The balance of Id3 and E47 determines neural stem/precursor cell differentiation into astrocytes

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Abstract

Adult neural stem/precursor cells (NSPCs) of the subventricular zone (SVZ) are an endogenous source for neuronal replacement in CNS disease. However, adult neurogenesis is compromised after brain injury in favor of a glial cell fate, which is mainly attributed to changes in the NSPC environment. Yet, it is unknown how this unfavorable extracellular environment translates into a transcriptional program altering NSPC differentiation. Here, we show that genetic depletion of the transcriptional regulator Id3 decreased the number of astrocytes generated from SVZ-derived adult NSPCs in the cortical lesion area after traumatic brain injury. Cortical brain injury resulted in rapid BMP-2 and Id3 up-regulation in the SVZ stem cell niche. *Id3*^{-/-} adult NSPCs failed to differentiate into BMP-2-induced astrocytes, while NSPCs deficient for the Id3-controlled transcription factor E47 readily differentiated into astrocytes in the absence of BMP-2. Mechanistically, E47 repressed the expression of several astrocyte-specific genes in adult NSPCs. These results identify Id3 as the BMP-2-induced transcriptional regulator, promoting adult NSPC differentiation into astrocytes upon CNS injury and reveal a molecular link between environmental changes and NSPC differentiation in the CNS after injury.

Keywords astrocyte-specific genes; basic helix–loop–helix transcription factor; bone morphogenetic protein; traumatic brain injury; vascular damage

Subject Categories Neuroscience

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Introduction

The mammalian central nervous system (CNS) has an inherent ability to generate functional neurons after injury or in neurodegenerative disease (Arvidsson *et al*, 2002; Thored *et al*, 2006). The

subventricular zone (SVZ) serves as a stem cell niche in the adult mammalian brain, where type B1 stem cells continuously generate mobile doublecortin (DCX)+ neuroblasts via type C transit amplifying cells (Doetsch *et al*, 1999; Alvarez-Buylla & Garcia-Verdugo, 2002; Doetsch, 2003). In the healthy brain, neuroblasts migrate long distances through the rostral migratory stream (RMS) to the olfactory bulb (OB) to become interneurons (Altman, 1969; Belvindrah *et al*, 2009). However, upon CNS injury or disease, such as stroke, multiple sclerosis and trauma, adult neural stem/precursor cells (NSPCs) of the SVZ increase their proliferation, redirect their migration path to the lesion area, and preferentially differentiate into glial cells or remain in a precursor state (Picard-Riera *et al*, 2002; Goings *et al*, 2004; Li *et al*, 2010; Benner *et al*, 2013). Continuous neurogenesis is controlled by a fine-tuned NSPC microenvironment (Lim *et al*, 2000; Ihrie & Alvarez-Buylla, 2011; Ming & Song, 2011; Robel *et al*, 2011). BMP signaling in the adult SVZ is neurogenic at basal levels (Colak *et al*, 2008) and inhibits neurogenesis at elevated levels (Lim *et al*, 2000; Gajera *et al*, 2010). Thus, the magnitude of BMP signaling is tightly regulated in the SVZ stem cell niche, such as by the BMP inhibitor noggin, which can be released from ependymal cells to control NSPC behavior (Lim *et al*, 2000). During CNS disease with vascular damage, extracellular environmental changes, such as the leakage of vascular system components (Schachtrup *et al*, 2007), as well as growth factor and cytokine abundance (Hampton *et al*, 2007; Schachtrup *et al*, 2010; Xiao *et al*, 2010; Logan *et al*, 2013; Schildge *et al*, 2014) are thought to modify NSPC fate. However, transcriptional changes evoked by the altered NSPC environment in CNS disease remain poorly characterized.

Inhibitor of DNA binding (Id) proteins function as crucial regulators of cell fate determination and differentiation in mammals (Ruzinova & Benezra, 2003). Id proteins (Id1–4) are helix–loop–helix proteins that function as dominant-negative transcriptional regulators by binding to basic helix–loop–helix (bHLH) transcription factors of the E protein family (E47, E12, HEB, and E2–2). Id proteins lack the basic DNA binding region, and thus, DNA binding of an E–Id protein heterodimer is prevented (Benezra *et al*, 1990; Murre,

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2005). In embryonic NSPCs, high levels of Id proteins facilitate self-renewal and inhibition of differentiation (Lyden *et al*, 1999; Jung *et al*, 2010; Niola *et al*, 2012). In the adult brain, Id protein expression declines in most cells; however, it persists in adult NSPCs (Kitajima *et al*, 2006; Havrda *et al*, 2008; Nam & Benezra, 2009; Gajera *et al*, 2010). Id protein abundance is controlled by members of the TGF- β superfamily (Hollnagel *et al*, 1999; Miyazono & Miyazawa, 2002; Kang *et al*, 2003), and thus, Id proteins act as key molecules in mediating biological responses downstream of the TGF- β superfamily. The expression levels of members of the TGF- β superfamily and downstream signaling in stem cell niches are tightly regulated to preserve NSPC maintenance and differentiation in healthy conditions (Lim *et al*, 2000; Colak *et al*, 2008; Mira *et al*, 2010; Porlan *et al*, 2013; He *et al*, 2014). However, after traumatic brain injury (TBI) with vascular rupture, members of the TGF- β superfamily are up-regulated contributing to altered cellular functions (Hampton *et al*, 2007; Schachtrup *et al*, 2010; Xiao *et al*, 2010).

Here, we find that Id3 is necessary for BMP-2-induced differentiation of adult NSPCs into astrocytes. We show that after cortical brain injury, rapid BMP-2 up-regulation in the SVZ stem cell niche is accompanied with increased Id3 expression in NSPC subpopulations. Furthermore, we show that the differentiation of SVZ-derived NSPCs into astrocytes in the lesion area was reduced in adult *Id3*^{-/-} compared to wild-type (WT) mice. Mechanistically, we show that the Id3-controlled bHLH transcription factor E47 functioned as a transcriptional repressor of a subset of astrocyte-specific genes. Thus, we propose that Id3 serves as a molecular link between vascular damage and neuronal regeneration in the CNS after injury.

Results

Genetic depletion of *Id3* alters NSPC differentiation after traumatic brain injury

Different NSPC subpopulations of the SVZ can be distinguished by differential marker expression (Fig 1A) (Menn *et al*, 2006; Ming & Song, 2011; Codega *et al*, 2014). To investigate the role of Id3 in adult NSPCs of the SVZ, we first examined the expression of Id3 in different types of NSPCs in the medial region of the SVZ (Fig 1B and C). Among these cells, almost all B1 and C cells express Id3 (Fig 1D and E). A minor subpopulation of C cells characterized by expression of the bHLH transcription factor (TF) Olig2 is capable to migrate and leave the SVZ stem cell niche and to differentiate into oligodendrocytes and astrocytes (Marshall *et al*, 2005; Menn *et al*, 2006). We found that this Olig2⁺ subpopulation can be further segregated according to its Id3 expression into Olig2+Id3⁺ and Olig2+Id3⁻ NSPCs (Fig 1D and E). In addition, Id3 is expressed in a small percentage of DCX⁺ type A neuroblasts (Fig 1D and E) and almost absent from CD24⁺ ependymal cells lining the lateral ventricle. Therefore, NSPCs residing in the SVZ are predominantly Id3-positive. However, variegated Id3 expression in the Olig2⁺ subpopulation of C cells and in DCX⁺ type A neuroblasts suggests further heterogeneity of these cell types. We thus conclude that NSPCs with the potential to leave the stem cell niche and the potential to differentiate into oligodendrocytes and astrocytes (C cells) and neurons (neuroblasts, A cells) are composed of Id3-positive and Id3-negative cell populations.

