Published in final edited form as:
Glia. 2013 September ; 61(9): 1533–1541. doi:10.1002/glia.22539.

Aldh1L1 is expressed by postnatal neural stem cells *in vivo*

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Abstract

The metabolic enzyme for folate, *Aldh1L1,* has been shown to be expressed robustly in astrocytes of the brain. It is now well accepted that astrocytes in certain regions of the adult brain also serve as neural stem cells. Here, we examined whether *Aldh1L1* is also expressed in postnatal neural stem cells. *In vitro*, cells in neural stem cell culture conditions have robust *Aldh1L1* promoter activity. *In vivo,* in the adult brain, astroctyes in neurogenic regions express *Aldh1L1* in a pattern consistent with inclusion in neural stem cells, and analysis of *Aldh1L1+* cell transcriptome profiles from neurogenic regions reveals a robust enrichment of known regulators of neurogenesis. Genetic fate mapping with *Aldh1L1* BAC Cre animals reveals adult born neuroblasts of the rostral migratory stream are derived from *Aldh1L1* expressing cells, as are sporadic neurons in other regions of the brain. Combining these lines of evidence from transgenic animals, cell culture, transcriptome profiling, and fate mapping, we conclude that *Aldh1L1* is also expressed in neural stem cells in the brain. These findings may influence the future design of experiments utilizing *Aldh1L1* genetic tools, and also suggest existing *Aldh1L1* bacTRAP mice may be of use for further experiments profiling neural stem cells *in vivo*.

Introduction

The astrocyte is a markedly important cell for the normal functioning of the CNS: they provide essential metabolic support for neurons, key signals for synaptogenesis(Christopherson et al. 2005; Eroglu and Barres 2010; Ullian et al. 2001), and actively respond to neurotransmission(Sun et al. 2013). Classically, astrocytes were most commonly identified by staining with the marker Glial Fibrillary Acidic Protein (Gfap), however this particular antigen readily labels astrocytes in only a subset of brain regions, or during gliosis(Chiu and Goldman 1985; Eng et al. 2000). Recently new astrocyte markers, notably the Aldehyde dehydrogenase 1 family, member L1 (*Aldh1L1*) have become popular. By several accounts, *Aldh1L1* more robustly labels astrocytes in the brain, including all GFAP+ cells, but also with expression in parenchymal astrocytes poorly labeled by GFAP (Anthony and Heintz 2007; Cahoy et al. 2008; Dougherty et al. 2012b; Doyle et al. 2008; Neymeyer et al. 1997; Pfrieger and Slezak 2012; Yang et al. 2011), and there now exist both reliable antibodies for post hoc labeling(Krupenko and Oleinik 2002; Rhodes and Trimmer 2006), and validated a Bacterial Artificial Chromosome (BAC) for genetic targeting (Anthony and Heintz 2007).

Cells with features of astrocytes serve as neural stem cells in the neurogenic regions of the brain – the subventricular zone (SVZ) of the lateral ventricles and the subgranular zone (SGZ) of the dentate gyrus (Alvarez-Buylla and Garcia-Verdugo 2002; Kempermann 2002).

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In both regions, slowly dividing astrocytes (Gfap+), give rise to more rapidly cycling neuronal progenitors (transiently amplifying cells), which eventually give rise to cells destined to become neurons to the olfactory bulb or dentate gyrus granule cell layer, respectively (Doetsch 2003; Garcia et al. 2004). These adult stem cells arise from a transient developmental cell type, which can also serve as a neural stem cell, the radial glia (Merkle et al. 2004). Radial glia are also known to express *Aldh1L1*, largely starting as they decrease their proliferation after the period of neuronal production (Anthony and Heintz 2007). However, there are some remaining questions about these cells, both during development and adulthood.

