E-proteins orchestrate the progression of neural stem cell differentiation in the postnatal forebrain

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Abstract

Background: Neural stem cell (NSC) differentiation is a complex multistep process that persists in specific regions of the postnatal forebrain and requires tight regulation throughout life. The transcriptional control of NSC proliferation and specification involves Class II (proneural) and Class V (Id1-4) basic helix-loop-helix (bHLH) proteins. In this study, we analyzed the pattern of expression of their dimerization partners, Class I bHLH proteins (E-proteins), and explored their putative role in orchestrating postnatal subventricular zone (SVZ) neurogenesis.

Results: Overexpression of a dominant-negative form of the E-protein E47 (dnE47) confirmed a crucial role for bHLH transcriptional networks in postnatal neurogenesis by dramatically blocking SVZ NSC differentiation. In situ hybridization was used in combination with RT-qPCR to measure and compare the level of expression of E-protein transcripts (E2-2, E2A, and HEB) in the neonatal and adult SVZ as well as in magnetic affinity cell sorted progenitor cells and neuroblasts. Our results evidence that E-protein transcripts, in particular E2-2 and E2A, are enriched in the postnatal SVZ with expression levels increasing as cells engage towards neuronal differentiation. To investigate the role of E-proteins in orchestrating lineage progression, both in vitro and in vivo gain-of-function and loss-of-function experiments were performed for individual E-proteins. Overexpression of E2-2 and E2A promoted SVZ neurogenesis by enhancing not only radial glial cell differentiation but also cell cycle exit of their progeny. Conversely, knock-down by shRNA electroporation resulted in opposite effects. Manipulation of E-proteins and/or Ascl1 in SVZ NSC cultures indicated that those effects were Ascl1 dependent, although they could not solely be attributed to an Ascl1-induced switch from promoting cell proliferation to triggering cell cycle arrest and differentiation.

Conclusions: In contrast to former concepts, suggesting ubiquitous expression and subsidiary function for E-proteins to foster postnatal neurogenesis, this work unveils E-proteins as being active players in the orchestration of postnatal SVZ neurogenesis.

Keywords: Class I basic helix-loop-helix proteins, E-proteins, Neural differentiation, Postnatal neurogenesis, Stem cell plasticity

Background

The self-renewal versus differentiation of neural stem cells (NSCs) into new neurons is a highly complex process and depends on tight regulatory mechanisms throughout postnatal life. Early after birth, radial glia cells (RGCs) mature into type-B cells that reside in the germinal niche lining the lateral ventricles (LVs), namely the subventricular zone (SVZ). These NSCs proliferate slowly (infrequently) and by asymmetric division give rise to fast proliferating cells, defined as type-C cells (or transit amplifying progenitors). After 3 to 4 cycles of symmetric divisions, allowing the pool of type-C cells to enlarge, these progenitors start to differentiate and commit to specific fates [1]. At this stage, they are defined as type-A cells (or neuroblasts), which then migrate through the rostral migratory stream (RMS) into the olfactory bulb [2,3] to terminally differentiate into specific neuron subtypes (i.e., periglomerular neurons and granule cells) [4].

To balance NSC maintenance and precursor differentiation into neurons or glial cells, postnatal neurogenesis relies on the timely integration of intracellular signaling mechanisms such as the basic-helix-loop-helix (bHLH)
transcription factor machinery. bHLH proteins represent a large family of transcription factors that are categorized in several classes based on their structure and function [5] and are believed to play an essential role in multiple aspects of forebrain development. By virtue of their transcriptional activity, bHLH proteins can be subdivided into three groups, i.e., activators (Class II), repressors (Class I), and transactivators (Class I) [6].

The classical view of bHLH protein interaction and function is still largely based on drosophila studies [7-9] and murine developmental studies [5,10-12]. There, bHLH repressors (i.e., Hes and Id proteins) and activators (pro-neural proteins, i.e., Ascl1, Neurog2) mutually antagonize each other either by direct transcriptional repression [13,14], physical inhibition [8,15], or by competing for bHLH transactivators (E-proteins) [12,16]. This antagonistic balance defines whether NSCs or their progeny are differentiating or not, with bHLH repressors predominantly favoring the retention of cells in immature stages [17,18], while bHLH activators accelerate progenitor maturation [13,14,19-22].

Recent work done in drosophila, however, indicates that this established paradigm is likely to be over-simplified and incomplete. A more sophisticated spatial and temporal regulation of E-protein expression has been proposed, suggesting that E-proteins (E2-2, E2A, HEB) fulfill a more complex function than anticipated in germinal zones of the central nervous system (CNS) [23,24]. Furthermore, work in rodents implies that various E-proteins exhibit differential patterns of expression at distinct ages, resulting in the preferential formation of specific dimers [25-27].

This complex pattern of expression, paired with a recent study implicating Ascl1 in regulating both NSC proliferation and differentiation [22], suggests that E-proteins orchestrate NSC lineage progression, a hypothesis we examine in the present study.

