1 expression levels in highly proliferative neurosphere cultures indicate that

Runx1 is a pro-mitotic factor for adult NSPCs. Therefore, the novel expression of Runx1 that we described in adult NSPCs after brain injury could be involved in promoting their proliferation.

Runx1 overexpression induces neuronal-like morphology change and increases expression of neuronal marker proteins.

Increasing Runx1 expression caused a striking morphological change in our cultures, inducing cell adherence and causing many of the cells to adopt a morphology that was reminiscent of migratory intermediate neuronal progenitor cells. These cells bore a small spherical cell body, and usually one, or sometimes two, long thin processes. Concurrent with this morphological change was the appearance of a cell population which strongly expressed the neuronal marker TuJ1, and an increase in the cell population strongly expressing the neuroblast maker DCX, providing further evidence that these differentiated cells were adopting neuronal characteristics. Interestingly, we did not see any change in the cell proliferation after Runx1 over-expression. So although heightened Runx1 protein levels were activating the expression of mature neuronal proteins, the changes did not cause cell cycle exit. It is possible that at a later time point, if the differentiated cells were given more time to mature, these cells might exit the cell cycle.

Cell adherence and migration is mediated through the interactions of transmembrane proteins of a cell, such integrins, with proteins and molecules of the extracellular matrix or with the transmembrane proteins of another cell. Runx1 is capable of inducing the expression of integrins and other transmembrane proteins associated with cell adhesion, and it is possible that the induction of these proteins mediated the observed cell adherence and clustering that was observed after LV-Runx1 treatment. Runx1 binds to the promoter of integrin alpha 2b (*CD41*), and in murine HSC cultures Runx1 induces *CD41* expression in a synergistic manner with the cofactor Scl (174). Runx1 hemizygous knockout mice (bearing only one functional copy of the

Runx1 gene) have significantly reduced CD41 expression in blood cells *in vivo* (174). Runx1 mutation impairs the phosphorylation of the $\alpha_{II}b\beta3$ integrin and impairs its ability to bind fibrinogen (59), suggesting that overexpression of Runx1 may facilitate the binding of extracellular matrix molecules by integrins.

Runx1 is highly expressed in the SKOV3 epethilial adenocarcinoma cell line, and knockdown of Runx1 expression in these cells by shRNA interference significantly decreases cell migration (91). This inhibition of Runx1 expression coincided with changes in the expression of many genes related to cell adherence and migration, including several collagen genes, cadherin-1, CD34, CD44, and CXCL3 (91). Runx1 has also been shown to induce the expression of the gene for the collagen binding protein *CD36* (3). It is therefore possible that LV-Runx1 treatment might have induced expression and secretion of collagen into the culture dish, and also induced the expression of collagen binding proteins, thereby inducing the observed morphology changes that we observed after LV-Runx1 treatment (106).

In vivo, we only observed Runx1 protein staining in early NSPCs of the DG and SVZ, and not in mature neurons. It was not expressed by DCX+ or Mash1+ cells of either region, though was often expressed in Nestin+ putative progenitor cells immediately adjacent to DCX+ cells. Since Runx1 expression caused an increase in the DCX+ fraction, it suggests that Runx1 is capable of producing a differentiation response of the early NSPC population to become DCX+ intermediate neuroblasts. It could therefore be having this effect on the Nestin+ and Nestin+/GFAP+ cell population after injury *in vivo*, but Runx1 protein expression is then inactivated once the cells become Mash1+ or DCX+ progenitors. Although its expression was not seen in DCX+ cells *in vivo*, the enhanced Tuj1+ generation after LV-Runx1 treatment also suggests that if it were still expressed in these cells it would remain a pro-neuronal

differentiating factor, and perhaps it is inactivated in these neuronal progenitors to prevent the cells from progressing too rapidly through the intermediate progenitor stage and precociously differentiating into mature neurons.

Our results suggest that activating Runx1 signaling in adult NSPC population *in vivo* could potentially elevate NSPC proliferation and simultaneously produce increased DCX+ cell generation from primary stem cells, as well as increased neuronal differentiation from neuroblasts. In total, our work identifies Runx1 signaling as a potentially valuable regulatory pathway of the proliferation and neuronal differentiation of a subpopulation of adult NSPCs following brain injury.

Table 1. Primary (1°) and secondary (2°) antibodies.

1° Antibodies	Species	Dilution	Source	Reference	Cell type labeled
Runx1	Rabbit	1:2500	Dr. Jessell	Chen 2006	Runx1+ cells
BrdU	Rat	1:200	Accurate	OBT0030	Proliferative cells
Nestin	Mouse	1:200	Millipore	MAB353	NSCs, intermediate progenitors
NeuN	Mouse	1:200	Millipore	MAB377	Mature neurons
NF200	Mouse	1:2000	Sigma	N0142	Neuronal filaments
GFAP	Mouse	1:1000	Millipore	MAB360	NSCs, astrocytes
Sox2	Mouse	1:500	R&D	MAB2018	NSCs, intermediate progenitors
DCX	Mouse	1:500	Millipore	AB2253	Immature neurons
Iba1	Rabbit	1:750	Wako	019-19741	Microglia
Mash1	Mouse	1:300	R&D	A21244	Intermediate progenitors
S100β	Mouse	1:500	Sigma	S2532	Mature astrocytes
2° Antibodies	**Species**	**Dilution**	**Source**	**Reference**	**Conjugate**
Rat IgG	Goat	1:2000	Jack. Immuno.	112-485-167	Dylight 488
Mouse IgG	Goat	1:1000	Invitrogen	A21121	Alexa Fluor 488
Rabbit IgG	Goat	1:2000	Invitrogen	A11036	Alexa Fluor 568
Mouse IgG	Goat	1:2000	Invitrogen	A11031	Alexa Fluor 568
Mouse IgG	Goat	1:1000	Invitrogen	A21236	Alexa Fluor 647
Rabbit IgG	Goat	1:500	Invitrogen	A21244	Alexa Fluor 647

Table 2. The percentage change in mRNA levels of the listed genes in tissue samples of the DG taken at 1, 3, or 7 dpi as compared to the mRNA levels in uninjured naïve animals. * p<0.05, **p<0.01 ***p<0.001.

Dentate Gyrus	Average Fold change		
Gene Symbol	1 dpi	3 dpi	7 dpi
Acvr1	0.87	1.09	0.58*
Acvr2a	0.44**	0.76	0.32***
Acvrl1	1.68	1.15	1.35
Amh	1.45	1.86	0.61
Amhr2	1.69	1.21	1.60
Bambi	0.40	1.09	0.28*
Bglap2	0.34	0.96	6.11*
Bmp1	0.85	0.83	0.47*
Bmp2	2.20**	0.95	1.90*
Bmp3	0.53*	0.82	0.39*
Bmp4	0.46*	1.7*	2.74***
Bmp5	1.11	0.82	1.63
Bmp6	0.74	0.92	2.65
Bmp7	1.22	0.85	1.85
Bmper	0.51	1.11	1.09
Bmpr1a	0.69*	1.22	0.96
Bmpr1b	0.48**	1.22	1.36*
Bmpr2	0.68*	0.94	0.6***
Cd79a	0.81	0.48	0.94
Cdc25a	0.89	0.95	0.91
Cdkn1a	5.52	1.57	3.86**
Cdkn2b	1.92	1.28	2.72***
Chrd	0.38***	0.62**	0.23***
Col1a1	0.93	0.68	5.54
Col1a2	1.66	0.86	11.72**
Col3a1	3.14	1.50	12.64***
Dlx2	0.46	0.94	0.38*
Eng	1.51	1.34	1.49*
Mecom	1.02	0.63	0.69**
Fkbp1b	0.71	0.89	0.61**
Fos	3.68**	2.13*	2.63**
Fst	0.26***	0.62	0.08***
Gdf1	0.57**	0.90	0.49***
Gdf2	3.08	1.00	1.41
Gdf3	2.58	0.32	2.36
Gdf5	0.40	1.04	1.51
Gdf6	0.38	0.65	0.31*
Gdf7	0.85	0.61	0.28**
Gsc	2.50	0.57	2.00
Id1	1.75	1.11	1.20
Id2	1.22	1.06	1.03
Igf1	0.45	0.91	5.73**

Igfbp3	4.10	1.05	1.04
Il6	4.44	0.52	0.73
Inha	0.33**	0.69	0.26**
Inhba	1.62	0.72	0.46
Inhbb	1.25	1.06	0.92
Itgb5	0.92	2.43	4.7***
Itgb7	2.39*	1.05	1.89*
Jun	1.26	1.40	0.61
Junb	1.88	1.02	1.11
Lefty1	1.56	1.00	1.58
Ltbp1	2.05*	1.27	1.32
Ltbp2	2.27	0.57	0.57
Ltbp4	0.47	0.95	0.63*
Myc	2.54*	1.18	1.40
Nbl1	0.87	0.97	1.06
Nodal	1.12	0.45	0.91
Nog	0.79	0.56	0.79
Nr0b1	0.81	0.64	0.39
Pdgfb	0.94	0.97	0.87
Plat	1.50	1.31	1.62
Plau	4.54	1.30	15.93***
Runx1	6.59**	2.48	8.19***
Serpine1	67.61***	1.76	2.56
Smad1	1.03	1.06	0.86
Smad2	0.83	1.04	0.8*
Smad3	0.67	1.03	0.35**
Smad4	0.72	0.91	0.51***
Smad5	0.92	1.14	0.93
Smurf1	0.94	0.79	0.90
Sox4	1.00	1.05	1.02
Stat1	1.96	1.51	6.34***
Tdgf1	0.55	1.57	3.22***
Tgfb1	3.02**	2.52*	8.56***
Tgfb1i1	1.30	0.77	1.00
Tsc22d1	0.66*	1.04	0.6**
Tgfb2	0.85	0.85	0.47***
Tgfb3	0.92	1.40	1.26
Tgfbi	35.56***	2.33	4.56
Tgfbr1	0.63	1.18	2.08***
Tgfbr2	1.63	1.78***	4.48***
Tgfbr3	0.58**	0.76	0.47
Tgfbrap1	0.76	1.19	0.77

Table 3. The percentage change in mRNA levels of the listed genes in tissue samples of the SVZ taken at 1, 3, or 7 dpi as compared to the mRNA levels in uninjured naïve animals. * p<0.05, **p<0.01 ***p<0.001.