Previously, we have employed an *Aldh1L1* BAC for transgenesis, which reliably and robustly labels astrocytes in the adult brain, targeting these cells for Translating Ribosome Affinity Purification(TRAP) (Doyle et al. 2008; Heiman et al. 2008) and sophisticated multicolored genetic imaging (Dougherty et al. 2012b). Here, we address the question of whether the astrocytes labeled by *Aldh1L1* include the neural stem cells and map the fate of cells with *Aldh1L1* BAC transcriptional activity. Examination of both the BAC transgene expression, as well as antibody labeling and fate mapping, determines that postnatal neural stem cells, *in vivo,* express *Aldh1L1.*

Materials and Methods

Mouse models

All experiments with mice were approved by the animal care and use committee of Rockefeller University We utilized multiple lines generated with the same BAC (RP23-7M9): the TRAP line *Aldh1L1* JD130 (Doyle et al. 2008), the Prism lines driving either *Cerulean* (JD1849) or *DSredmax*(JD1989) (Dougherty et al. 2012b), and a *Cre* expressing line (JD1884) (Tien et al. 2012). All BAC transgenic mice were maintained as heterozygotes, and genotyped at each generation by tail tip PCR or fluorescence microscopy. *Cre* mice were crossed to reporter mice with a floxed stop in front of *Yfp*, in the Rosa locus (b6.129×1-GT(ROSA)26Sor tm1(EYFP)Cos/J, Jackson Laboratories) for fate mapping experiments.

Neuroanatomy

For immunohistochemistry, brains were processed using MultiBrain™ Technology (NeuroScience Associates) using a 1:75,000 dilution of Goat anti-EGFP serum (Heiman et al. 2008) following the Vectastain elite protocol (Vector Labs). Note: GFP antibodies cross react with the YFP expressed here. Serial sections were digitized with Zeiss Axioskop2 microscope at 10× magnification. For immunofluorescence, sections were blocked with 5% normal donkey serum, 0.25% triton and incubated with primary antibodies (Ng2, NeuN from Chemicon, and NF200 from Sigma, Aldh1L1, a gift from Dr. Krupenko), and appropriate Alexa dye conjugated secondary antibodies (Invitrogen). Prism mouse images were taken with a LSM 510 NLO inverted multiphoton system. For five color imaging, system was utilized as a standard confocal: Yfp was excited with a 514 nm laser, and detected with a Band Pass 540/20 filter in place. mCherry and DSredmax were excited with a 543 nm laser and detected with a 565-615 IP Band Pass filter. Cerulean was exited with a 458 nm laser, as detected with a 470-500 Band Pass filter. Far red Alexa dyes were excited with a 633 nm laser and collected with the Lambda mode of the Zeiss system. DAPI was visualized with a coherent chameleon 2 photon on the same system. Epifluorescence images were taken with a wide-field Nikon microscope.

Microarray analysis

We reanalyzed data from GEO: GSE13379(Doyle et al. 2008) as follows: Cel files for *Aldh1L1* TRAP microarray data from forebrain and cerebellum(3 replicates each from, each replicate from pooled adult animals) were normalized with GCMRA using Bioconductor within the statistical package R, and chip definition files based on Entrez Gene Ids(Dai et al. 2005). Data were filtered to remove genes with low expression (less than 50), and to keep only genes enriched in astrocytes(TRAP/Total tissue RNA fold change >1), prior to directly comparing TRAP samples from *Aldh1L1* Forebrain and *Aldh1L1* Cerebellum utilizing the LIMMA module with Benjamini-Hochberg multiple testing correction. A threshold for forebrain enriched genes was selected at $p<.05$ with a 2 fold enrichment. pSI was calculated only for forebrain *Aldh1L1* as described(Dougherty et al. 2010) comparing this sample to all of our other previously collected TRAP samples(Dalal et al. in press; Dougherty et al. 2013; Doyle et al. 2008). Analyzed data are available in Supplemental Table 1.