Results

Heterodimerization of Class I/II bHLH proteins is essential for normal NSC differentiation in vitro and in vivo

NS5 cell cultures contain homogenous populations of progenitors that divide indefinitely upon exposure to FGF2 and EGF and undergo neuronal and glial differentiation following mitogen withdrawal [28]. Overexpression of proneural proteins, such as Ascl1, in progenitors of various origins results in their rapid neuronal differentiation [19-22].

In the first instance, the role of Class I/II bHLH factors in mediating neurogenesis was assessed in vitro. The efficiency of neuronal differentiation following overexpression of Ascl1 alone or in combination with E-proteins when NS5 cells were grown in proliferative culture conditions was determined. Overexpression of Ascl1 induced a >3-fold increase in neuronal differentiation compared to an empty control plasmid, as revealed by elevated Map2 or CD24 transcript expression, both of which are immature neuron markers (Figure 1A). Cotransfection of Ascl1 with either E-protein, i.e., E2-2, E47 (E2A isoform), and HEB, further potentiated Ascl1-induced neuronal differentiation by approximately 50 to 80% when Map2 expression was measured, and to a lesser extent when CD24 transcription was probed (Figure 1A). In contrast, measurement of ABCG2, a transcript expressed in NSCs, showed opposite trends (Figure 1A). Together, these results illustrate the interchangeability of E-proteins in this cellular context and their potential to promote Ascl1-induced NS5 cell differentiation.

We next disrupted Class I/II bHLH transcriptional activity in vitro and in vivo to investigate its effect on NSC differentiation. We used a mutated form of the E2A isoform E47, which lacks its nuclear localization sequence and fails to translocate to the cell nucleus, and therefore sequesters its binding partners within the cytoplasm [29,30] (Figure 1B,D insert)). While overexpression of dominant-negative E47 (dnE47) alone did not influence Map2 transcript expression in proliferative culture conditions (Additional file 1A), it efficiently prevented Ascl1-induced neuronal differentiation of NS5 cells as shown by the partial blockade of Map2 induction (Figure 1C). We next tested the effect of dnE47 in SVZ, NSCs (i.e., radial glia cells (RGCs) at this early postnatal stage) by performing postnatal in vivo electroporation. Early after birth, NSCs can be easily distinguished from their progeny based on morphological criteria, i.e., an elongated cell body and the presence of a basal and apical process [31,32]. Quantification revealed a dramatic blockade of differentiation following dnE47 overexpression, with most electroporated RFP+ cells still presenting a clear RGC morphology (Figure 1D,E). Interestingly, cells that were already undergoing differentiation into non-radial glial cells (non-RGCs) exhibited an enhanced proliferative phenotype, as demonstrated by the doubling of the number of Ki67+/RFP+ non-RGCs (Figure 1F, Additional file 1B).

To confirm the accuracy in monitoring RGC differentiation progression by electroporation and analysis of morphological criteria, we next performed an in depth antigenic characterization of RGCs and non-RGCs. At 2 days post-electroporation (2 dpe), RGCs were highly positive for type-B cell markers (i.e., Vimentin and Hes5-EGFP) and completely devoid of the type-C cell marker Ascl1 (Figure 2A,B). In contrast, non-RGCs were characterized as a mix of type-C (Ascl1+, 50%) and type-A (Dcx+, 50%) progenitors (Figure 2A,B). Approximately half of the non-RGCs were proliferating, as indicated by expression of Ki67 (Figure 2B). Those proliferating cells were mostly Ascl1+ type-C cells (~60%, Figure 2C,
Additional file 1C), while only ~25% expressed the type-A cell marker Dcx (Figure 2C, Additional file 1D). Interestingly, ~10% of proliferating non-RGCs exhibited a transitory phenotype between type-C and type-A cell stages and were positive for both markers (Ascl1 + Dcx +; Figure 2C).

We next confirmed the blockade of RGC differentiation after ectopic dnE47 using these antigenic criteria. Overexpression of dnE47 resulted in an increase in the number of Vimentin+/RFP+ cells (Figure 2D, Additional file 1E), whilst the numbers of Ascl1+/RFP+ (type-C) and Dcx+/RFP+ (type-A) precursors were concomitantly decreased at 2 dpe compared to controls (Figure 2E,F, Additional file 1F,G). Strikingly, the retention of a more immature phenotype by the electroporated cells was further supported by the 3-fold increase of Dcx+ cells, still expressing type-C cell specific Ascl1 (Figure 2G).

Altogether, these experiments demonstrate that the progression of RGC differentiation can be analyzed based on morphological criteria and reveal a dependence of NSC differentiation on Class I/II bHLH transcriptional activity in the postnatal SVZ.

E-proteins exhibit a complex spatio-temporal pattern of expression in the postnatal forebrain

Based on these findings, we further explored both spatial and temporal E-protein expression in the postnatal and adult forebrain. We first analyzed the spatial pattern of expression by in situ hybridization (ISH) at postnatal day 6 (P6) and 60 (P60). All three genes coding for E-proteins...
Antigenic properties of radial glial as well as non-radial glial cells and confirmation of differentiation blockade by dnE47 overexpression. 