After injury, NSPCs originating from the SVZ change their migration path. Instead of migrating to the OB and becoming neurons, they migrate to the lesion area and differentiate mainly into glial cells (Picard-Riera *et al*, 2002; Goings *et al*, 2004; Li *et al*, 2010; Benner *et al*, 2013). To investigate the role of Id3 in regulating NSPC migration and differentiation after brain injury, we performed cortical stab wound injury (SWI), a model of brain trauma (Fig 2A; Appendix Fig S1A and B). WT and *Id3*^{-/-} mice were treated with 5-bromo-2'-deoxyuridine (BrdU) for 3 consecutive days before SWI with the last injection 6 h before the cortical injury to track NSPCs originating from the SVZ (Sundholm-Peters *et al*, 2005). Colocalization analysis of BrdU in combination with cell type-specific markers was performed to determine the cell fate of the SVZ-derived NSPC in the lesion area 10 days after SWI (Appendix Fig S1C). The applied BrdU injection regime resulted in exclusive labeling of NSPCs in the SVZ stem cell niche and their progeny, as no proliferating astrocytes in the cortex lesion area (BrdU+GFAP⁺ cells) were detectable at early timepoints after SWI (Appendix Fig S2), which is in accordance with the literature that the half-life of systemic BrdU is ~2 h (Deng *et al*, 2010) and that astrocyte proliferation starts ~3 days after cortical injury (Buffo *et al*, 2005; Bardehle *et al*, 2013; Wanner *et al*, 2013). Immunolabeling of SVZ-derived NSPCs with BrdU revealed migration of SVZ-derived NSPCs (BrdU⁺, white arrowheads) to the lesion area 10 days after SWI (Fig 2B). Remarkably, loss of Id3 using the *Id3*^{-/-} mouse line revealed a 45% reduction of BrdU⁺ cells as well as a 70% reduction in the number of SVZ-derived adult NSPCs differentiating into astrocytes (BrdU+GFAP⁺) in the lesion area compared to control mice (Fig 2C–E). Accordingly, using S100b as a marker for mature astrocytes that have lost their neural stem cell potential (Raponi *et al*, 2007), we revealed that *Id3*^{-/-} mice showed a 65% reduction in the number of BrdU+S100b⁺ astrocytes in the lesion area than WT mice (Fig EV1). Interestingly, *Id3*^{-/-} mice showed an increased number of SVZ-originated BrdU⁺ cells that migrated to the glomerular layer of the OB (Fig EV2). Furthermore, an increased number of the BrdU⁺ cells differentiated into neurons in the healthy *Id3*^{-/-} animals and 10 days after SWI (Fig EV2). Lack of Id3 did not affect astrocyte activation of resident astrocytes in the cortex lesion area at day 3 after SWI (Appendix Fig S3) and no significant differences in SVZ cell composition or proliferation and cell death of NSPCs *in vitro* between healthy *Id3*^{-/-} and WT mice were detected (Appendix Fig S4). In addition, *Id3*^{-/-} adult NSPCs showed no significant change in cell migration and adhesion *in vitro* (Appendix Fig S5). Overall, these results reveal that Id3 promotes the abundance of SVZ-derived newborn astrocytes in the lesion area after brain injury.

Id3 expression is differentially regulated in NSPCs after injury

Extracellular factors in the adult SVZ niche are finely balanced to propagate continuous neurogenesis of NSPCs (Lim *et al*, 2000; Colak *et al*, 2008). To address the question whether a potentially altered NSPC environment after cortical brain injury with vascular rupture regulates Id3 abundance, we examined the expression of Id3 in SVZ NSPC subpopulations at the ipsilateral and contralateral side at different timepoints after SWI. NSPC subpopulations derived of the SVZ, such as neuroblasts (DCX⁺), a small fraction of C cells (Olig2⁺), and SVZ-generated astrocytes (Thbs4⁺), have been shown

Figure 1. Id3 expression in the adult SVZ niche.

A Scheme illustrating SVZ cell type-specific markers.

B Scheme showing the brain region examined for the cell type-specific Id3 expression in the SVZ.

C Low-magnification images showing immunolabeling for Id3 (green) in the SVZ niche in coronal brain sections of adult mice. White box indicates the area of quantification.

D Immunolabeling for Id3 (green) in combination with CD24, GFAP, Sox2, nestin, Ki67, Mash1, Olig2 or DCX (all red) in cells of the adult SVZ in coronal brain sections of adult mice.

E Quantification of Id3+ SVZ cells per individual cell type.

Data information: Nuclei are stained with DAPI (blue). CC, corpus callosum; DH, dorsal horn; LV, lateral ventricle; SVZ, subventricular zone. Scale bars: 200 μ m (C), and 12 μ m (D). Values are mean $\pm$ SEM ($n = 4$ mice).

to migrate to the lesion area upon CNS disease (Goings *et al*, 2004; Sundholm-Peters *et al*, 2005; Menn *et al*, 2006; Benner *et al*, 2013) (Fig 3A). Our results revealed that BrdU+Thbs4+ SVZ-generated astrocytes as well as BrdU+Olig2+ C cells co-express GFAP in the lesion area at 10 days after SWI and therefore contribute to the cortical lesion astrocyte population and are the potential source for Id3-regulated NSPC differentiation into astrocytes after injury (Fig 3A and B). DCX+ neuroblasts are already fate-restricted

to become neurons and therefore do not contribute to the cortical lesion astrocyte population (Liu *et al*, 2009; Obernier *et al*, 2014).

Interestingly, the number of GFAP+ B cells in the SVZ already increased significantly at day 1 after SWI (Fig EV3B), and thereafter, the number of GFAP+ B cells and Thbs4+ cells significantly decreased until day 3 and 10 after SWI, respectively, while the total cell number and the number of Olig2+ cells in the SVZ did

Figure 2. Decreased number of SVZ-derived NSPCs differentiating into astrocytes in the lesion area 10 days after SWI in *Id3*^{-/-} mice.

A Scheme illustrating the area of analysis after stab wound injury (SWI).

B Representative images of uninjured and injured WT mice 10 days after SWI immunolabeled for BrdU (green). White arrowheads indicate migration of SVZ-derived BrdU+ NSPCs to the lesion area. Yellow dotted line indicates the lesion border.

C Double-IHC for BrdU (green) and GFAP (red, marker for astrocytes) in the lesion area in *Id3*^{-/-} mice compared to WT mice. White box indicates the area of quantification, shown in (D).

D High magnification images of double-IHC for BrdU (green) and GFAP (red) in the quantification area in *Id3*^{-/-} mice compared to WT mice. Enlargements of regions indicated by rectangles show BrdU+GFAP- cells (left) and BrdU+GFAP+ cells (middle and right, indicated by an asterisk).

E Quantification of BrdU+ cells and BrdU+GFAP+ cells.

Data information: CC, corpus callosum; CTX, cortex; LV, lateral ventricle; n.d., not detected; OB, olfactory bulb; RMS, rostral migratory stream; SVZ, subventricular zone. Scale bars: 50 μ m (B), 75 μ m (C), and 50 μ m (D). Values are mean $\pm$ SEM (P -values calculated by one-way ANOVA; $n = 6$ mice per group).

not change significantly at any of the investigated timepoints after SWI (Fig EV3A and B). Importantly, the percentage of Id3-expressing cells among Thbs4+ cells increased significantly at day 1 after SWI compared to uninjured control animals (Fig 3E and

H) and the percentage of Id3 immunoreactivity increased significantly in Id3+GFAP+ B cells, and in Id3+Thbs4+ SVZ-generated astrocytes, suggesting a role for Id3 in the fate control of these cells at early timepoints after SWI (Fig 3C and F, bottom; Fig 3E

Figure 3. Differentially regulated Id3 expression in NSPC subpopulations of the adult SVZ niche after cortical lesion.