Cell culture

Postnatal day six mice of line Prism JD1989 were euthanized, and cortices were dissociated with Trypsin and fire polished pipettes, and seeded in either Neurosphere media (DMEM/F12, 1% Penicillin Streptomycin, 2% B27 Supplement (Invitrogen), 10ng/ml bFGF, 10 ng/ml EGF (New England Biolabs), 5 ug/ml Heparin(Sigma)) or traditional astrocyte media (DMEM/F12, 1% Penicillin Streptomycin, 10% Fetal Bovine Serum(Sigma)), at 50,000 cells per ml. Growth factor was added to neurospheres two times a week, and cells were passaged at one week by dissociation with trypsin on seeding on poly-ornithine fibronectin coated plates as described (Nakano et al. 2005).

Results

Aldh1L1 transcriptional activity in neurosphere cultures

Previously, we generated triple colored transgenic mice, with three BACs in a single genomic locus, to label neurons, astrocytes and oligodendrocytes with distinct fluorophores *in vivo (Dougherty et al. 2012b)*. Here, we tested one of these mouse lines, Prism JD1989, for the same activity *in vitro.* The three cell types are labeled by BACs covering *Aldh1L1*(astrocytes, *DSredmax*), *Snap25(*neuron, *YFP-L10a*), and *Mobp(*oligodendrocytes, *Cerulean*). From post-natal day 6 mouse cortices, we cultured both neural stem cells, with the classic ‘neurosphere’ technique (Reynolds and Weiss 1992) including the mitogens EGF and bFGF, and also cultured cortical astrocytes, by plating these cells in traditional astrocyte conditions (McCarthy and de Vellis 1980; Nakano et al. 2005). Contrary to our expectations, after one week *in vitro*, only a fraction of the cells plated under astrocyte conditions demonstrated expression of the *DSredmax* transgene from the *Aldh1L1* BAC promoter (Figure 1A). In contrast, nearly all of the neurospheres (Figure 1B) showed bright *DSredmax* expression, though some spheres had varying degrees of *DSredmax* negative cells within them, suggesting some mosaicism.

Neurospheres are floating cultures, while traditional astrocyte conditions grow as adherent cultures. To determine if the *Aldh1L1* BAC activity was simply suppressed by plating, we dissociated the neurospheres and plated them as neural progenitor cultures, still in the presence of mitogen. Under these conditions the cells sustained bright expression of *DSredmax* from the *Aldh1L1* BAC, indicating that adherent culture conditions do not suppress *Aldh1L1* BAC activity (Figure 1C). Likewise, induction of differentiation by withdrawal of mitogen did result in the emergence of transcriptional activity from the *Mobp* BAC, as evidenced by *Cerulean* positive cells with oligodendroglial morphology(not shown), but did not substantially decrease *DSredmax* fluorescence. It is possible that the transgene *in vitro* was not accurately reflecting the expression of the endogenous gene. To

provide a more direct assessment of Aldh1L1 gene expression, we also examined *Aldh1L1* expression by RT-qPCR on cells from the same culture conditions but derived from E14.5 wildtype mouse cortex. *Aldh1L1* mRNA was robustly detected in the neural stem cell culture conditions, crossing threshold of detection around 21 cycles, not much later than the neural stem cell marker *Nestin* (19 cycles). Analyzing the data with the ddCT method relative to *Actb(*Beta Actin) mRNA, expression of *Aldh1L1* decreased with differentiation approximately 3 fold. In total, our experiments suggested the *Aldh1L1* may be expressed in neural stem cells under a variety of culture conditions.