(A, B) At 2 days post electroporation (dpe), 40% of the labeled cells showed morphological criteria of radial glial cells (RGCs, i.e., bipolar processes, apical connection to lateral ventricle (LV) lumen), while 60% presenting a spherical morphology were distant from the LV and were defined as non-RGCs. Morphologically-defined RGCs express the NSC marker Vimentin (88.4 ± 0.7) and were EGFP positive in Hes5-EGFP reporter mice (94.2 ± 2.4), but negative in Ascl1-EGFP reporter mice, in which type-B or type-C cells are labelled, respectively [33,34]. In contrast, non-RGCs consisted of a heterogeneous population of type-C (i.e., Ascl1+, 53.6 ± 4.3) and type-A cells (i.e., Dcx+, 45.9 ± 0.2). Non-RGCs were frequently proliferative (i.e., Ki67+, 58.2 ± 5.7).

(C) A subsequent characterization of cycling non-RGCs revealed that most Ki67+ cells were type-C cells (Ascl1+, 60.1 ± 4.4), whereas fewer were type-A cells (Dcx+, 22.9 ± 4.0). Notably, a minor population of cycling cells were double-positive for Ascl1 and Dcx (10.1 ± 1.4), probably representing a transition phase in between type-C and type-A phenotypes.

(D–G) Antigenic assessment of RGC differentiation blockade following dnE47 overexpression. Electroporation of dnE47 in the postnatal SVZ reduced RGC differentiation, as revealed by the increased number of Vimentin+/RFP+ RGCs (D) and concomitant decrease of Ascl1+/RFP+ (E) and Dcx+/RFP+ (F) non-RGCs, respectively, when compared to an empty RFP control plasmid 2 dpe (Vimentin: 100 ± 3.2 vs. 150.5 ± 1.6; Ascl1: 100 ± 8.4 vs. 48.2 ± 3.1; Dcx: 100 ± 3.3 vs. 39.5 ± 6.3). This blockade of differentiation was further supported by an increase in Dcx+/RFP+ type-A cells retaining expression of the type-C cell marker Ascl1 (Dcx+/Ascl1+, 100 ± 25.7 vs. 308.5 ± 50.3) (G). P values: *P <0.05; **P <0.01; ***P <0.001. Quantifications were normalized to control conditions (D–G). Scale bars: A, 10 μm.
(E2-2, E2A, and HEB) were abundant in the postnatal forebrain and were enriched in germinai zones, i.e., dentate gyrus, SVZ, and RMS at P6 (Figure 3A,B). These results were confirmed by real-time quantitative polymerase chain reaction (RT-qPCR) measurement from the microdissected lateral SVZ and adjacent parenchyma (i.e., striatum). All E-protein transcripts were enriched in the SVZ, with a clear predominance of E2-2 and E2A (Figure 3C). At P60, E-protein transcripts were generally less abundant compared to earlier postnatal ages. While E2-2 expression was still enhanced in the SVZ and the RMS, E2A and HEB expression levels were only marginally higher than in non-germinai regions (Figure 3A,B), thus implying that E2-2 may be required for neuronal differentiation continually during development and into adulthood.

Having demonstrated an enrichment of E-protein transcripts in germinai regions of the postnatal forebrain, regulation during early neuronal differentiation was assessed. In vitro differentiation of the NS5 cell line increased the expression of all E-protein transcripts. Up-regulation of E2-2 and HEB had occurred following 24 h, before any increase of doublecortin (Dcx), a neuronal differentiation marker, could be detected. After 48 h of differentiation, all E-protein transcripts were significantly upregulated (Figure 3D).

A similar upregulation was observed in vivo. Early progenitors and committed neuroblasts were isolated based...
on their differential expression of the cell surface markers Prominin-1 and PSA-NCAM, respectively. Measurements of cell-specific markers confirmed the efficiency of the approach by showing that Prominin-1+ sorted cells were enriched for the stem cell markers GFAP and Id1, while Dcx and CD24 enrichment was prominent in PSA-NCAM+ sorted cells. Measurements of E-protein transcripts by RT-qPCR revealed that E2-2 and, to a lesser extent, E2A and HEB, were strongly upregulated in the postnatal SVZ during NSCs differentiation (Figure 3E).

Altogether, our results indicate that E-protein expression is spatially and temporally regulated in a pattern that matches NSC differentiation during postnatal forebrain development.

E-protein manipulation alters the progression of NSC differentiation in the postnatal SVZ

We next performed further E-protein gain-of-function (GoF) and loss-of-function (LoF) experiments in the postnatal lateral SVZ, by means of plasmid electroporation (as above) (Figure 4A). We focused this functional characterization onto E2-2, which appears to be the most prominent E-protein expressed in the postnatal and adult SVZ (see above), and E2A, which is the best studied E-protein in CNS development [23,25,29,35-37].