- A** Scheme illustrating potential SVZ-derived NSPCs contributing to astrocytes in the cortical lesion area 10 days after SWI.
- B** Triple-IHC for BrdU (green), and GFAP (blue) in combination with marker for SVZ-generated astrocytes (Thbs4, red), and a subpopulation of C cells (Olig2, red) in the lesion area in WT mice 10 days after SWI. Enlargements of regions indicated by rectangles show BrdU+GFAP+Thbs4+ and BrdU+GFAP+Thbs4- cells (left), and BrdU+GFAP+Olig2+ cells (right). Triple-positive cells are indicated by an asterisk.
- C–E** Immunolabeling for Id3 (green) in combination with GFAP (C), Olig2 (D), or Thbs4 (E) (all red) in the ipsilateral SVZ niche 1, 3, 8, and 10 days after cortical SWI compared to uninjured control in coronal brain sections of WT mice. Enlargements of regions indicated by rectangles depict representative Id3 immunoreactivity of Id3+GFAP+ (C), Id3+Olig2+ (D), or Id3+Thbs4+ (E) cells at the different timepoints after SWI and uninjured control (high magnification images at the bottom).
- F–H** Quantification of Id3+ cells per total GFAP+ cells (F, top), Olig2+ cells (G, top), or Thbs4+ cells (H, top) and Id3 immunoreactivity per total Id3+GFAP+ cells (F, bottom), Id3+Olig2+ cells (G, bottom), or Id3+Thbs4+ cells (H, bottom).
- I** Immunolabeling for BMP-2 (red) in combination with GFAP (green) in the ipsilateral SVZ niche 1, 3, 8, and 10 days after cortical SWI compared to uninjured control in coronal brain sections of WT mice (top). White box indicates representative BMP-2 colocalization with GFAP+ cells (white arrowheads) and secreted BMP-2 (yellow arrowheads) at 3 days after SWI (high magnification images at the bottom).
- J** Quantification of BMP-2 immunoreactivity in the SVZ per area.

Data information: CTX, cortex; LV, lateral ventricle; RMS, rostral migratory stream. Scale bars: 18 μ m (B), 27 μ m (C–E, I, all top), 9 μ m (C–E; all bottom), and 7 μ m (I, bottom). Values are mean $\pm$ SEM [P-values calculated by one-way ANOVA; $n = 4$ mice per group (C–H); $n = 5$ mice per group (I, J)].

and H, bottom). Subsequently, the percentage of Id3+ cells of all investigated NSPC subpopulations and the percentage of Id3 expression levels per Id3+ cell subpopulation decreased and returned to basal level or below at later timepoints after SWI (Fig 3C–H). Overall, SWI resulted in an ipsilateral and contralateral SVZ stem cell niche activation, with a rapid up-regulation of Id3 in NSPC subpopulations (Figs 3 and EV3), suggesting prompt changes in the SVZ NSPC microenvironment after cortical brain injury.

Cytokines and growth factors, especially members of the TGF- β superfamily, regulate *Id* gene expression (Hollnagel et al, 1999; Miyazono & Miyazawa, 2002; Kang et al, 2003). In previous work, we linked vascular damage with the immediate bioavailability of active TGF- β , and other groups revealed BMP up-regulation after TBI in the lesion area (Hampton et al, 2007; Schachtrup et al, 2010; Xiao et al, 2010). Surprisingly, immunolabeling for BMP-2 (red) and GFAP (green) revealed increased BMP-2 expression colocalizing with GFAP+ cells (Fig 3I top and bottom, white arrowheads) and secreted BMP-2 in the SVZ NSPC extracellular environment (Fig 3I top and bottom, yellow arrowheads) at both the ipsilateral and contralateral side at days 1 and 3 after SWI (Figs 3I and J, and EV3D), while BMP-4 level in the SVZ niche is not altered at different timepoints after SWI (Fig EV3E). Id3 and BMP-2 are expressed in resident astrocytes (Appendix Fig S6A–D) and Id3 is also expressed in reactive, proliferating BrdU-Olig2+GFAP+ astrocytes (Appendix Fig S6E) (Chen et al, 2008) in the cortical lesion area at day 10 after SWI. In addition, SVZ-derived NSPCs in the cortical lesion area are still expressing Id3 (BrdU+Id3+GFAP+, BrdU+Id3+Thbs4+, both cell types ~90% Id3+; BrdU+Id3+Olig2+, ~50% Id3+) (Appendix Fig S6F), suggesting that, beside a potential role of BMP-2 and Id3 in resident astrocyte proliferation and activation (Tzeng et al, 1999; Hampton et al, 2007), BMP-2 and Id3 might still be important for further maturation of SVZ-derived NSPC differentiation into astrocytes in the cortical lesion area. Overall, a cortical lesion can result in increased BMP-2 level in the SVZ stem cell niche, which might be translated into rapid Id3 up-regulation altering NSPC fate toward astrocyte differentiation.

Id3 promotes BMP-2-induced differentiation of NSPCs into astrocytes

Id3 expression has been shown to be differentially regulated by members of the TGF- β superfamily in various cell types, including embryonic stem cells (Hollnagel et al, 1999). Since BMP-2 and Id3 are both up-regulated in NSPC subpopulations in the SVZ stem cell niche after SWI and BMP-2 induces the differentiation of embryonic NSPCs into astrocytes (Gross et al, 1996), we sought to determine the direct role of Id3 in BMP-2-induced adult NSPC differentiation into astrocytes. While BMP-2 treatment of WT NSPCs induced a robust increase in the appearance of GFAP+ astrocytes 4 days after initiation of differentiation *in vitro*, *Id3*^{-/-} NSPCs were resistant to BMP-2-induced differentiation into astrocytes (Fig 4A and B). Interestingly, those *Id3*^{-/-} NSPCs, which differentiated into astrocytes in the untreated and BMP-2-treated cultures, displayed a different morphology than WT astrocytes, being mainly bipolar (Fig 4A). Furthermore, *Id3*^{-/-} NSPCs showed greater differentiation into class III beta-tubulin (Tuj-1)+ neurons than BMP-2-treated WT cells (Fig 4B). Accordingly, BMP-2 treatment of *Id3*^{-/-} NSPCs failed to

increase the protein abundance of typical astrocyte genes such as *GFAP*, *Aqp4*, and *Aldh1l1* 2 days after the initiation of differentiation (Fig 4E), suggesting that *Id3*^{-/-} NSPCs are refractory to BMP-induced differentiation of adult NSPCs into astrocytes. Besides BMP, ciliary neurotrophic factor (CNTF) also promotes the differentiation of cortical precursor cells into astrocytes, while TGF- β has been shown to promote the differentiation of embryonic radial glia cells into astrocytes (Bonni et al, 1997; Stipursky et al, 2014). Remarkably, CNTF treatment induced robust astrocyte differentiation both in WT and in *Id3*^{-/-} NSPCs, revealing that the refractory response of the *Id3*^{-/-} NSPCs is specific for BMP-2-induced differentiation into astrocytes (Fig 4C). In addition, TGF- β treatment did not induce the differentiation of WT and *Id3*^{-/-} adult NSPCs into astrocytes (Fig 4D). *In vitro* cultured primary neurospheres derived from the adult SVZ mouse tissue express Id3 and single adult NSPCs cultured under differentiation conditions revealed a nuclear Id3 expression pattern (Fig 4F). Treatment of primary NSPCs with BMP-2 induced a strong and rapid up-regulation of Id3 mRNA and protein, compared to untreated control cells (Fig 4G), while CNTF treatment had no effect and TGF- β treatment resulted in the repression of Id3 expression in NSPCs (Fig 4H and I). Overall, these results show that the transcriptional regulator Id3 is strongly up-regulated by BMP-2 and that Id3 is necessary for BMP-2-driven astrocyte differentiation of adult NSPCs.