Subventricular Zone	Average fold change		
Gene Symbol	1 dpi	3 dpi	7 dpi
Acvr1	0.98	0.68*	0.82
Acvr2a	0.71**	0.65***	0.80***
Acvrl1	1.04	0.59**	0.64*
Amh	1.01	1.13	1.38
Amhr2	2.21**	1.30	1.45
Bambi	1.15	1.60	1.10
Bglap2	0.63	0.71	2.04
Bmp1	1.10	0.76*	1.02
Bmp2	0.83	0.65*	1.37*
Bmp3	1.79	0.09*	0.68
Bmp4	0.64	1.88	1.66
Bmp5	1.59	6.69**	3.21
Bmp6	0.50	2.41**	1.96*
Bmp7	1.14	4.71	2.38***
Bmper	0.62*	1.04	1.23
Bmpr1a	0.89	1.02	1.03
Bmpr1b	0.64*	0.75	1.4*
Bmpr2	1.11	0.94	1.00
Cd79a	0.95	0.33**	1.12
Cdc25a	0.89	1.14	1.17
Cdkn1a	2.83***	1.66*	1.46
Cdkn2b	1.10	1.36	1.20
Chrd	0.77	0.44**	0.86
Col1a1	1.68	1.33	1.94
Col1a2	0.71	2.83***	1.73*
Col3a1	2.38*	2.43*	2.42*
Dlx2	0.47*	0.99	1.35
Eng	1.00	1.21	1.20
Mecom	1.55	1.01	1.52
Fkbp1b	0.76*	0.54*	0.81
Fos	3.07	2.26	0.52
Fst	0.79	0.56*	0.25**
Gdf1	1.02	0.62*	0.96
Gdf2	1.42	0.61	1.15
Gdf3	2.32	0.80	2.23
Gdf5	0.49	2.65	3.11*
Gdf6	1.56	1.08	1.29
Gdf7	1.35	1.12	1.37
Gsc	2.06	3.22*	1.51
Id1	1.14	2.07**	1.39
Id2	0.99	1.00	1.22

Igf1	0.96	0.78	1.10
Igfbp3	2.04	2.22*	1.05
Il6	4.99**	1.05	2.19
Inha	0.83	0.55***	0.81
Inhba	2.30	1.09	0.98
Inhbb	0.92	1.14	1.38
Itgb5	0.82	2.02**	2.09**
Itgb7	0.79	0.6***	0.81
Jun	2.65***	1.10	1.35
Junb	1.61	0.90	0.76
Lefty1	1.61*	1.08	1.15
Ltbp1	1.65*	0.81	1.26
Ltbp2	1.76	0.77	0.84
Ltbp4	0.66**	1.08	1.14
Myc	2.49***	1.00	0.94
Nbl1	1.02	1.81	1.51
Nodal	1.79	0.44	1.53
Nog	1.35	1.05	1.73
Nr0b1	1.38	0.53	1.45
Pdgfb	1.46**	1.4**	1.37*
Plat	1.01	0.75	1.21
Plau	2.32	0.92	2.17
Runx1	2.86***	1.39	2.18**
Serpine1	10.86***	1.86	1.30
Smad1	0.88	0.92	0.94
Smad2	0.75*	0.97	0.91
Smad3	1.01	0.89	0.98
Smad4	0.95	0.74**	0.89
Smad5	0.71*	1.01	0.96
Smurf1	0.96	0.64**	0.95
Sox4	0.62	0.69	1.51
Stat1	1.14	1.07	2.31***
Tdgf1	1.31	1.76	1.96
Tgfb1	1.67**	1.55**	2.02***
Tgfb1i1	1.58*	0.91	1.19
Tsc22d1	0.81	0.98	1.00
Tgfb2	0.83	2.76***	2.11
Tgfb3	1.26	0.96	1.24
Tgfbi	3.35***	2.31**	1.98*
Tgfbr1	0.85	0.81	1.52**
Tgfbr2	1.19	1.49	2.25**
Tgfbr3	1.02	1.73	1.96*
Tgfbrap1	0.98	0.99	0.90

Table 4. Runx1 expression in the microglial cell populations of the DG and SVZ.

		Control	*1 dpi*	*3 dpi*	*7 dpi*	*14 dpi*	*30 dpi*
DG	% Runx1+/Iba1+ out of total Iba1+ population	46.6±6%	99.0±0.6%	83.2±6%	81.5±5%	69.3±13%	80.2±7%
	% of BrdU+/Iba1+ cells expressing Runx1	n/a	100%	94%	100%	100%	100%
	Runx1+/Iba1+/BrdU+ cells/mm^2	0±0	42.3±13.6	74.5±41.8	5.6±1.8	2.0±0.69	2.7±1.0
SVZ	% Runx1+/Iba1+ out of total Iba1+ population	10.6±1%	64.4±2%	28.6±5%	23.4±5%	30.2±4%	16.7±2%
	% of BrdU+/Iba1+ cells expressing Runx1	n/a	100%	92%	94%	100%	100%
	Runx1+/Iba1+/BrdU+ cells/mm^2	0±0	4.3±1.5	4.5±3.9	4.3±1.2	2.2±0.72	3.8±1.9

Table 5. Colocalization of Runx1 with cell-type markers in the neurogenic regions.

Colocalization of Runx1 with progenitor/stem cells markers								
		Control	***1 dpi***	***3 dpi***	***7 dpi***	***14 dpi***	***30 dpi***	***60 dpi***
DG	Nestin	-	++	++	++	+/-	-	-
	GFAP	-	+	+	+	+/-	-	-
	Sox2	-	++	++	++	-	-	-
	Mash1	-	-	-	-	-	-	-
	DCX	-	-	-	-	-	-	-
	NeuN	-	-	-	+/-	++	+++	++
	Nestin/BrdU	-	+	+	+	+	-	-
SVZ	Nestin	-	+	++	+	+/-	+/-	-
	GFAP	-	+/-	+/-	-	-	-	-
	Sox2	-	-	-	-	-	-	-
	Mash1	-	-	-	-	-	-	-
	DCX	-	-	-	-	-	-	-
	NeuN	-	-	-	-	-	-	-
	Nestin/BrdU	-	-	-	-	-	-	-