GoF experiments resulted in the rapid transformation of RGCs into non-RGCs. Thus, whilst E2-2 and E2A overexpression increased the transformation of RGCs into non-RGCs by 30% and 40%, respectively, both E2-2 and E2A silencing decreased it by 20% (Figure 4B,C, Additional file 1H). We next focused our analysis on non-RGCs, which, at this early timepoint (2 dpe), consisted of transient amplifying progenitors (type-C) and neuroblasts (type-A) (Figure 2A,B). E-protein overexpression significantly reduced the proliferation of non-RGCs, as demonstrated by a ~45% decrease in the number of green fluorescence protein positive (GFP+) non-RGCs expressing the cell cycle marker Ki67. In contrast, LoF enhanced the proliferation capacity of non-RGCs (Figure 4D). Interestingly, these effects were more pronounced following E2-2 silencing, consistent with its higher expression in the postnatal SVZ compared to E2A (see above).

Altogether, these data are in agreement with the results obtained with the dpe (Figure 1D–F) and suggest that E-proteins control the differentiation of NSCs, and influence the cycling behavior of their progeny.

We next tested whether the changes in proliferation observed following E-protein GoF could be explained by a cell cycle exit induction. Dividing cells in S-phase that incorporated 5-ethyl-2‘-deoxyuridine (EdU) 24 h prior to sacrifice, but did not re-enter another round of cell division (i.e., cell cycle exit), would no longer express Ki67. We quantified the number of EdU+Ki67–/EdU+ cells and found a doubling in the number of progenitors that had exited the cell cycle, when E2-2 was overexpressed (Figure 5A), whereas a smaller number of progenitors exited the cell cycle upon E2-2 silencing (Figure 5B). Finally, we tested whether E2-2-induced cell cycle exit resulted in an abortive differentiation or whether cells appropriately completed their differentiation into Dcx+ neuroblasts. We observed a ~30% increase of Dcx+ neuroblasts (type-A) within the postnatal forebrain at 2 dpe (Figure 5C, Additional file 1I), which paralleled the reduction of the number of RGCs described above, whereas the number of Ascl1+ type-C cells remained unaltered (Figure 5D, Additional file 1J).

Taken together, these results highlight an important role of E-proteins in controlling the timing and progression of NSC differentiation in the postnatal SVZ. Thus, upon E2-2 overexpression, a concomitant decrease of type-B RGCs and increase of Dcx+ type-A cells was observed. These observations, together with the stable number of Ascl1+ type-C cells, suggest a more rapid differentiation of E2-2 overexpressing cells as schematized in Figure 5E,F.

E47 disruption is not sufficient to mediate the switch of Ascl1-dependent transcription

E-proteins induce NSC differentiation by transactivation of BH3LH proneural proteins. Among those is Ascl1, expressed mainly by progenitor cells in the postnatal forebrain [19,38]. We confirmed the interdependence of E-proteins and Ascl1 by NSC differentiation induction, combining GoF and LoF experiments in isolated progenitors in vitro. Ascl1 knock-down resulted in a complete loss of Map2 mRNA transcription, when E2-2 or E47 were concomitantly overexpressed. This confirms that E-protein activity is largely dependent on Ascl1 expression. On the other hand, Ascl1 is still able to exert its function although to a lesser extent upon silencing of E2-2, but not after silencing E2A, illustrating both redundancy but also a strong dependency on E2A expression in SVZ-derived progenitor cells (Figure 6A).

The BH3LH protein Ascl1 has recently been shown to control proliferation and differentiation in neural progenitors by sequentially activating cell cycle progression and cell cycle arrest genes in a context-dependent manner [22]. Therefore, we next assessed whether modifications in E-protein expression levels that occur during neural progenitor cell differentiation could mediate this molecular switch. E2A, in the form of E47, known to be the main E-protein binding to Ascl1 [21], was overexpressed or silenced in isolated neurosphere cultures and the level of expression of Ascl1 targets, reported to be differentially expressed during cell cycle progression (FoxM1, Cdk4) and cell cycle arrest (Gadd45g, Ccng2, Btg2, Prmt2) [22], was measured by RT-qPCR. No consistent change in expression of genes differentially
targeted by Ascl1 was detected following E2A GoF or LoF (Figure 6B).

In summary, the divergent context-dependent function of Ascl1 in promoting cell proliferation and in triggering differentiation, as described recently [22], is apparently independent of E-protein activity. Rather, these findings propose that E-protein upregulation during NSC differentiation is likely to act in parallel with other signaling pathways to fine-tune differentiation progression in an Ascl1-dependent manner [39].