The bHLH transcription factor E47 prevents the BMP-2-induced NSPC differentiation into astrocytes

Id3 functions by preventing DNA binding and thus target gene regulation of class I bHLH transcription factors, called E proteins (Benezra et al, 1990). E proteins are the obligate partners of the proneurogenic bHLH transcription factors, such as Mash1, Ngn, and NeuroD, but their role in adult NSPC differentiation into astrocytes after brain injury has not yet been described (Ruzinova & Benezra, 2003). The E protein E47 is expressed in various NSPC subpopulations of the SVZ, although no significant changes in E47 expression in the different NSPC subpopulations were detectable between *Id3*^{-/-} and WT uninjured mice and 3 days after SWI (Fig 5A and B). E47 was expressed in primary NSPCs under proliferative and under differentiation conditions (Fig 5C). Endogenous co-immunoprecipitation experiments using lysate of WT NSPCs cultured under differentiation conditions revealed that Id3 interacts with E47 in adult NSPCs (Fig 5D), indicating that the rapid increase in Id3 expression results in reduced E47 transcriptional activity immediately upon Id3 induction. At later timepoints, we found that additionally, BMP-induced differentiation of NSPCs into astrocytes led to a down-regulation of E47 by twofold (Fig 5C), leading to a more sustained decrease in E47 transcriptional activity. Next, we determined if the balance of Id3 and E47 regulates the BMP-2-induced differentiation of adult NSPCs into astrocytes by using NSPCs from *E47*^{-/-} mice. Remarkably, already 2 days after the initiation of differentiation, more untreated *E47*^{-/-} NSPCs differentiated into GFAP+ astrocytes than WT NSPCs in the absence of BMP-2 (Fig 5E and F). BMP-2 treatment further increased the differentiation of *E47*^{-/-} NSPCs into astrocytes (Fig 5E and F). These data suggest that Id3 promotes astrocyte differentiation by restricting the gene regulatory activity of E47.

Figure 4. Id3 regulates the BMP-2-induced differentiation of adult NSPCs into astrocytes.

A Representative images of immunolabeled GFAP+ astrocytes (green) of untreated and BMP-2-treated *Id3*^{-/-} and WT NSPCs cultured for 4 days on poly-D-lysine. B–D Graphs showing quantification of GFAP+ astrocytes (B, left; C, D) and Tuj-1+ neurons (B, right) of differentiated WT and *Id3*^{-/-} NSPCs (untreated) and NSPCs treated with BMP-2 (B), CNTF (C) or TGF-β (D). E Immunoblot protein expression analysis for astrocyte-specific markers in BMP-2-treated *Id3*^{-/-} and WT NSPCs cultured for 2 days on laminin. F Immunolabeling for Id3 (green) in neurospheres and NSPCs *in vitro*. G–I Expression of *Id3* mRNA in NSPCs after BMP-2 (G), CNTF (H), or TGF-β (I) treatment determined by quantitative PCR and normalized to *GAPDH* (top). Protein expression of Id3 in NSPCs after BMP-2 (G), CNTF (H), or TGF-β (I) treatment determined by Western blotting (bottom).

Data information: Nuclei are stained with DAPI (blue). Scale bars: 45 μm (A), 40 μm (F, left), and 6 μm (F, right). Values are mean ± SEM (*P*-values calculated by one-way ANOVA; ns, not significant) of at least three independent experiments (A–D). A minimum of 250 cells per condition were analyzed. Representative Western blots are shown from three independent experiments (E, G–I). Quantitative PCR results are of three (G and H) and of two (I) independent experiments performed in duplicate.

Source data are available online for this figure.

E47 restricts NSPC differentiation into astrocytes by repressing the expression of astrocyte-specific genes

To identify which genes are regulated by the Id3–E47 axis during BMP-induced NSPC differentiation into astrocytes, we used microarrays to analyze gene expression in WT and *Id3*^{−/−} adult NSPCs 3 h after BMP-2 treatment. Recently, a transcriptome database provided an astrocyte-specific transcriptional signature (Cahoy et al, 2008). By comparing our microarray gene expression data to the astrocyte transcriptional signature we found that upon BMP-2 treatment, *Id3*^{−/−} NSPCs failed to increase a subset of astrocyte-specific genes, such as *GFAP*, *Aldh1l1*, *Slc1a2*, *Aqp4*, and *Slc1a3* (also known as *GLAST*), although these genes were all up-regulated in WT control NSPCs upon BMP-2 treatment (Fig 6A and Appendix Table S1; gray shadow). We then clustered selected genes showing

the most robust and consistent differences in expression in *Id3*^{−/−} NSPCs treated with BMP-2 for 3 h compared to WT control cells to identify astrocyte-specific genes being regulated by E47 (Fig 6B and Appendix Table S2). After comparison to the astrocyte transcriptional signature, a striking match was found for a group of genes belonging to the solute carrier (SLC) family (Appendix Table S2; gray shadow), including *GLAST*, which is expressed at significant lower levels

(1.6-fold) in *Id3*^{−/−} NSPCs treated with BMP-2 than in WT cells.

To identify genes that are potentially regulated by E47, we overexpressed E47 in primary adult NSPCs and revealed that E47 repressed the gene expression of *GFAP* and *GLAST* (Fig 6C). E proteins either act as transcriptional activators or repressors by directly binding to specific DNA sites, called E-boxes (CANNTG). Next, we analyzed the promoter or putative enhancer regions of

Figure 5. Id3 interacts with the bHLH transcription factor E47 to regulate BMP-induced NSPC differentiation into astrocytes.

A Immunofluorescence for E47 (green) in combination with GFAP (top), Olig2 (middle), or Thbs4 (bottom) (all red, marker for different NSPC subpopulations) in the adult SVZ niche in *Id3*^{−/−} mice compared to WT mice 3 days after SWI compared to uninjured mice.
 B Quantification of E47+ cells per GFAP+ (B cells, top), Olig2+ (C cells, middle), and Thbs4+ cells (SVZ-generated astrocytes, bottom).
 C E47 expression in primary NSPCs cultured under proliferation conditions, differentiation conditions and differentiation conditions treated with BMP-2 determined by Western blotting.
 D Endogenous co-immunoprecipitation of Id3 with E47. Primary NSPC lysate was immunoprecipitated with anti-Id3 antibody, and Western blots were developed with anti-E47 (top) and anti-Id3 (bottom) protein antibody.
 E Representative images of immunolabeled GFAP+ astrocytes (green) of untreated and BMP-2-treated *E47*^{−/−} and WT NSPCs cultured for 2 days on poly-D-lysine.
 F Graph showing quantification of GFAP+ astrocytes of differentiated WT and *E47*^{−/−} NSPCs (untreated) and NSPCs treated with BMP-2.

Data information: LV, lateral ventricle. Nuclei are stained with DAPI (blue). Scale bars: 24 μm (A), and 60 μm (E). Representative Western blots are shown from three independent experiments (C). Values are mean ± SEM [P-values calculated by one-way ANOVA; ns, not significant; n = 3 mice per group (A, B), n = 5 independent experiments (E, F)]. A minimum of 400 cells per condition were analyzed.

Source data are available online for this figure.