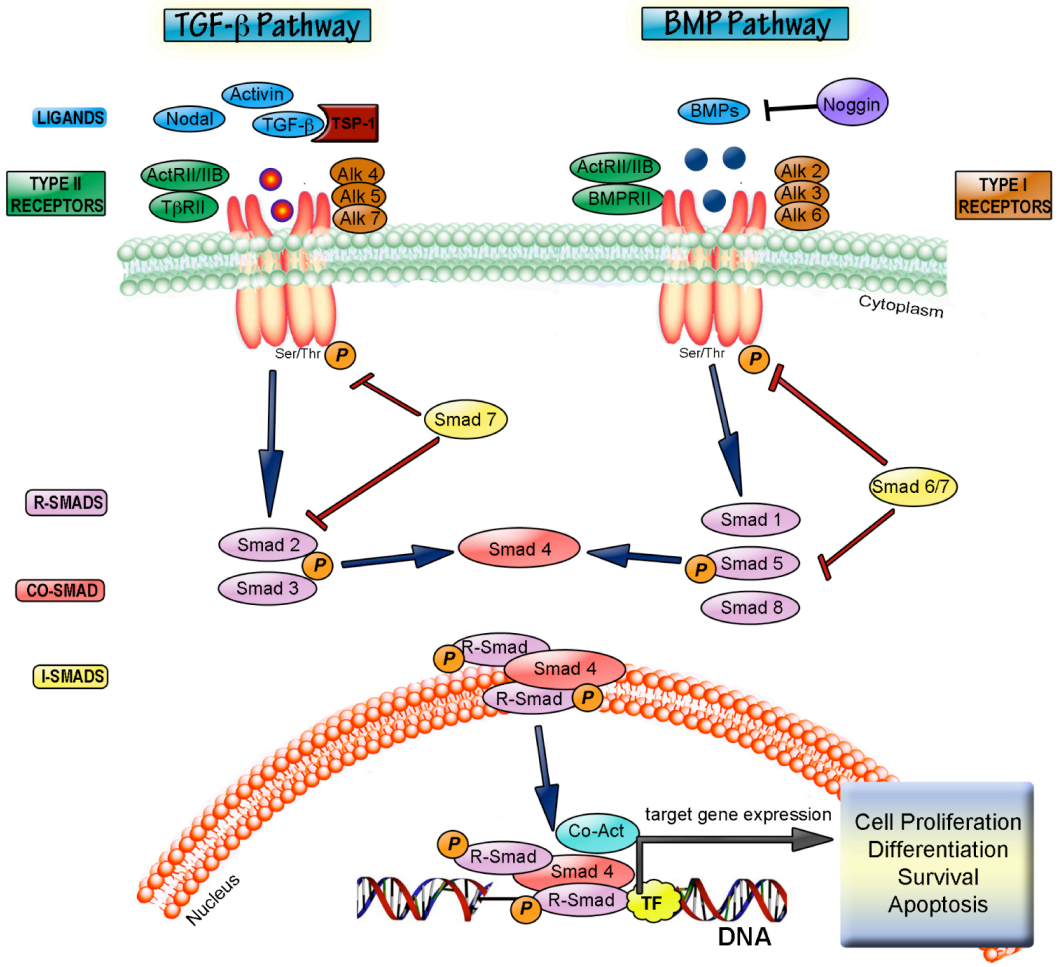


Figure 1. TGF-β, nodal or activin ligands bind to Type II receptors, which then recruit Type I receptors leading to transphosphorylation of type 1 receptors. Activated type I receptors phosphorylate Smad 2/3 (i.e. R-Smads) which then complex with the co-Smad, Smad4 and translocate to the nucleus to bind DNA at specific DNA motifs. Smad proteins activate or repress transcription through association with various co-activator (Co-Act) or co-repressor proteins. This pathway is inhibited by Smad7. BMP signaling operates by a similar paradigm. BMP6 and BMP7 bind to their Type II receptor before the complex recruits the Type I receptors, Alk-3 or Alk-6. BMP2 and BMP4, however bind first to their type I receptor before recruiting the type II receptor BMPRII. BMP binding to either receptor can be inhibited by first binding to various extracellular inhibitor proteins, such as noggin. Activation of the receptor complex leads to phosphorylation of the receptors and subsequent phosphorylation of Smad1, Smad5, or Smad8, allowing them to form a complex with Smad4. This heteromeric complex translocates to the nucleus, to target BMP-regulated genes through interaction with co-activators or repressors. Smad 6 and Smad7 may act similarly to inhibit the BMP pathway through interactions with the receptor complex and thus inhibiting R-Smad activation. TGF-β and BMP pathways induce the expression of proteins involved in proliferation, differentiation, survival and apoptosis (138).

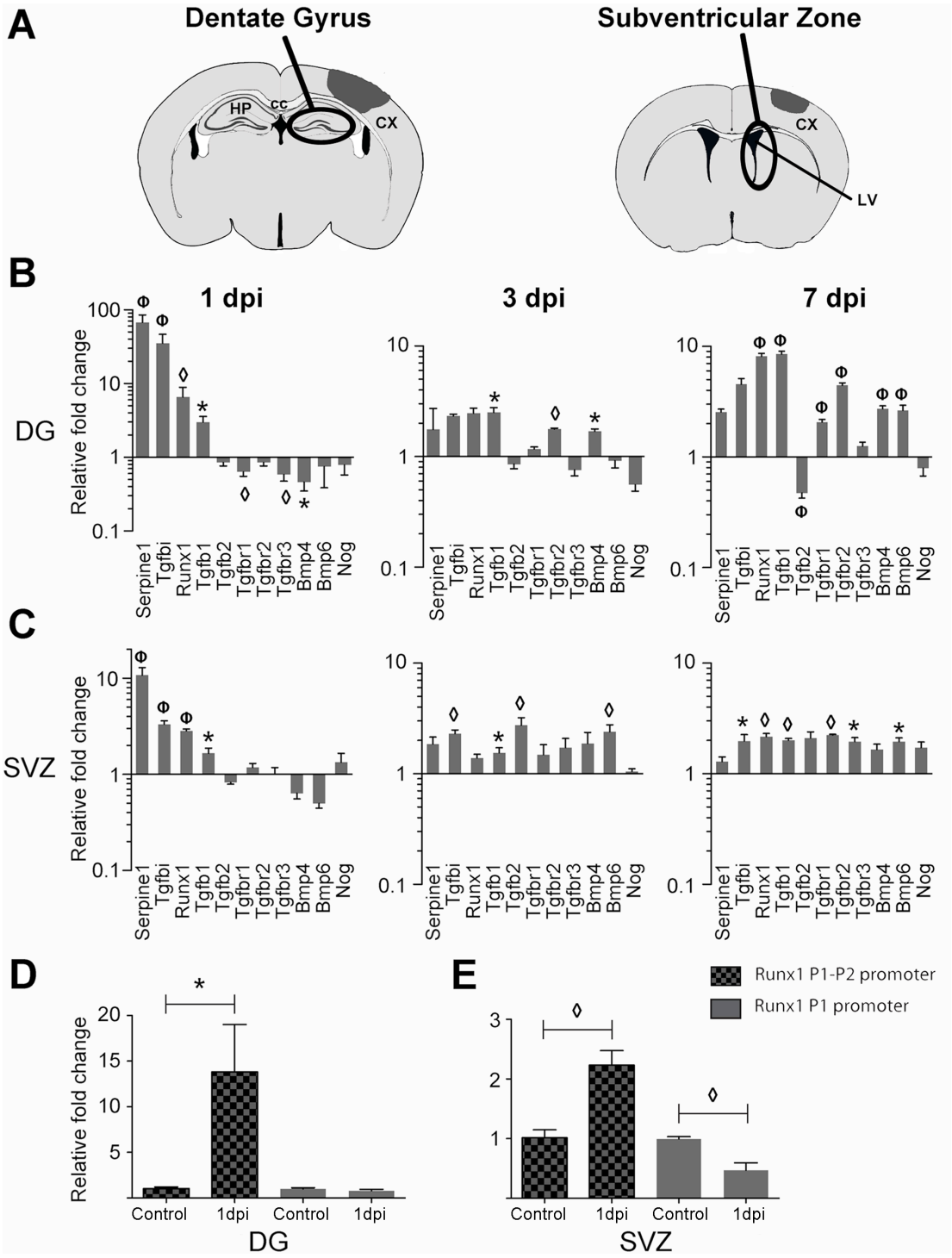


Figure 2. Cortical brain injury increases mRNA expression of TGF-β and BMP related molecules in the adult neurogenic areas. (A) Brain diagrams show the approximate area of the lesion penumbra at -1.82 mm from bregma for DG and +0.26 mm for SVZ, (adapted from Paxinos & Franklin, 2007). (B, C) QPCR analysis with TGF-β related PCR arrays indicates changes in RNA expression of specific genes at different times after CCI injury in either the DG (B) or the SVZ (C). Graphs (log_{10} scale) show genes whose expression was significantly altered

by injury (n=3 ± s.e.m.). (D, E) Runx1 mRNA expression is significantly increased at 1 and 7 dpi in both regions and is specifically upregulated in the DG (D) and SVZ (E) at 1 dpi when measured by P1-P2 primers, but not by P1 primers (n=3 ± s.e.m.). Gene expression is displayed as the relative fold change in mRNA levels as compared to the level in control animals. *$p<0.05$, ◊ $p<0.01$, Φ $p<0.001$. Abbreviations: dentate gyrus (DG), subventricular zone (SVZ), cortex (CX), corpus callosum (CC), hippocampus (HP), lateral ventricle (LV), and days post-injury (dpi).

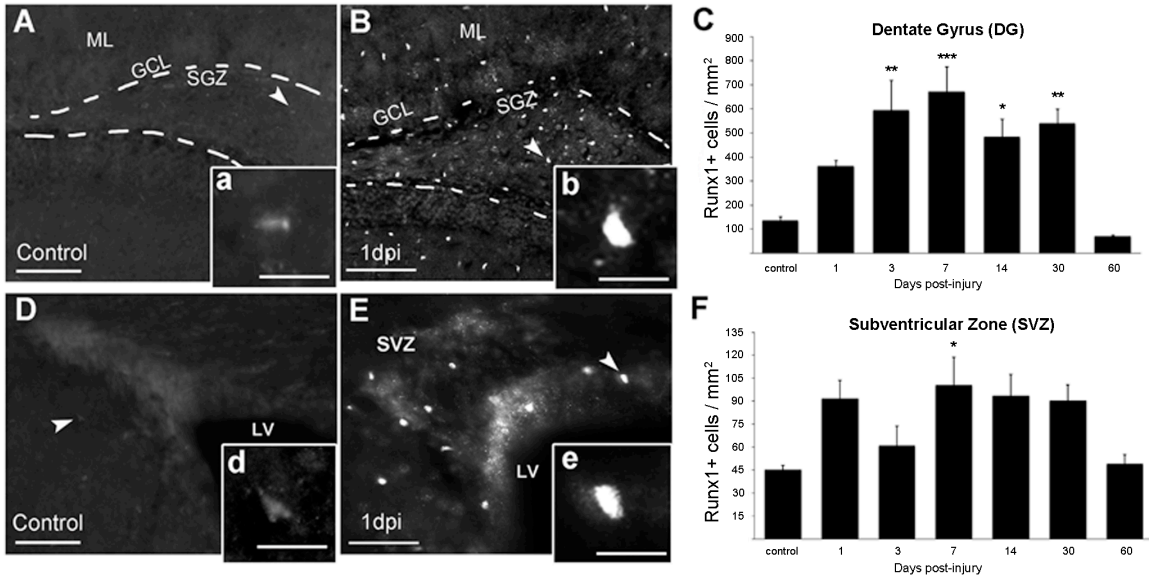


Figure 3. Runx1 expression in the two neurogenic areas of the adult brain. Runx1 is present at low levels in control animals in the DG (A, inset a) and SVZ (D, inset d). Runx1 expression increases in both regions after injury (DG; B, inset b and SVZ; E, inset e). (C, F) The number of cells expressing Runx1 is significantly increased in the DG at 3, 7, 14, and 30 days after injury and significantly increased in the SVZ at 7 days after injury compared with control animals. The peak of Runx1 expression in both regions was seen at 7 dpi. *p<0.05, **p<0.01 ***p<0.001, n=5 ± s.e.m. Scale bars for A, B, D, E = 50 μm; for a, b, d, e = 20 μm. Abbreviations: subventricular zone (SVZ), dentate gyrus (DG), molecular layer (ML), granule cell layer (GCL), subgranular zone (SGZ), lateral ventricle (LV), and days post-injury (dpi).