Discussion

In the present study, we demonstrate enriched expression of E-proteins in postnatal germinal regions of the murine forebrain and highlight their key role as transcriptional orchestrators of postnatal SVZ neurogenesis.

bHLH family members are known to regulate a variety of developmental programs in several tissues of the organism, such as myogenesis, hematopoiesis, and pancreatic and heart development, as well as neurogenesis [5]. In the CNS, proneural Class II proteins are the most studied
members of the bHLH family. They were shown to be indispensable and sufficient to induce neurogenesis in drosophila [40], C. elegans [41,42], and mice [43]. During telencephalic development, Neurog2 and Ascl1 confer an opposing dorso-ventral expression gradient to specify distinct neuronal populations [44]. However, less is known regarding Class I bHLH protein expression patterns and their role during neurogenesis. In drosophila, Daughterless (E-protein homolog) knock-out results in defective precursor differentiation [45,46]. In contrast, deletion of single Class I bHLH protein members in rodents led to no gross anatomical phenotype [26] or spatially restricted developmental deficits [47]. Deletion of multiple E-proteins resulted in stronger phenotypes highlighting some degree of redundancy in between these genes [26]. Our co-expression experiments confirmed the potential interchangeability of E-proteins, since E2-2, E47, and HEB were equally potent in transactivating Ascl1, even though the LoF analysis revealed Ascl1 to preferentially bind E47. Sequence analysis studies revealed extremely high sequence conservation within, as well as beyond, the HLH domains supporting possible redundancy among individual E-proteins [23]. Indeed, functional replacement of the mouse E2A gene with an E2A promoter-driven human HEB cDNA rescued B-cell commitment and differentiation during B-lymphopoiesis [37].

Furthermore, expression studies have proposed a regulation of E-protein transcription during development, with a gradual restriction to germinal regions in the CNS [25], supporting key neurogenesis functions. Our ISH combined

Figure 5 E2-2 alteration influences cell cycle exit of progenitors in vivo. (A) E2-2 overexpression increased cell cycle exit (EdU⁺Ki67⁻/EdU⁺) among the progenitor cell population (non-RGC) compared to control conditions at 2 dpe (100 ± 7.5 vs. 184.9 ± 18.0). Confocal micrographs show representative non-RGCs (GFP⁺) having cycled within 24 h prior to sacrifice (EdU⁺) and having re-engaged (Ki67⁺) or exited the cell cycle (Ki67⁻), respectively. (B) In contrast, knock-down of E2-2 decreased cell cycle exit within the progenitor pool as illustrated by the increased Ki67 immunoreactivity among EdU⁺ non-RGCs, when compared to control conditions (Scrambled) (100 ± 7.9 vs. 65.8 ± 1.3). (C, D) E2-2 overexpression also increased the number of Dcx⁺ neuroblasts (100 ± 0.5 vs. 128.1 ± 2.9) (C), whereas the number of Ascl1⁺ type-C cells remained unchanged (100 ± 6.4 vs. 94.6 ± 9.2) (D). (E) Percentage of cell type composition (i.e., type-B = RGC, type-C = Ascl1⁺, type-A = Dcx⁺) upon E2-2 overexpression, when compared to an empty control plasmid at 2 dpe (30.7 ± 4.6 vs. 14.2 ± 2.3, 30.0 ± 1.2 vs. 35.6 ± 2.5, 39.2 ± 0.3 vs. 50.2 ± 1.8, respectively). (F) Summary model: E2-2 orchestrates neurogenesis progression within the murine forebrain. P values: *P <0.05; **P <0.01; ***P <0.001. Quantifications were normalized to control conditions (A–D). Scale bars: A & B, 20 μm.
with RT-qPCR gene expression analysis confirmed an enrichment of all E-protein transcripts in postnatal and adult germinal regions of the forebrain when compared to non-neurogenic regions. Notably, these expression experiments uncovered E2-2 as the most abundant E-protein in the SVZ, followed by prominent expression for E2A and HEB. Additionally, E-proteins seem not only to be restricted spatially but also temporally during lineage progression. More differentiated progenitors exhibited increased transcription levels in vitro as well as in vivo, further implicating a specialized function in maintaining proper neurogenesis behavior. Altogether, these results highlight a complex regulation of E-protein expression in germinal regions of the postnatal forebrain.

The dramatic blockade of NSC differentiation and subsequent neurogenesis observed following dne47 overexpression in vitro as well as in vivo underlines the importance of a functional bHLH network for lineage progression. Based on these findings, we further aimed at manipulating E-protein expression more concisely in the lateral SVZ using overexpression and shRNA plasmids within an established postnatal electroporation approach [31,32]. Major alterations in progenitor proliferation and cell cycle exit were evident. These results are in agreement with a previously described role of E2A in cell cycle regulation in transiently transfected NIH3T3 fibroblasts, by activating cyclin-dependent kinase inhibitors [27,48,49]. Moreover, Id proteins, known E-protein antagonists (see above), enhance cell cycle duration and prevent cell cycle exit, which was further confirmed with a decreased cyclinD1 and increased p27Kip1 (cyclin-dependent kinase inhibitor 1B) expression within the VZ of triple knock-out mice [18]. In line with Id protein mediated anchorage to the neurogenic niche [18], E-proteins were also reported to actively repress expression of E-cadherin, a cell adhesion molecule [50,51]. Recent studies in drosophila, revealing a direct transcriptional regulation of Emc (Id protein homolog) by Daughterless, coupled
with the primary sequestration of E-proteins by Class V bHLH members [6], results in an autoregulatory feedback [24,52]. In addition, E-protein expression and therefore lineage progression seems to be initially mediated by extracellular signaling pathways, which is consistent with earlier reports, suggesting a general role for Shh and BMPs in SVZ neurogenesis [53–55]. In drosophila, it was demonstrated that Hh (Shh homolog) and Dpp (BMP homolog) specifically repress Id expression and thus increase E-protein activity [24], ultimately leading to germinal niche detachment and altered cell cycle behavior.