GFAP and the highly regulated SLC family members, including *GLAST* for the presence of conserved E-boxes by using the rVISTA genome browser. Interestingly, we identified conserved E-boxes in the *GFAP* gene and in six genes of the SLC family, including the gene encoding for the glutamate transporter *GLAST* (Fig 6D). To determine whether E47 binds to the *GFAP* and *GLAST* loci, we used chromatin immunoprecipitation with an E47-recognizing antibody in NSPCs and subsequent quantitative real-time PCR. This

analysis revealed that E47 was bound to a region spanning the putative regulatory region of the *GFAP* and *GLAST* genes in adult NSPCs (Fig 6E), suggesting a direct regulation of *GFAP* and *GLAST* expression by E47. Since we found conserved E-boxes in the first intron, the 5'UTR and the putative promoter region of *GLAST*, we performed luciferase assays to analyze, if the activity of these putative regulatory regions is altered by E47. Therefore, we cloned a 2,400-bp fragment, including the first intron, the first non-coding

Figure 6. E47 regulates BMP-2-induced astrocyte-specific genes in NSPCs.

- A** Microarray gene expression profile of WT NSPCs compared to *Id3*^{-/-} NSPCs after treatment with BMP-2 for 3 h. Displayed are signal intensity values in WT (y-axis) or *Id3*^{-/-} (x-axis) NSPCs. Differentially expressed genes lie on the left or right of the diagonal line representing equally expressed genes.
- B** Heatmap showing genes that are changed by a factor of at least 1.5 between BMP-2-treated WT and *Id3*^{-/-} NSPCs.
- C** Expression of *GFAP* (top) and *GLAST* (bottom) mRNA in NSPCs 24 h after electroporation with E47 plasmid or control plasmid determined by quantitative PCR and normalized to *GAPDH*.
- D** Comparison of mouse and human *GFAP* and *Slc1a3* (*GLAST*) genomic region, displaying conserved regions and E-boxes.
- E** ChIP assay using anti-E2A antibody on NSPC lysate from adult WT mice. Relative enrichment of the bound DNA over input was determined by quantitative PCR using specific primers for the promoter region of *GFAP* and 5'UTR region of *GLAST*. The *Arbp* gene, which does not contain an E-box, served as a negative control.
- F** Luciferase reporter assay in HEK293T cells using the indicated *GLAST* luciferase reporter construct.

Data information: Data are derived from four microarray replicates using cells from independent preparations (A, B). Values are mean ± SEM [*P*-values calculated by Student's *t*-test (C, E) or by one-way ANOVA (F); *n* = 3 (C, E); *n* = 5 (F)].

exon, and the putative promoter upstream of the luciferase gene (Fig 6D), and transfected HEK293T cells with this construct and simultaneously with increasing amounts of E47. We found that the transcriptional activity of the reporter gene was 20-fold higher with the putative *GLAST*-regulatory element than with the empty control plasmid, suggesting that this DNA segment contains the *GLAST* promoter and probably an additional enhancer element (Fig 6F). Furthermore, the transcriptional activity of these elements was decreased by E47 in a dose-dependent manner (Fig 6F). In summary, these results suggest that E47 prevents the differentiation of NSPCs into astrocytes by directly repressing the expression of a subset of astrocyte-specific genes, such as *GFAP* and *GLAST*.

Discussion

The impaired differentiation of SVZ-derived NSPCs into neurons in the lesion site after TBI or in CNS disease is thought to be caused by a changed extracellular environment, directing NSPCs to differentiate into astrocytes. Our study showed that cortical brain injury leads to environmental changes within the SVZ stem cell niche, resulting in an altered balance of the transcriptional regulators Id3 and E47 in NSPCs, leading to preferential differentiation of NSPCs into astrocytes. Our study suggests the following model for the role of Id3 in adult NSPC differentiation after TBI: (i) TBI results in increased BMP-2 abundance in the SVZ. (ii) Elevated BMP-2 levels are translated into an increase in Id3 expression in adult NSPC subpopulations. (iii) Id3 then heterodimerizes with the bHLH transcription factor E47 and prevents E47-mediated repression of astrocyte-specific gene expression. (iv) Consequently, adult NSPCs preferentially differentiate into astrocytes. The key event in this model is the BMP-2-induced upregulation of Id3 expression in NSPCs of the SVZ, and thus, E47-mediated repression of astrocyte-specific genes will only be released under pathological conditions with increased BMP-2 abundance.

The extracellular environment of the SVZ niche is instrumental for instructing NSPC maintenance and differentiation (Ihrie & Alvarez-Buylla, 2011; Fuentealba et al, 2012). After TBI, the extracellular environment within the damaged brain parenchyma changes due to the increased leakage of blood factors as well as locally produced cytokines and growth factors (Hampton et al, 2007; Schachtrup et al, 2007, 2010; Xiao et al, 2010; Logan et al, 2013). Surprisingly, we found that a cortical lesion can result in a globally changed extracellular environment of NSPCs in the SVZ stem cell niche. Our data revealed rapid appearance of BMP-2 in the ipsilateral and contralateral SVZ upon cortical brain injury, which results in elevated Id3 protein level in NSPCs. Consequently, the gene regulatory activity of E47 is restricted allowing NSPC differentiation into astrocytes. While BMP-4 expression level was not affected by the cortical injury, our data suggest that SVZ GFAP+ astrocytes or SVZ B1 stem cells are the main source of extracellular BMP-2. However, we cannot rule out other SVZ cell types expressing and secreting BMP-2 at this point.

Previous studies demonstrated that TBI with vascular rupture leads to the immediate leakage of the blood protein fibrinogen into the brain parenchyma. Fibrinogen is a carrier of latent TGF- β , which was processed to active TGF- β upon interaction with astrocytes, leading to the activation of the TGF- β /Smad signaling pathway in

astrocytes (Schachtrup et al, 2010). Here, we found that TGF- β represses Id3 expression in adult NSPCs *in vitro* and that Id3 expression levels are downregulated after the initial rapid BMP-2-induced up-regulation within the SVZ stem cell niche after SWI, suggesting that potential TGF- β in the SVZ stem cell niche might down-regulate Id3 at later timepoints after SWI. While TGF- β has been shown to induce the differentiation of embryonic stem cells into astrocytes (Stipursky et al, 2014), we find here that TGF- β signaling does not induce the differentiation of adult NSPCs into astrocytes *in vitro*. It would be intriguing to examine whether the niche vascular system is a source for fibrinogen-bound latent TGF- β in the SVZ stem cell environment after cortical injury, as it has already been shown that the SVZ vascular system is already permeable for circulating small molecules under homeostatic conditions (Tavazoie et al, 2008), and whether NSPCs or SVZ astrocytes are able to activate this fibrinogen-bound latent TGF- β , which might then down-regulate Id3 expression in NSPCs. Therefore, the initial up-regulation of BMP-2, which results in elevated Id3 protein level in NSPCs, restricts the gene regulatory activity of E47 allowing NSPC differentiation into astrocytes. At later timepoints, the potentially TGF- β -mediated low levels of Id3 expression within the SVZ stem cell niche might result in higher transcriptional activity of E47 leading again to the repression of astrocyte-specific genes and adjusting the SVZ stem cell niche back to homeostatic NSPC maintenance and differentiation.

Other pathways, such as the STAT3-signaling pathway, are activated after injury (Okada et al, 2006) and promote astrogenesis by up-regulating astrocyte-specific genes (Kessaris et al, 2008; Freeman, 2010). CNTF injection into the adult mouse forebrain increases the number of proliferating cells in the SVZ and increases the number of BrdU+GFAP+ cells, raising the possibility that CNTF stimulates the formation of GFAP precursors in the SVZ (Emsley & Hagg, 2003). Notably, the activation of STAT signaling by CNTF induced comparable adult NSPC differentiation into astrocytes of *Id3*^{-/-} and WT cells and CNTF did not change Id3 expression levels in NSPCs *in vitro*, indicating that the BMP-2-driven differentiation of adult NSPCs into astrocytes strictly depends on increasing Id3 levels, while NSPC differentiation into astrocytes can also occur through Id3/E47-independent pathways.