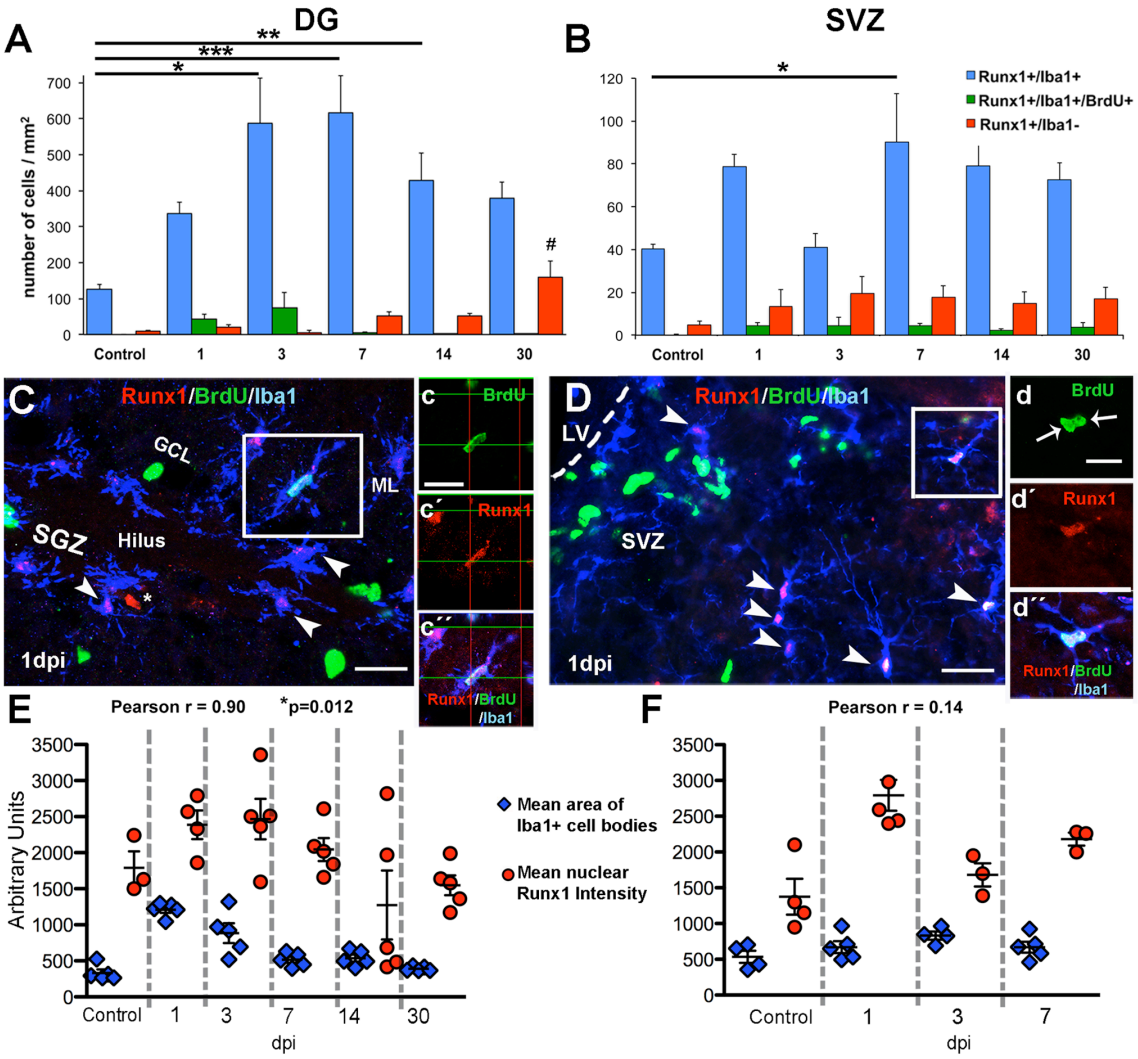


Figure 4. Runx1 is expressed predominantly in microglia after CCI injury in the neurogenic regions of the adult mouse. (A, B) The number of Runx1+ cells/mm^2 identified as Iba1+ microglia (blue bars), Iba1+ microglia incorporating BrdU (green bars), or Iba1- cells (red bars) are shown in the DG and SVZ. No Iba1+ cell proliferation was observed in control animals. Asterisk indicates statistically significant difference as compared to control Runx1+/Iba1+ cell counts and number symbol indicates difference as compared to control Runx1+/Iba1- cell counts. *p<0.05, **p<0.01 ***p<0.001, and #p<0.01. n = 5 ± s.e.m. (C, D) Confocal micrographs of triple labeling for Runx1 (red), Iba1 (blue), and BrdU (green) positive hypertrophic microglia in the DG (C and insets c, c´, c´´) and intermediate microglia in the SVZ (D and insets d, d´, d´´) at 1 dpi. Arrowheads denote Runx1+ microglia (C, D), and an asterisk (C) denotes a non-microglial, Runx1+ cell. Scale bars for C and D = 50 μm; for c and d = 20 μm. (E, F) Graphs show the mean area of Iba1+ cell body (total pixels) and mean Runx1 immunoreactivity (pixel intensity) at different time points post-injury and in control in the DG (E) and SVZ (F). Each point represents combined data from one animal. Abbreviations: subventricular zone (SVZ),

dentate gyrus (DG), granule cell layer (GCL), subgranular zone (SGZ), molecular layer (ML), lateral ventricle (LV), and days post-injury (dpi).