Our findings suggest that the effects of E-proteins on SVZ NSC differentiation are entirely dependent on *Ascl1*, which is expressed in all postnatal SVZ progenitors [19,56]. Knock-down of *Ascl1* entirely abrogated the proneural activity of *E2-2* and *E2A*, demonstrating that E-proteins converge with the same Class II bHLH protein. The incomplete loss of *Ascl1* activity when *E2-2* is silenced is likely due to a persistent endogenous E47 activity, as *Ascl1* seems to rely more on *E2A* than on *E2-2*, demonstrated by the absence of *Ascl1* activity upon *E2A* silencing. Recent findings propose that sustained *Ascl1* expression favors cell differentiation, whereas oscillatory expression correlates more with proliferative cell fates [57]. Given that E-proteins, in competition with Id proteins, tightly regulate Ascl1 protein stability [58] and that *Ascl1* has recently been demonstrated to target two subsets of genes, namely proliferation- and differentiation-associated genes [22], we investigated a role for E-proteins in mediating this switch in binding bias. However, in the present study, E2A alterations did not induce directed and systematic gene expression changes, at least for the *Ascl1* target genes tested, most likely mitigating the probability of E-proteins to induce differentiation through promoting Ascl1 binding and activation of cell cycle arrest genes. This implies that E-proteins are likely to operate in parallel with other signaling pathways to fine tune differentiation progression in an Ascl1-dependent manner [39].

Although our results did not reveal significant differences in the expression pattern of distinct E-proteins during postnatal SVZ neurogenesis, and an interchangeability of E-proteins in the context of Ascl1-induced SVZ NSC differentiation, it should be noted that the dimerization properties of bHLH proteins can be affected at the post-translational level. For example, GSK3β phosphorylation of Neurog2 was described to influence its transcriptional activity [59]. Similar mechanisms might be at play in the postnatal SVZ to fine tune the choice of proneural/E-protein-binding partners and regulate individual aspects of NSC differentiation, such as, for example, specification. Previous studies have indeed demonstrated a specific role of E2-2 for the generation of a unique subset of neural progenitors during pontine development [47], despite the presence of other E-proteins. Also, B- and T-lymphocyte specification was shown to be orchestrated by E2A and HEB [60,61]. Whether Class I/II bHLH protein dimerization is dynamically regulated in the postnatal SVZ to influence fate specification is currently unknown and will be the subject of future investigations. Performing such experiments in the postnatal SVZ is particularly attracting due to the regional expression of some proneural proteins as, for example, Neurog2, which shows dorsal enrichment in the postnatal SVZ in a population that gives rise to glutamatergic neurons [62,63].

Conclusions

In the present work, we demonstrate a diverse expression pattern of Class I bHLH proteins and underpin their underestimated significance in orchestrating early neuronal lineage progression. However, the precise mechanism behind E-proteins regulating NSC differentiation remains to be elucidated, but is unlikely to rely solely on *Ascl1* heterodimerization.

Methods

Cell culture and in vitro nucleofection

The mouse embryo-derived and immortalized RGC line NS5 was cultured as described earlier [28] and was nucleofected following the manufacturer’s instructions using a 4D-nucleofector device with SG solution (Lonza, Basel, Switzerland) and the EN150 program with a maximum of 0.5 μg of plasmid DNA per 1 × 10⁶ cells. NS5 cell differentiation was achieved by plating cells into polyornithin-coated (0.001% in H₂O, Sigma-Aldrich, St. Louis, MO, USA) and laminin-coated (20 μg/mL in H₂O, Life Technologies, Carlsbad, CA, USA) flasks in D1 medium (Euromed-N stem cell medium; Lucerna Chem, Lucerne, Switzerland) supplemented with 1% penicillin/streptomycin, 0.25% L-glutamine, 1% B27 (Life Technologies), 0.5% N2 (Life Technologies), and 5 ng/mL FGF2 (Peprotech, Hamburg, Germany) [64].