Under homeostatic conditions, we found predominant Id3 expression in NSPC subpopulations (B cells, C cells) and SVZ-generated Thbs4+ astrocytes. This observation agrees with recent findings that Id proteins contribute to NSPC identity in the SVZ stem cell niche under healthy conditions (Nam & Benezra, 2009; Niola et al, 2012). Simultaneous inactivation of the three Id genes *Id1*, *Id2*, and *Id3* in NSPCs triggered detachment of embryonic and postnatal NSPCs from the ventricular and vascular niche. Our study revealed no difference in the cell composition of the SVZ in *Id3*^{-/-} mice in the uninjured condition, suggesting that Id proteins can compensate for their role in anchorage of neural stem cells to the niche. Our study revealed that an Olig2+ NSPC subpopulation of C cells and DCX+ neuroblasts are composed of an Id3-positive and Id3-negative population. We could show that shifting the Id3–E47 balance toward high E47 activity (either in *Id3*^{-/-} or in E47 overexpression experiments) resulted in decreased NSPC differentiation into astrocytes. As it has been demonstrated that C cells can contribute to oligodendrocytes and astrocytes and that type A neuroblasts are fate-restricted to become neurons, future studies will determine whether the Id3-negative NSPC subpopulations might be prone to

differentiate into oligodendrocytes and neurons, respectively. Indeed, *Id3*^{-/-} mice showed increased neurogenesis in the OBs in uninjured mice.

SVZ-generated Thbs4⁺ astrocytes and Olig2⁺ C cells have been shown to be capable to leave the SVZ stem cell niche after brain injury and contribute to the cortical astrocyte population (Menn *et al*, 2006; Benner *et al*, 2013). Indeed, we detected SVZ-derived NSPCs that co-expressed Thbs4 or Olig2 with GFAP in the cortical lesion area. Interestingly, Id3 was up-regulated rapidly in Thbs4⁺ cells and Olig2⁺ C cells in the SVZ upon injury and its expression persisted in these SVZ-derived cells in the cortical lesion area. Furthermore, the higher percentage of Id3-expressing cells among all Thbs4⁺ SVZ-generated astrocytes as well as the higher percentage of Id3 immunoreactivity per Id3⁺Thbs4⁺ cells was observed in the ipsilateral SVZ side compared to the contralateral side at day 1 after SWI, suggesting that although a cortical lesion can result in a globally changed extracellular environment of NSPCs in the SVZ stem cell niche, the magnitude of the response to cortical injury is different between the ipsilateral and contralateral side. Until now, it is not known whether B cells or their progeny other than Thbs4⁺ astrocytes and Olig2⁺ C cells contribute to the cortical astrocyte population after injury and whether the Id3–E47 axis also regulates their differentiation into astrocytes. Yet, based on our data, we suggest that Id3 plays a major role in the migration and differentiation of Olig2⁺ and Thbs4⁺ cells into astrocytes contributing to the cortical astrocyte population after injury.

Even though genes specifying the astrocyte-lineage were reported (Cahoy *et al*, 2008), their transcriptional regulation during adult NSPC differentiation into astrocytes is not known. Our study suggests that in adult NSPCs, the expression of at least a subset of these astrocyte-signature genes, including the astrocyte marker GFAP and SLC family members, is actively repressed by E47. During development, embryonic NSPC differentiation into astrocytes is regulated by transcriptional activation of the GFAP promoter through the JAK-STAT pathway (Sun *et al*, 2001; Freeman, 2010). This pathway probably also activates GFAP upon CNTF treatment in adult NSPCs and is independent of Id3–E47, suggesting that E47 might get degraded or displaced from the astrocyte-specific promoters. We showed in adult NSPCs of the SVZ that E47 repressed a subset of astrocyte-specific genes by direct binding to regulatory regions of these genes, by repression of mRNA expression of astrocyte-specific genes in E47-overexpressing primary NSPCs and by the genetic depletion of E47, which resulted in increased adult NSPC differentiation into astrocytes. Accordingly, *Id3*^{-/-} mice, which might have increased E47 activity and thus increased active repression of astrocyte-determining genes, showed an increased NSPC differentiation into neurons within the OBs compared to WT mice. However, we detected no alteration of SVZ-derived NSPC differentiation into neurons after injury. Therefore, under pathological conditions, high Id3 expression in a subset of NSPCs might then release E47 from the repressed astrocyte-specific genes, directing the SVZ-derived NSPCs toward the lesion area and astrocyte differentiation, while at the same time, another Id3-negative subset migrates toward the OBs to generate neurons. Future studies will show whether neurogenesis in the OB might be independent of the BMP-2–Id3 axis in the SVZ after injury.

Since NSPCs from the SVZ niche are an endogenous source for repair mechanisms (Lindvall & Kokaia, 2006; Benner *et al*, 2013;

Gage & Temple, 2013), identification of novel mechanisms regulating adult NSPC differentiation into neurons and astrocytes after CNS injury is considered essential for the design of therapies to initiate CNS regeneration. The first studies investigating the role of Id proteins in adult NSPCs (Havrdá *et al*, 2008; Nam & Benezra, 2009) including this study identify distinct roles of the individual Id proteins in adult NSPC identity, suggesting a targeted therapy against specific Id proteins. Id-specific siRNA and Id-binding peptides to target the protein–protein interaction properties of Id proteins with bHLH transcription factors have been developed and showed therapeutic efficacy in models of cancer (Lasorella *et al*, 2014). Thus, the importance of the BMP-2–Id3–E47 axis in determining adult NSPC fate decisions could provide novel targets for therapeutic intervention with the unfavorable NSPC differentiation into astrocytes after injury.

Materials and Methods

Animals

C57BL/6J mice (Jackson Laboratory), C57BL/6J-inbred mice deficient for inhibitor of DNA binding 3 (*Id3*^{-/-}) (Rivera *et al*, 2000) and deficient for the E2A splice variant E47 (*E47*^{-/-}) (Beck *et al*, 2009) were used. All animal experiments were approved by the Federal Ministry for Nature, Environment and Consumer Protection of the state of Baden-Württemberg and were performed in accordance with the respective national, federal, and institutional regulations.

Cortical stab wound injury and BrdU labeling regime

Stab wound injury (SWI) was performed as described (Schachtrup *et al*, 2010). To analyze SVZ-derived adult NSPCs after SWI on sagittal brain sections and BMP-2, BMP-4, and Id3 expression in the SVZ after SWI in coronal brain sections, a 30-gauge needle was stereotactically inserted (sagittal: anteroposterior (AP), 1.5 mm; mediolateral (ML), –0.5 mm; dorsoventral (DV), –3.0 mm; coronal: AP, 0 mm; ML, –1.2 mm; DV, –1.5 mm from bregma, according to Paxinos and Franklin) and left in place for 5 min. To track NSPCs of the SVZ, adult mice were injected intraperitoneally with a daily dose of BrdU (30 mg/kg body weight, Life Technologies) for three consecutive days. On the third day, SWI was performed 6 h after the last injection, and mice were sacrificed 2 h after SWI (day 0), or at days 1, 3, 8, and 10 after SWI.