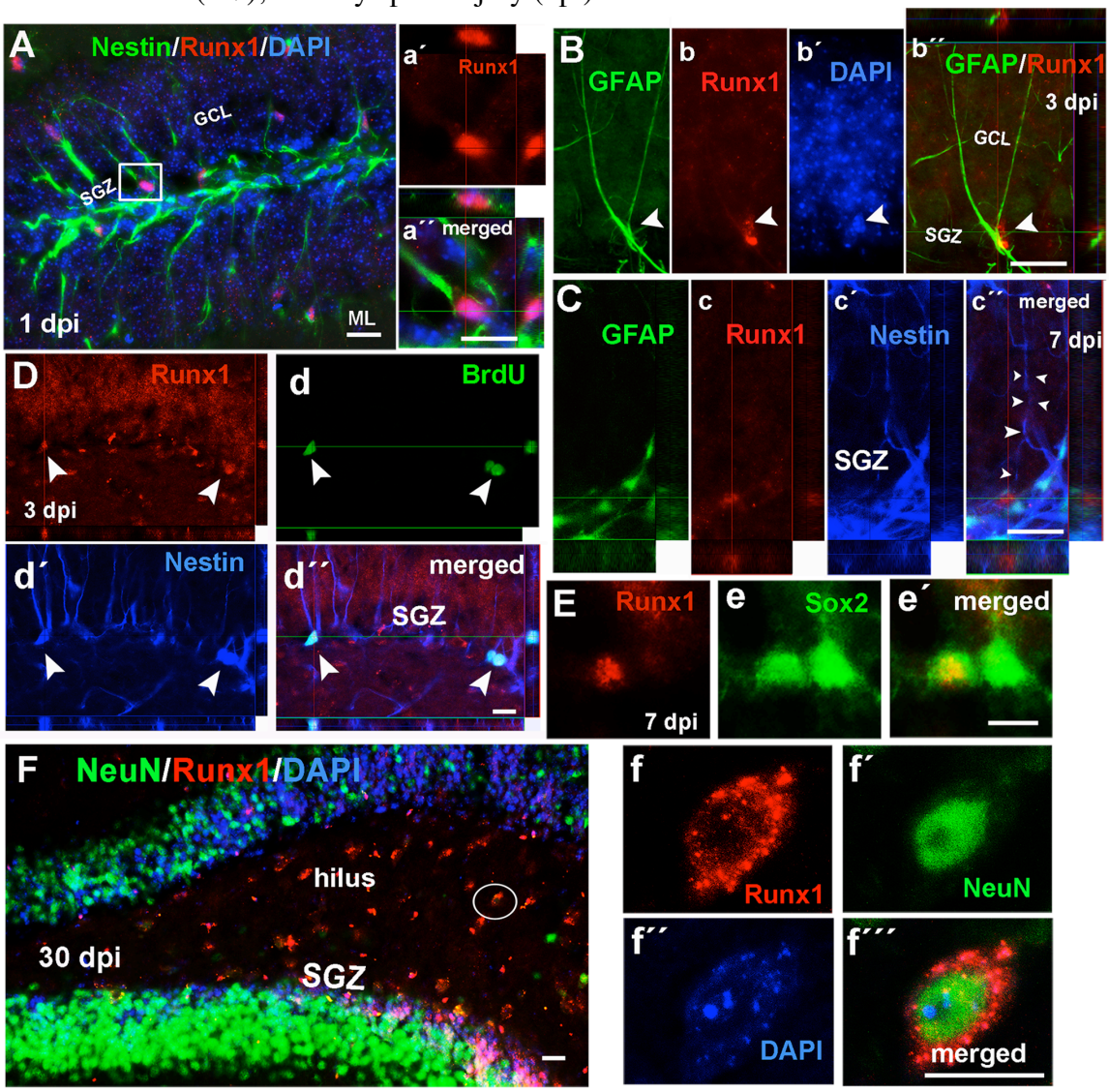


Figure 5. Adult hippocampal radial glia-like cells and mature neurons express Runx1. Confocal microscopy images of adult hippocampal mouse sections showing Runx1 (red) colocalized with Nestin+ (green) radial glia-like cells projecting radially from the dentate gyrus subgranular zone (SGZ) after injury (A, inset a´, a´´, DAPI in blue). These Runx1+ cells (red) often expressed GFAP (green) shown at 3 (B, b, b´, b´´, DAPI in blue) and 7 days post-injury (dpi) (C, c, c´, c´´, Nestin in blue), and extended processes through the granule cell layer (GCL) (an example is highlighted by the arrowheads in c´´). Some of these Runx1+/Nestin+ cells in the SGZ incorporated BrdU (D, d, d´, d´´). Nuclear Runx1 was also found to colocalize with Sox2 (green) expression in radial glia-like cells of the SGZ after injury (E, e, e´). At late time points (30 and 60 dpi), Runx1 was rarely expressed in the SGZ, but widely expressed in mature neurons (NeuN - green) of the dentate gyrus hilus (F, inset in f, f´, f´´, f´´´). Scale bars fo A, a'', b'', c'', e', f'''=20 μm; for d'' and F = 50 μm.

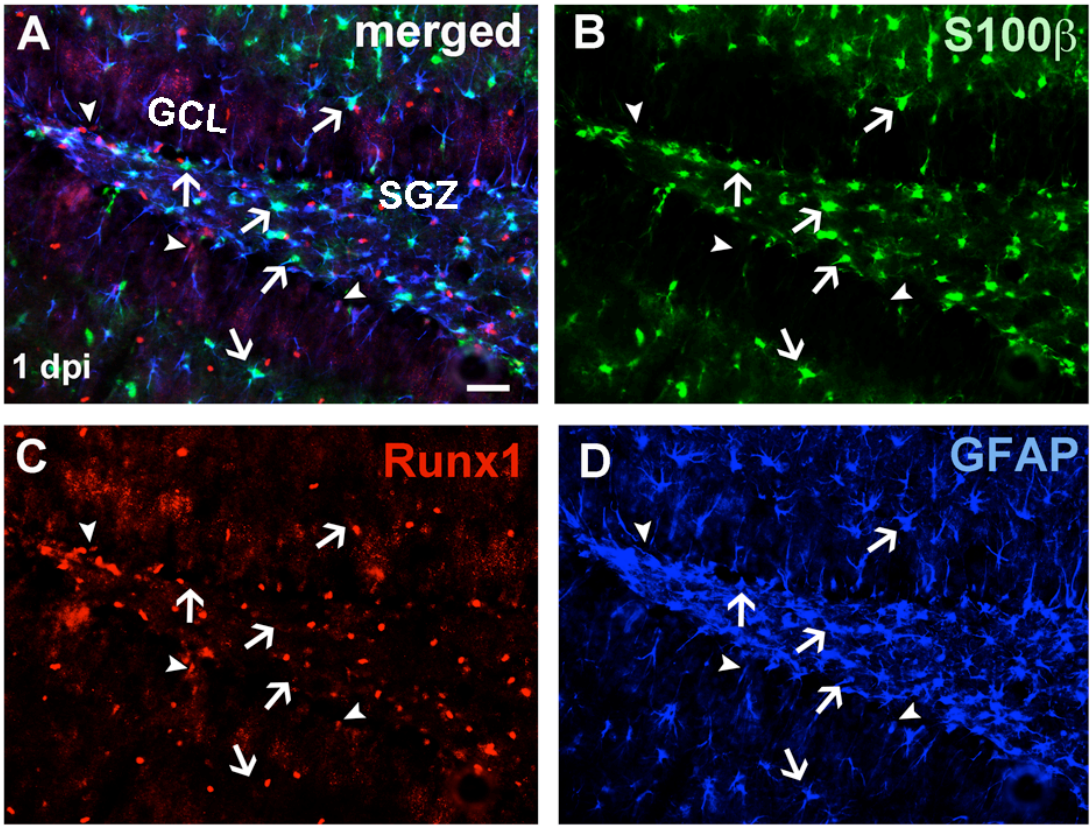


Figure 6. Reactive astrocytes do not express Runx1 protein in dentate gyrus of the adult mouse. Merged image (A) showing reactive astrocytes which did not express Runx1 protein (arrows) (Runx1-/GFAP+/S100β+) and progenitor/stem cells expressing Runx1 protein (arrowheads) (Runx1+/GFAP+/S100β-) in the dentate gyrus (DG) at 1 day post-injury (dpi). S100β immunoreactivity (B, green) is present in most GFAP-expressing reactive astrocytes, but is not colocalized with Runx1 protein in the SGZ (C, red). GFAP immunoreactivity (D, blue) is detected in both S100β+ astrocytes, and with Runx1 protein in progenitor/stem cells. Scale bar = 50 μm. Abbreviations: subgranular zone (SGZ), granule cell layer (GCL).

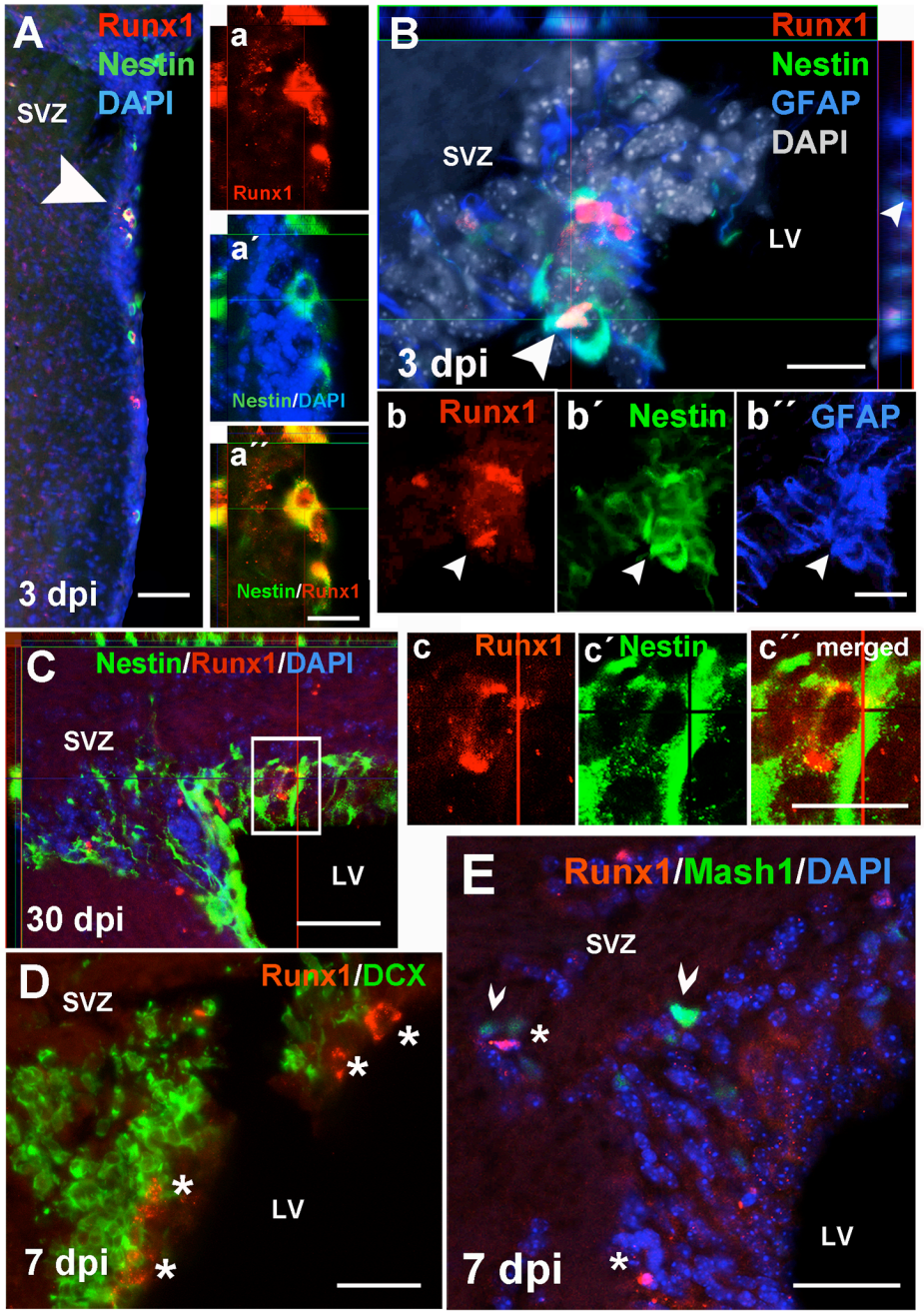


Figure 7. Runx1 is expressed by a subpopulation of putative neural stem cells in the subventricular zone. Runx1 (red) was co-expressed with Nestin (green) in cells of the lateral and apical subventricular zone after injury, between 3 dpi (A, inset a, a´, a´´) and 30 dpi (C, inset c, c´, c´´). Some of these cells also expressed GFAP (B, inset b, b´, b´´). Runx1 was not expressed by DCX+ (D, green) or Mash1+ (E, green) cells after injury, but was often immediately adjacent to clusters of DCX+ cells (D). Scale bars for B, a´´, b´´, c´´ = 20 μm; for A, C, D, and E = 50 μm. Abbreviations: subventricular zone (SVZ), lateral ventricle (LV), days post-injury (dpi).