Neurosphere-forming progenitor cells were isolated from the SVZ of P4 aged animals. The SVZ was micro-dissected and the tissue digested with a solution of DNAsel I (Worthington, Lakewood, NJ, USA), Papain (Worthington), and Dispase II (Roche, Basel, Switzerland), subsequently triturated and filtered through a 70 μm cell strainer (BD Biosciences, Franklin Lakes, NJ, USA). Cells were plated into DMEM/F12 medium supplemented with 1% N2, EGF, FGF2 (both 20 ng/mL), Peprotech), heparin (1 μg/mL), and 1% penicillin/streptomycin. For nucleofections, a mouse neural stem cell solution (Lonza), program DS113, and a maximum of 1 μg of plasmid DNA per 1 × 10⁶ cells were used.

Both NS5 and progenitor cells were subjected to RNA isolation and subsequent RT-qPCR 24 to 48 h post-nucleofection.
Animals and postnatal electroporation

All animal experiments were performed in agreement with the Canton of Zurich veterinary office guidelines (authorization 182/2011). All mice used in this study were of the CD1 (Swiss mice) strain and were obtained from the Charles River laboratory.

Electroporation of RGCs lining the lateral ventricle was performed on postnatal-day-2 mice (P2), as described previously [31,32]. In brief, following anesthesia by hypothermia, P2 pups were injected with 2 µL of plasmid solution (5 µg/µL in PBS). Fast Green 1% (Sigma-Aldrich) was used to assess accuracy of the intra-ventricular injections. Successfully injected mice were subjected to five electrical pulses (95 V, 50 ms, separated by 950 ms intervals) using the ECM 830 BTX electroporator (Harvard Apparatus, Holliston, MA, USA) and tweezer electrodes (5 mm diameter; BTX Tweezertrodes, Harvard Apparatus) coated with conductive gel (Siga gel, Parker Laboratories, Fairfield, NJ, USA). For proliferation studies, 50 mg/kg 5-ethyl-2′-deoxyuridine (EdU) were administered subcutaneously 24 h prior to sacrifice.

Tissue processing

Two days post-electroporation, mice were deeply anesthetized (150 mg/kg pentobarbital, i.p.) and transcardially perfused with Ringer solution supplemented with heparin (250 mg/L, Sigma-Aldrich) followed by 4% paraformaldehyde (PFA) (Sigma-Aldrich). Then, 50 µm thick vibratome slices (Leica, Wetzlar, Germany) were collected as a series of six encompassing the entire rostro-caudal extent of the lateral ventricle. Sections were stored at −20°C in antifreeze solution (25% Glycerol, 25% Ethyleneglycol and 50% 0.1 M PB).

Immunostaining

All immunostainings were performed on a complete series of free-floating sections. Blocking and permeabilization were achieved by incubating the sections for 2 h in 0.1 M PB supplemented with 0.4% Triton-X100 and a TNB buffer (thereafter mentioned as 0.4% TNB-TX100) consisting of 0.05% Casein (Sigma-Aldrich), 0.25% bovine serum albumin (Sigma-Aldrich), and 0.25% Topblock (Lubio Science, Lucerne, Switzerland) at room temperature. Afterwards, primary antibody incubations were performed in 0.4% TNB-TX100 at 4°C overnight (M α Ki67, 1:300 (BD Biosciences); Rb α Ki67, 1:500 (MM France, Francheville, France); G α Dcx, 1:300 (Santa Cruz Biotechnology, Santa Cruz, CA, USA); Ch α Vimentin, 1:1000 (Millipore, Darmstadt, Germany); and M α Ascl1, 1:300 (BD Biosciences)). Then, incubation with species-matched secondary antibodies (Life Technologies) was performed for 2 h at 4°C in 0.4% TNB-TX100. GFP signal was amplified using a biotin-conjugated secondary antibody against the GFP primary antibody (Ch α GFP, 1:1,000 (Aves Labs, Tigard, OR, USA)). In addition, the tissues were incubated for 15 min with a biotin-specific streptavidin Alexa 488 complex. Finally, sections were counterstained using 4,6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich) and coverslipped with antifading mounting medium (Vectashield, Vector Labs, Burlingame, CA, USA).

For nuclear stainings, an antigen retrieval protocol was applied. Sections were incubated with 10 mM tri-sodium citrate buffer (+0.05% Tween 20, pH 6.0) for 30 min at 80°C and 20 min at room temperature.

Detection of EdU incorporation was performed using the Click-it EdU system (Life Technologies) according to the manufacturer’s instructions.

In situ hybridization

In situ RNA hybridization was performed as previously described [65]. After dissection, postnatal or adult brains were fixed in 4% PFA at 4°C for 48 or 24 h, respectively, cryoprotected in 30% sucrose at 4°C overnight, cryosectioned at 20 µm on consecutive slides, and stored at −80°C. Sections were acetylated with 0.1 M triethanolamine and 2.5 µL/mL acetyanhydride for 10 min and additionally fixed in 4% PFA at RT for 15 min before prehybridization for 4 h followed by 1 to 2 µg/mL probe hybridization for 16 h at 65°C. The antisense E2-2 [NM_013685], E2a [NM_011548], and HEB [NM_011544] probes were derived from plasmid constructs, whereas linearized templates were transcribed using a T7 RNA polymerase (Thermo Fisher Scientific, Waltham, MA). Anti-DIG FAB (1:2000 (Roche)) immunostaining was applied at 4°C overnight and subsequently developed in buffer containing BCIP (175 µg/mL) and NBT (100 µg/mL) for 12-48 h.