Immunohistochemistry

Mice were transcardially perfused with ice-cold saline, followed by 4% paraformaldehyde in phosphate buffer, and 30-μm sections were cut after cryoprotection. Analysis of the brain tissue was performed as described (Schachtrup *et al*, 2010). For BrdU detection, sections were pretreated with 2 N HCl for 1 h at 37°C. Primary antibodies used were mouse anti-BMP-2 and rabbit anti-Id3 (1:200; Abcam), goat anti-BMP-4 and goat anti-nestin (1:100; Santa Cruz Biotechnology), rat anti-BrdU (1:300; Abcam), rat anti-CD24 (1:100; Abcam), guinea pig anti-DCX (1:1,000; Millipore), rabbit anti-E2A (sc-349, recognizing the E2A splice variants E12 and E47) (1:500; Santa Cruz Biotechnology), rabbit anti-GFAP

(1:2,000; Abcam), rat anti-GFAP (1:1,000; Life Technologies), mouse anti-human Ki67 (1:500; Abcam), mouse anti-Mash1 (1:500; BD Pharmingen), rabbit anti-Mash1 (1:1,000; Abcam), mouse anti-Olig2 (1:500; Millipore), rabbit anti-S100b (1:1,000; Abcam), mouse anti-Sox2 (1:500; Abcam), and sheep anti-Thbs4 (1:1,000; R&D Systems). Secondary antibodies used were conjugated with FITC and Alexa Fluor 405, 488, or 594 (1:200; Jackson ImmunoResearch Laboratories). Sections were coverslipped with DAPI (Southern Biotechnology).

Adult neural stem/precursor cell culture

NSPCs were isolated and cultured as described (Reynolds & Weiss, 1992). Briefly, NSPCs were cultured in Neurobasal A/B27 without vitamin A (2%), Pen/Strep (1%), GlutaMax (1%), glutamine (0.5%), rhFGF2 (20 ng/ml) (all from Invitrogen), and rhEGF (20 ng/ml; Sigma-Aldrich). For differentiation assays, NSPCs were plated on poly-D-lysine (Millipore)-coated eight-well culture slides (BD Falcon) at a density of 40,000 cells per well in NSPC culture medium without rhFGF2 and rhEGF. NSPCs were treated with BMP-2 (2 ng/ml; Peprotech), TGF- β 1 (2 ng/ml; R&D Systems), or CNTF (2 ng/ml; Peprotech). NSPCs were differentiated as indicated, fixed in 4% paraformaldehyde in phosphate buffer, and processed for immunocytochemistry. Quantification was performed as described with slight modifications (Reynolds & Weiss, 1992; Bonaguidi *et al*, 2005). Briefly, NSPC differentiation into neurons and astrocytes was defined by morphology and antigenic properties. An arbitrary threshold was defined for the GFAP immunoreactivity as a measure for an astrocyte cell. For cell migration assays, neurospheres were plated on laminin (1 μ g/ml; Sigma-Aldrich)-coated 22 $\times$ 22-mm cover glasses (Corning) and cultured in NSPC culture medium without rhFGF2 and rhEGF. NSPCs migrated out of neurospheres for 24 h and were fixed in 4% paraformaldehyde in phosphate buffer and processed for immunocytochemistry. The quantification was performed as described (Morales-Garcia *et al*, 2011). Briefly, the farthest distance of cell migration was calculated from the edge of the sphere. At least 10 plated neurospheres per situation were analyzed. For E47 overexpression experiments, WT NSPCs were transfected by electroporation using the Mouse Neural Stem Cell Nucleofector Kit (Lonza) following manufacturer's instructions with pCAGGS-hE47-IRES-GFP or pCAGGS-IRES-GFP control plasmids (3 μ g) and cultured for 1 day. Subsequently, transfected cells (GFP+) were sorted by flow cytometry and RNA was isolated with the RNeasy kit (Qiagen), according to the manufacturer's instructions.

Immunocytochemistry

Cells were rinsed with ice-cold PBS, fixed in 4% paraformaldehyde in phosphate buffer for 30 min at 4°C, washed three times with PBS, blocked with 5% BSA, permeabilized with 0.1% Triton X-100 for 10 min at 4°C, and washed three times in PBS. The cells were then incubated with rabbit anti-Id3 (1:200; Abcam), goat anti-nestin (1:200; Santa Cruz Biotechnology), rat anti-GFAP (1:1,000; Life Technologies), or mouse anti- β 3-tubulin (1:100; Millipore) in PBS with 1% BSA overnight. After three washes in PBS, the cells were incubated with a secondary antibody conjugated with FITC, Alexa Fluor 488 or 594 (1:200; Jackson ImmunoResearch Laboratories) for

1 h in PBS with 1% BSA at RT, washed three times in PBS, and coverslipped with DAPI (Southern Biotechnology).

RNA isolation and quantitative PCR

RNA was isolated from primary adult NSPCs, and quantitative real-time PCR was performed as described (Schachtrup *et al*, 2010). The following primers were used: *GAPDH*: Fwd 5'-CAAGGCCGA GAATGGGAAG-3', Rev 5'-GGCCTCACCCATTGTGT-3'; *GFAP*: Fwd 5'-ACCAGCTTACGGCCAACAG-3', Rev 5'-CCAGCGATTCAA CCTTTCTCT-3'; *Id3*: Fwd 5'-GAGGAGCTTTTGCCACTGAC-3', Rev 5'-GCTCATCCATGCCCTCAG-3'; *Slc1a3*: Fwd 5'-CCGACCGTATAA AATGAGCTACC-3', Rev 5'-ATTCCTGTGACGAGACTGGAG-3'.

Microarray analysis

RNA from primary adult NSPCs for microarray analysis was isolated with the RNeasy Micro Kit (Qiagen), according to the manufacturer's protocol. RNA samples were further processed by the Ambion WT Expression kit as described by the manufacturer (Life Technologies). Fragmentation and labeling of the amplified cDNAs was done with the GeneChip WT Terminal Labeling and Controls Kit (Affymetrix). Labeled fragments were hybridized to Affymetrix GeneChip ST 1.0 arrays for 16 h at 45°C with 60 rpm in an Affymetrix Hybridization oven 645. After washing and staining, the arrays were scanned with the Affymetrix GeneChip Scanner 3000 7G. CEL files were produced from the raw data with Affymetrix GeneChip Command Console Software version 4.0. Partek Genomics Suite software (version 6.13.0412) was used for further analysis. CEL files were imported, including control and interrogating probes, pre-background adjustment was set to adjust for GC content and probe sequence, and RMA background correction was performed. Arrays were normalized using quantile normalization, and probe-set summarization was done using median polish. Probe values were log2-transformed. To identify differentially expressed genes between the groups, one-way ANOVA was performed in Partek.

CFSE labeling and flow cytometry

For proliferation assays, dissociated adult NSPCs were labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE) by incubating 2×10^6 cells/ml in PBS containing 1 μ M CFSE for 10 min at 37°C. Cells were washed and cultured at 50,000 cells in 4 ml per 25-cm² flask. Adult NSPCs were harvested at different timepoints, resuspended in FACS buffer (PBS+1% BSA) containing propidium iodide (1 μ M), and immediately analyzed by flow cytometry. Data were collected with the LSRII flow cytometer (BD Biosciences) and analyzed with FlowJo software (Tree Star).