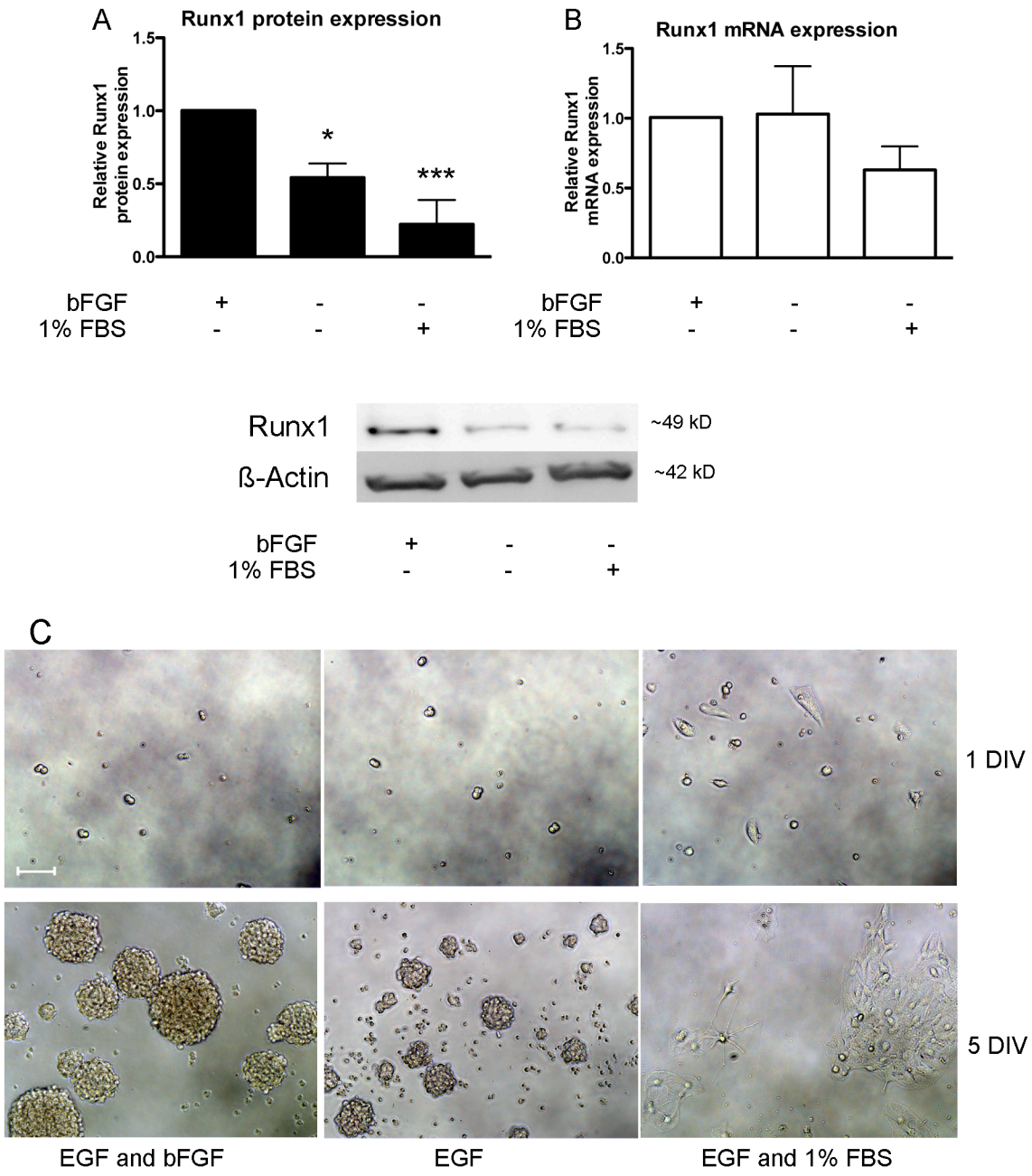


Figure 8. Neurosphere Runx1 protein expression drops in the absence of bFGF or presence of 1% FBS. A) Runx1 expression in neurosphere cells growing in different medias for 24 hours ±SEM, normalized to the expression of β-actin. *p<0.05, ***p< 0.001 , mean ±SEM, n=6). B) QPCR analysis of Runx1 mRNA expression in neurospheres ±SEM, cultured in indicated media for 24 hours. Runx1 mRNA was normalized to GAPDH mRNA, (mean ±SEM, n=3). C) Bright field images of cells after one or five days in vitro (DIV) containing the indicated growth factors or serum. Scale bar = 50μm.

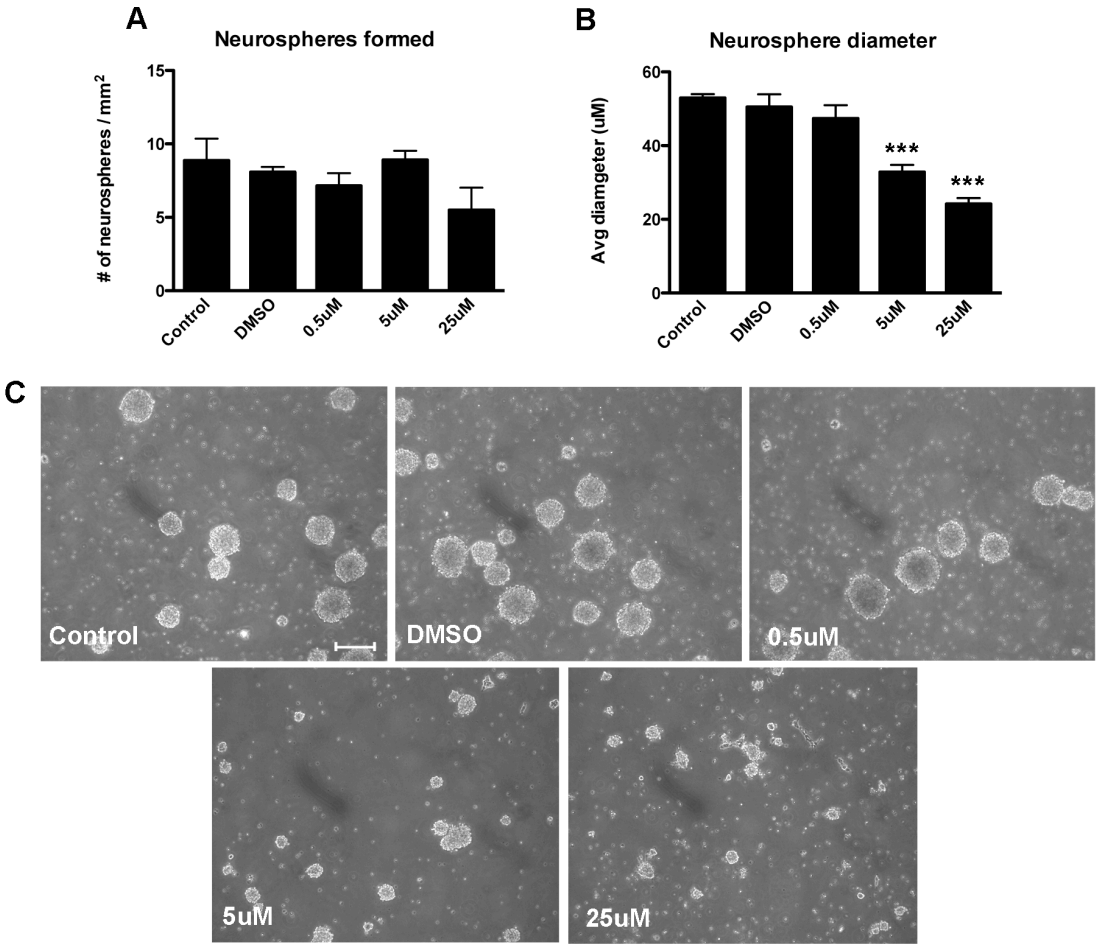


Figure 9. Ro5-3335 inhibitor treatments decrease neurosphere diameter. A) Different concentrations of Ro5-3335 did not significantly alter the number of floating multicellular neurospheres formed over 5 days in growth media. B) The average diameter of the neurospheres was changed ($p<0.001$ $F=32.39$, $n=3$), decreasing at the 5 ($p<0.001$) and 25 μM ($p<0.001$) concentrations. C) Bright field images of the cells after 5 days in the indicated concentrations of Runx1 inhibitor. Error bars indicate SEM. Scale bar = 100μm.