Microdissection and MAC sorting

To determine the spatio-temporal E-protein expression pattern, the SVZ or the striatum were microdissected from mice aged P6 as described in previously published protocols [66]. In brief, NSCs and neuroblasts were isolated by magnetic affinity cell sorting (MACS) using conjugated antibodies against the transmembrane proteins Prominin-1, also known as CD133, and PSA-NCAM. Dissected tissues were pooled in Hanks buffered salt solution (Life Technologies) and dissociated using a trypsin-based kit following manufacturer’s guidelines (Miltenyi Biotec, Bergisch Gladbach, Germany). Then, anti-Prominin-1 or anti-PSA-NCAM antibodies conjugated with magnetic beads (Miltenyi Biotec) were applied to the cell suspension for 30 min at 4°C before they were passed through MACS separator columns (Miltenyi Biotec). Sorted cells were snap-frozen for subsequent RT-qPCR. One litter represents 1 ‘n’ number.
Gene expression analysis

RNA was extracted, processed, and amplified from tissues/cells as previously described [32]. For RT-qPCR, 20 ng of cDNA were loaded with 5 μl of forward and reverse primers (Map2: fw_GGAAAGGCTCCAGGCACTCGTGA, rv_GTGGAGGTAAGCTGTGG; E2A: fw_GTGGGCTGACAGAAGGACTGA, rv_CCAGGTCTTCCATGG; HERB: fw_CCATCCCCAAATTCGAGAT, rv_GCTGGCTGATTTACCAGAC, rv_TTCGGGCTGAGTGGCTGAGTA; CD24: fw_CCACTCAGGTAACGACCAAGAC, rv_TTCGATGGCAGAGAGACAG; E2-2: fw_GGACCTGAAGCCAGAACTCCTT, rv_CCCATGGTTCTTACTCCTT) was used as reference gene. Relative gene expression was determined using the ΔΔCT method [67].

Analysis

Images were acquired with a laser scanning confocal microscope (Olympus IX 81, Olympus, Tokyo, Japan) equipped with a 20x or 40x objective, or with an epifluorescence microscope (Leica DM5500 B, Leica). Quantifications following in vivo electroporations are based on an average cell count of 254 successfully transfected cells per animal (n ≥5). Each animal represented 1 ‘n’ number. Morphological criteria to define RGCs were bipolar morphology and apical connection to the LV lumen. Morphological criteria to define non-RGCs were absence of apical and basal processes (i.e., roundish appearance), and absence of contact to the LV lumen [31,32].

All data are expressed as mean and standard error of the mean (±SEM). P values were determined using unpaired t-test or two-way ANOVA followed by Dunnett’s post hoc test (GraphPad Prism v5 software, San Diego, CA, USA).

Additional file

Additional file 1: (A) In contrast to the blockade of Ascl1-induced neuronal differentiation shown in Figure 1C, dne47 overexpression alone did not affect Map2 expression in NS5 cells kept in proliferative culture conditions (100 ± 1.0 vs. 93.9 ± 5.3). (B) E67 immunoreactivity revealed an increased non-RGC proliferation after dne47, when compared to empty control conditions, as shown in Figure 1E. (C & D) Antigenic characterization of E67 non-RGCs, demonstrated that both Ascl1 type-C (E, Figure 2C) and, to a lesser extent, Dcx type-A cells (D, Figure 2C) proliferate. (E–G) Representative immunostainings for detected changes in the expression of cell type specific markers; i.e., RGC = Vimentin” (E), type-C = Ascl1” (F), type-A = Dcx” (G), following dne47 expression, as quantified in Figure 2D–G. (H) Absence of cross-reactivity between shRNAs against E2A and E2-2 transcripts (see also Figure 4B). E2A mRNA expression was not reduced when shRNA against E2-2 was applied in vitro (100 ± 7.6 vs. 111.5 ± 1). Similarly, E2-2 mRNA expression was not reduced when shRNA against E2A was used (100 ± 2.7 vs. 148.6 ± 8.7). (I, J) Representative immunostainings for Dcx and Ascl1 illustrating increased Dcx expression (J, quantified in Figure 5C) and unaltered Ascl1 expression (J, quantified in Figure 5D), when E2-2 was overexpressed. Arrows identify representative cells for each experimental condition. Quantifications were normalized to control conditions. Scale bars: B–G, I, J, 20 μm.

Competing interests

The authors declare that they have no competing interests.

Authors’ contributions

BF, KA, SR, and MF carried out the experimental work. AHC contributed expertise for the subcloning of plasmids. BF and OR wrote the manuscript. BF, KA, SR, and MF carried out the experimental work. AHC contributed expertise for the subcloning of plasmids. BF and OR wrote the manuscript.

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