Chromatin immunoprecipitation assays

ChIP assays were performed as described (Beck *et al*, 2009). Briefly, neurospheres from adult NSPCs ($\sim 10 \times 10^6$ cells) were fixed with 1.5 mM ethylene glycol bis (succinimidyl succinate) for 30 min at RT and subsequently with 1% formaldehyde for 10 min at RT, lysed and sonicated. ChIP was performed overnight at 4°C with rabbit anti-E2A (1.5 μ g; sc-349, Santa Cruz Biotechnology) or control

normal rabbit IgG bound to protein A Dynabeads. After washing and elution of the beads, crosslinking was reversed and protein digested by proteinase K treatment. Isolated genomic DNA and DNA from the sonicated input chromatin (1:100 of the material used for ChIP) were analyzed by quantitative PCR with the iCycler iQ, the MyiQ Optical Module (Bio-Rad), and the SYBR Green PCR Master Mix Kit (Life Technologies) with 5 μ l of DNA template in a 20- μ l reaction mixture. The following primers were used: *Arbp*: Fwd 5'-AGTGAGGCAGGACAGTCGAT-3', Rev 5'-CCCTCCCAACAAAA CAA-3'; *GFAP*: Fwd 5'-GCTTGAGACTTCAGGCCAT-3', Rev 5'-AGCAGAGCTGTCTGTGTTC-3'; *Slc1a3*: Fwd 5'-TGCCTGATAG TAAGTGGCGG-3', Rev 5'-AGTAACAGCTTAGCGAGCGG-3'.

Luciferase reporter transactivation assays

The luciferase reporter construct was generated by cloning a 2,400-bp fragment, including the first intron, the first non-coding exon and the putative promoter of the *GLAST* gene into the basic pGL3 (empty) plasmid (Promega). The fragment was isolated by PCR amplification using C57BL/6J mouse genomic DNA. Primers were designed to span the entire conserved region (between mouse and human) surrounding predicted E-boxes (using the rVISTA genome browser). Cotransfection of the reporter construct and the constitutively active *Renilla reniformis* luciferase-producing vector pRL-CMV (Promega) into HEK293T cells was done using calcium-phosphate co-precipitation. Cells were lysed 24 h after transfection using the passive lysis buffer PLB (Promega). Luciferase activity was determined in duplicates using a 96-well plate reader (PerkinElmer) with automatic substrate injection by injecting 100 μ l substrate and measurement for 10 s after a 2-s delay. As substrates we used the "Renilla Juice Kit" (PJK) for measuring *Renilla* luciferase activity, according to the manufacturer's instructions. For the quantification of firefly luciferase activity, a luciferin-containing solution was used. To generate the reporter construct, the following primers for *GLAST* were used: Fwd 5'-GGTACCTTCTAACTCCTGAAAA CGGGGA-3', Rev 5'-CTCGAGTTATAGCCTCCTGGGCTGAG-3'.

Immunoblots and co-immunoprecipitation

For the detection of Id3 expression in adult NSPCs, primary cells were treated with BMP-2 (2 ng/ml; Peprotech), CNTF (2 ng/ml; Peprotech), or TGF- β 1 (2 ng/ml; R&D Systems) for various times. For the detection of astrocyte-specific marker expression (GFAP, Aqp4, and Aldh1 l1) in *Id3*^{-/-} and WT NSPCs, primary cells were treated with BMP-2 (2 ng/ml; Peprotech) or left untreated and cultured for 2 days in NSPC culture medium without rhFGF2 and rhEGF (differentiation medium). To detect E47 expression in adult NSPCs, primary neurospheres were cultured in NSPC culture medium, in NSPC differentiation medium, and NSPC differentiation medium treated with BMP-2 (2 ng/ml; Peprotech). For co-immunoprecipitation, 1 mg of NSPC cell lysate was prepared in 1% Nonidet P-40, 150 mM NaCl, 1 mM EDTA, and 20 mM Tris-HCl, pH 8.0, and incubated with rabbit anti-Id3 antibody (5 μ g; CalBioReagents) bound to protein A Sepharose beads for 4 h at RT. After three washes, the beads were resuspended in sample buffer, boiled for 10 min, and centrifuged. Protein extracts were separated by electrophoresis on 13 and 16% SDS-PAGE gels as described (Schachtrup et al, 2010) with the following antibodies: rabbit

anti-GFAP (1:1,000; Abcam), rabbit anti-Aqp4 (1:500; Santa Cruz Biotechnology), rabbit anti-Aldh1 l1 (1:500; Abcam), mouse anti-E47 (1:6,000; BD Pharmingen), rabbit anti-Id3 (1:2,000; CalBioReagents), and rabbit anti-GAPDH (1:1,000; Cell Signaling).

Microscopy and imaging analysis

Images were acquired on a Leica TCS SP8 confocal laser scanning microscope with 20 $\times$, 40 $\times$ or 63 $\times$ oil immersion objectives and the LAS AF image analysis software; on an Axioplan 2 Imaging epifluorescence microscope and dry Plan-Neofluar objectives (10 $\times$ /0.30 NA; 20 $\times$ /0.50 NA), an Axiocam HRC CCD camera, and the AxioVision image analysis software (Carl Zeiss); on an Axio Imager.M2 epifluorescence microscope and dry Plan-Apochromat (10 $\times$ /0.45 NA; 20 $\times$ /0.80 NA) or Plan-Neofluar (40 $\times$ /0.75 NA) objectives, an Axiocam 506 mono CCD camera, an Apotome.2 module and the ZEN image analysis software (Carl Zeiss); or on a Primovert microscope (Carl Zeiss). Quantitative image analysis was performed on an area of 290 $\times$ 290 μ m in the cortex lesion for colocalization experiments, 500 $\times$ 300 μ m for resident astrocyte activation in the cortex, 92 $\times$ 10 μ m in the SVZ for the cellular Id3 expression in uninjured mice, 290 $\times$ 10 μ m in the SVZ for the cellular Id3 expression in injured mice, 290 $\times$ 60 μ m in the SVZ niche area for the cellular Thbs4 expression in injured mice, BrdU short-term labeling regime and BMP-2 expression, 850 $\times$ 200 μ m in the glomerular layer of the OB, and 125 $\times$ 125 μ m (dorsal horn SVZ) or 300 $\times$ 25 μ m (medial SVZ) for the cell type-specific analysis between uninjured WT and *Id3*^{-/-} mice. For colocalization analyses, images were acquired in green, red, and blue channels simultaneously, generating z-stack projections (0.5–1 μ m per stack) through a distance of 16–18 μ m per brain section. z-stacks were displayed as maximum intensity projections, and analysis was performed by the use of axis clipping and the rotation of the 3D-rendered images. For immunoreactivity analysis, Id3+ cells colocalizing with NSPC subpopulations were randomly selected and the number of pixels with an intensity above a predetermined threshold level was quantified and normalized to the respective WT uninjured control side. For BMP-2 expression in the SVZ or cell composition analyses between WT and *Id3*^{-/-} mice in the SVZ, defined areas as indicated above were analyzed. Total immunoreactivity was calculated as percentage area density defined as the number of pixels (positively stained areas) divided by the total number of pixels (sum of positively and negatively stained area) in the imaged field. All immunoreactivity measurements were performed using the ImageJ software (National Institutes of Health). At least three brain sections per animal through the level of the SVZ or five brain sections per animal in the cortex were analyzed. Representative images of colocalization experiments were acquired with the Leica TCS SP8 confocal laser scanning microscope; representative images of differentiation, migration, and adhesion assays *in vitro* were performed with the Axioplan 2 Imaging epifluorescence microscope or the Primovert microscope (Carl Zeiss). All quantitative analyses were performed in a blinded manner.

Statistical analysis

Data are shown as means $\pm$ SEM. Differences between groups were examined by one-way ANOVA followed by Bonferroni's post-test

for multiple comparisons. Differences between isolated pairs were examined by Student's *t*-test.

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Author contributions

CB, SP, KM, SS, LPI, MH, LPO, KSR, VID, DP and KS performed research; CB, SP, KM, DP, JD, KS and CS analyzed data; MK contributed crucial reagents and advice; CB and CS designed the study, coordinated the experimental work, and wrote the manuscript with contribution from all authors.

Conflict of interest

The authors declare that they have no conflict of interest.

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