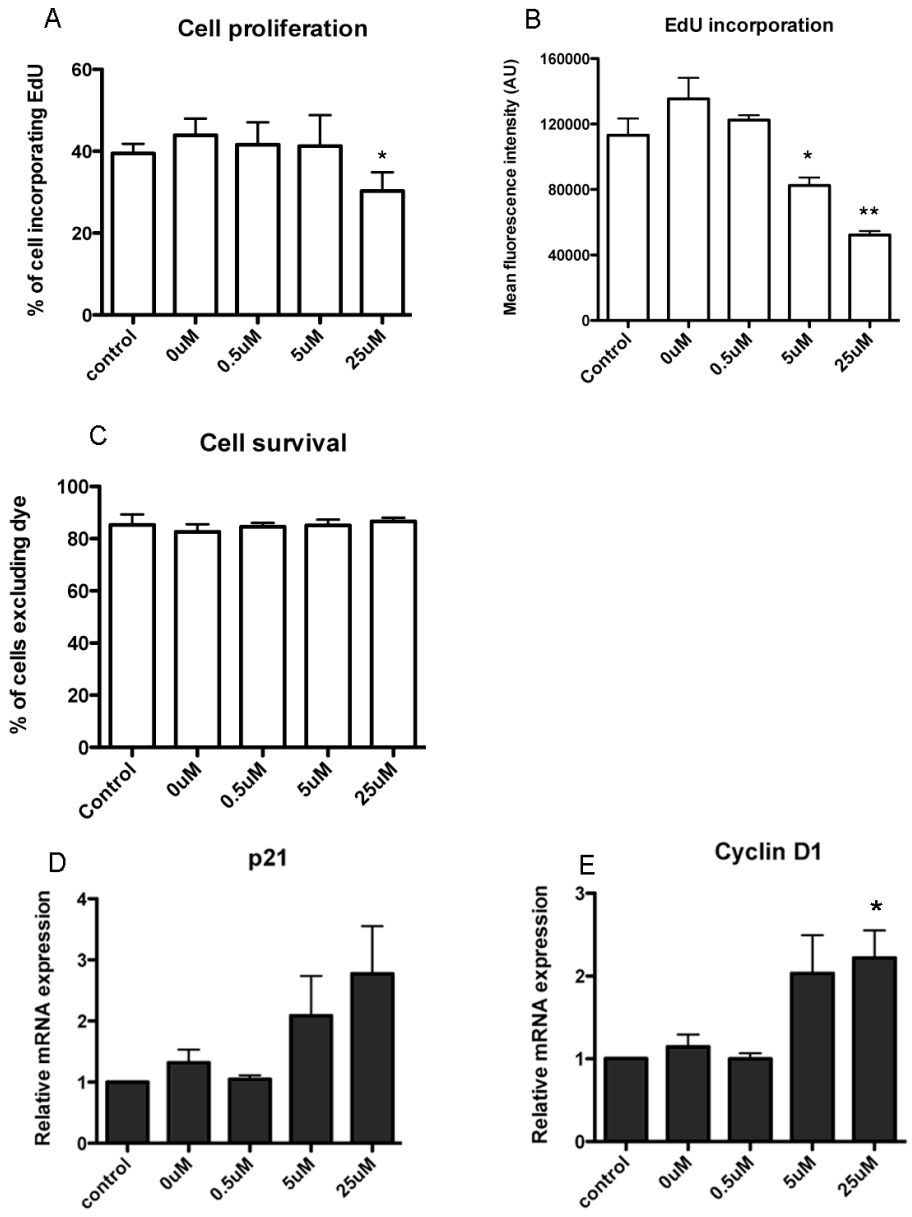
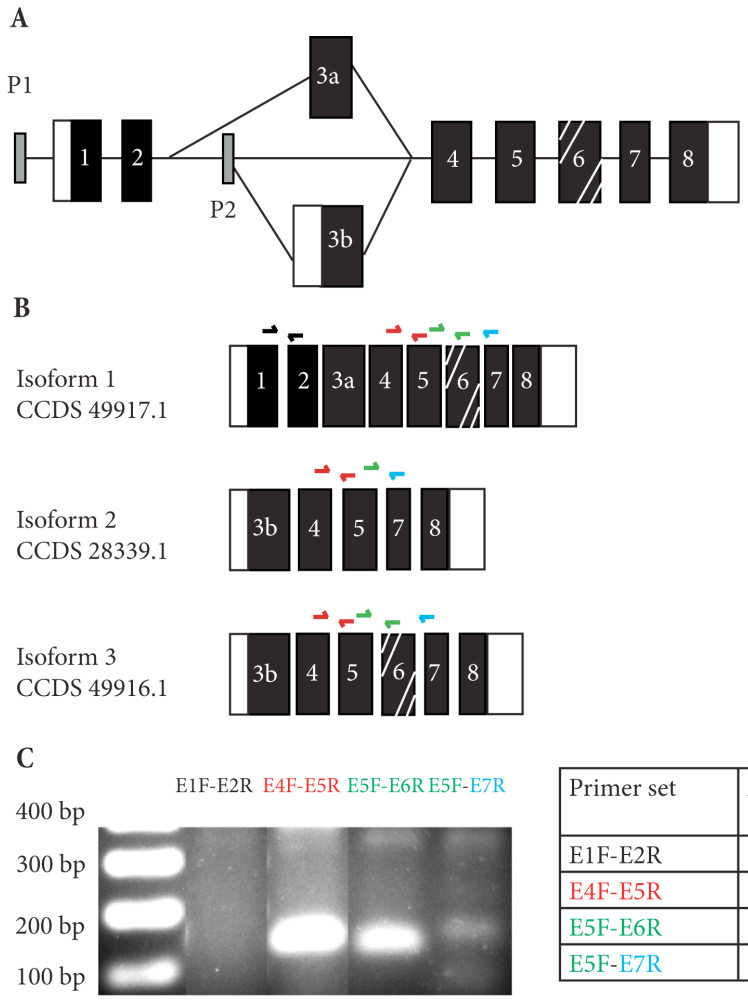


Figure 10. Ro5-3335 inhibitor treatment decreases neurosphere EdU incorporation and induces expression of p21 and cyclin D1 without increasing cell death. A) EdU was pulsed for 7 hours in neurosphere cultures treated with the indicated concentrations of Ro5-3335 for five days and quantified by flow cytometry. The total percentage of cells (±SEM) incorporating EdU was significantly altered by 25μM Ro5-3335 ($p<0.05$). B) The mean fluorescence intensity of EdU signal (±SEM) was also decreased by approximately half on treatment with 25 μM Ro5-3335, (*$p<0.05$, **$p<0.01$, n=3). C) The level of trypan blue exclusion (±SEM) by the cells was unchanged at any Ro5-3335 concentrations, indicating no increase in cell death by Ro5-3335. D) qPCR analysis of expression of p21 and Cyclin D1 mRNA, showed increased expression with 25μM Ro5-3335 (Cyclin D1, $p<0.05$, p21: $p=0.0505$, n=3).



Primer set	Amplicon size	Transcripts amplified
E1F-E2R	118	Isoform 1
E4F-E5R	149	Isoforms 1, 2, 3
E5F-E6R	123	Isoform 3
E5F-E7R	140, 332	Isoforms 2 and 3

Figure 11. Adult mouse neurospheres express Runx1 isoforms 2 and 3. A) Schematic diagram of the Runx1 gene showing the distal (P1) and proximal (P2) promoters (gray boxes), as well as untranslated and translated regions of exons (white, black boxes). B) Diagrams of the three identified mouse Runx1 mRNA transcripts. Colored arrows indicate the binding sites of exon specific primer sets used in the PCR shown in (C), with forward arrows indicating the forward primer set and backwards arrows indicating the reverse. The E1F-E2R exon set did not produce an amplicon, indicating the absence of isoform 1 mRNA. The E4F-E5R primer set, which could bind all three isoforms, produced a strong band of the expected size. The E5F-E6R primer set also produced a strong band, specifically indicating the presence of mRNA for isoform 3. The E5F-E7R primer set produced two bands, one band of around 140bp, indicating the presence of isoform 2, and one band of between 300 and 400bp, indicating the presence of isoform 3.

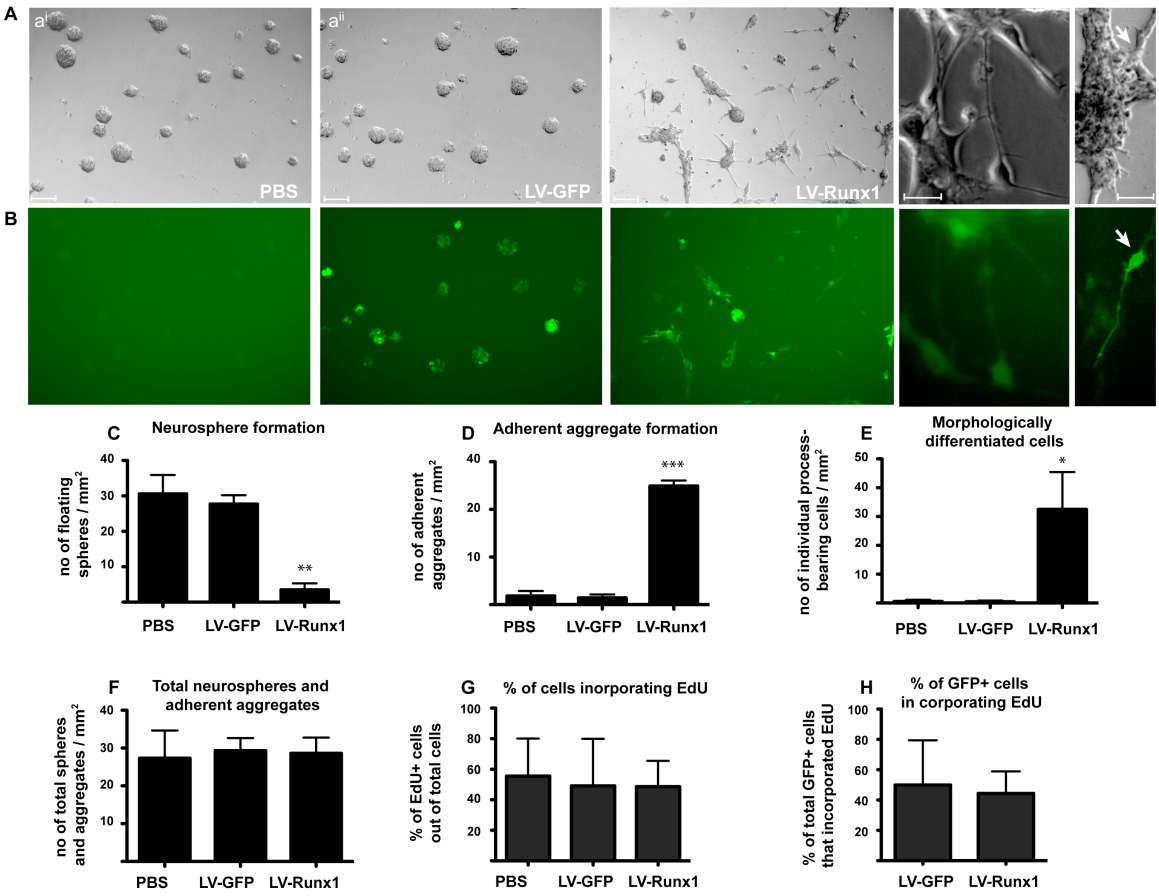
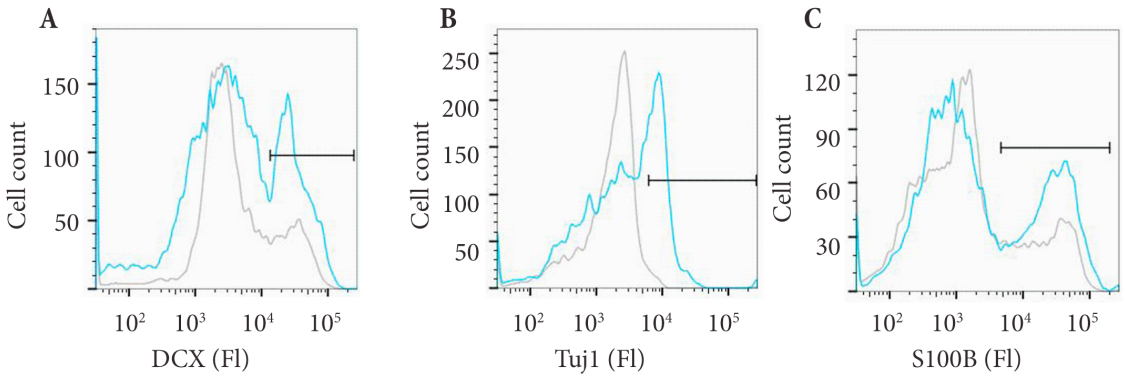


Figure 12. Runx1 over-expression induces adherence and morphological differentiation of neurospheres, without affecting proliferation A) Bright field images and (B) their corresponding GFP-fluorescent images of neurospheres in growth media treated with PBS, LV-GFP, or LV-Runx1. C) Quantification showing the formation of floating neurospheres over the course of 5 days in growth media was greatly reduced by transduction with LV-Runx1 ($p<0.01$), while D) the formation of adherent cell aggregates was increased ($p<0.01$), and E) the formation of morphologically differentiated, individual, adherent, process-bearing cells appeared after LV-Runx1 transduction ($p<0.05$) . F) The total number of cell aggregates, either as floating neurospheres or adherent colonies, was unchanged by either lentiviral treatment (n=3). EdU incorporation over a 7-hour pulse, 5 days after lentiviral treatment was not changed by either treatment, as quantified by flow cytometry, either in the total cell population (H), nor specifically in GFP+ lentiviral transfected cells (I) (n=2). a^{i}-a^{iii} scale bar = 100μm; a^{iiv} , scale bar = 25μm; a^{iv}, scale bar = 50μm. Error bars in graphs C-G indicate SEM. Error bars in H indicate SD.



D

Antigen	Treatment	% of cells under high gate	% increase after LV-Runx1 treatment
DCX	LV-GFP LV-Runx1	20.3 27.3	7.3
Tuj1	LV-GFP LV-Runx1	2.2 32.9	30.7
S100B	LV-GFP LV-Runx1	24.4 35.3	10.9

LV-GFP treated

LV-Runx1 treated

Figure 13. Runx1 over-expression strongly increases Tuj1 expression, as well as increasing DCX and S100β expression. Flow cytometry analysis indicates expression of different cell-specific markers in lentiviral transduced neurosphere cultures. Histograms depict the distribution of cells expressing A) DCX, B) Tuj1, C) S100b transduced for 5 days with either LV-GFP (grey lines) or LV-Runx1 (blue lines). The black bars in the graphs indicate the cell populations with strong antigen staining. D) The percentages of cells in the neurosphere culture expressing cell-specific markers in each lentiviral transduced population.

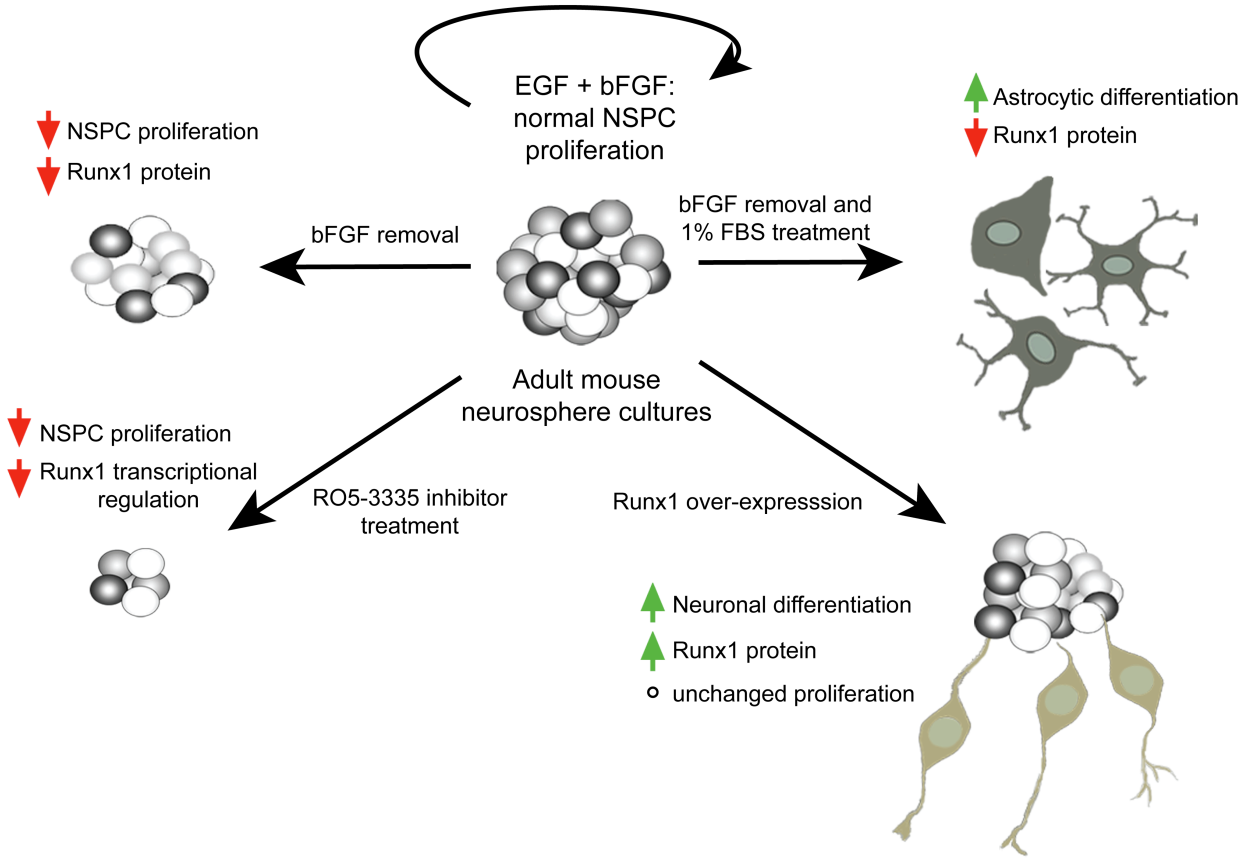


Figure 14. Model of the effects of various treatments on the proliferation rates and differentiation of cultured neurospheres. Removal of bFGF from growth media causes a drop in endogenous Runx1 protein expression, and concurrently decreases neurosphere proliferation. Inhibition of Runx1 activity with Ro5-3335 also decreases NSPC proliferation. Treatment with 1% FBS in the absence of bFGF further decreases Runx1 protein expression, and induces neurosphere cultures to differentiate almost entirely into astrocytes. Overexpression of Runx1 with LV-Runx1 causes a large percentage of cells to differentiate into neuronal cells, without affecting cell proliferation.

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