Aldh1L1 is expressed in neurogenic niches in vivo

Gene expression in culture does not always reflect the *in* vivo state. To determine if *Aldh1L1* could be expressed in post-natal neural stem cells *in vivo* we examined its pattern of expression in regions of adult neurogenesis across multiple BAC transgenics and using an antibody to the endogenous protein. In the neurogenic SVZ, *Cerulean* from an *Aldh1L1* BAC (in Prism line JD1849) is found in a pattern consistent with known localization of 'Type B' neural stem cells (Doetsch 2003; Dougherty et al. 2005; Nakano et al. 2007): *Cerulean+* cells surround pockets of *Cerulean*−, DAPI+ nuclei(presumptive neuroblasts) (Figure 2B). These *Cerulean* expressing cells show no overlap with *Mobp* BAC activity (oligodendrocytes, *Yfp*), *Snap25* BAC activity (mature neurons, *mCherry*), or Ng2 antibody staining (oligodendrocytes progenitor cells, OPCs). Likewise, in the SGZ, the *Aldh1L1* BAC labels the astrocytes found immediately beneath the granule cell layer, consistent with a 'Type B' neural stem cell (*not shown)*. We see this neurogenic labeling across all of our *Aldh1L1* transgenic lines. For example, in mouse line *Aldh1L1* JD130, in which the *Aldh1L1* BAC drives expression of the *eGFP-RpL10a* (TRAP) construct for transcriptome profiling, we have previously shown that all Gfap+ cells express *Aldh1L1* (Doyle et al. 2008). In the SVZ, this transgene is expressed in the same pattern as in Prism JD1849, as is the endogenous *Aldh1L1* protein, demonstrating the expression pattern is not an artifact of transgenesis (Figure 2B). To systematically determine if the SVZ *Aldh1L1* cells include neural stem cells, we next examined the expression of neural stem cell markers with a comprehensive transcriptomic approach.

Comparative analysis of forebrain astrocytes reveals enrichment for neural stem cell and forebrain development genes

Previously, we utilized the TRAP to profile transcripts from *Aldh1L1* expressing cells from multiple regions in the brain (Doyle et al. 2008): notably the cortex/forebrain and the cerebellum, with the former dissection including the dorsal surface of the lateral ventricles and some ventricular zone (Figure 3A). Comparative analysis of these two regions reveals 127 transcripts significantly enriched in *Aldh1L1* expressing cells from the forebrain by at least 2 fold (Figure 3B) using conservative criteria. Careful examination of this list revealed a robust enrichment of several transcription factors, such as *Nr2e1(Tlx1)*, *Sox21*, *Id1*, *Hes5*, *Dmrta2*, *Emx2*, and *Lhx2* which are known to regulate either telencephalic development and/or adult neurogenesis (Boncinelli et al. 1993; Galli et al. 2002; Hatakeyama et al. 2004; Lyden et al. 1999; Monuki et al. 2001; Qu et al. 2010; Sandberg et al. 2005; Yoshizawa et al. 2011) (Figure 3C). Two of these(*Nr2e1 and Lhx2*) were also previously shown to be enriched in astrocytes cultured specifically from the forebrain(Yeh et al. 2009). To determine if this enrichment in neural stem cell genes was statistically significant, we compared the current analysis to our former screen for neural stem cell genes (Geschwind et al. 2001). The ~50 genes discovered in that early screen were significantly overlapped with the 127 genes discovered here($p<.0001$, Fisher Exact Test) and included the functionally validated Psph (Nakano et al. 2007).

In contrast, it is worth noting that we do not see enriched expression of a large set of proliferation genes such as the Cyclins, *Pcna*, or *Ki-67* nor related markers of the transient amplifying cells such as *Eomes*(Tbr2) (Englund et al. 2005), *Ascl1*(Mash1) (Parras et al. 2004), or *Pbk* (Dougherty et al. 2005). Likewise, in our previous profiling of *Pdgfra* expressing 'oligodendrocyte progenitor cells' from a similar dissection, we did see robust enrichment of cyclins, and proliferating progenitor genes such as *Pbk*, but not of *Aldh1L1*, nor the neurogenesis markers described above (Dougherty et al. 2012a). This suggests that relatively quiescent *Aldh1L1+* cells give rise to a more rapidly proliferating *Aldh1L1−* neural progenitor, which then differentiates into the neuroblasts of the rostral migratory stream. To confirm this, we utilized Cre-Lox mediated fate mapping to examine the fate of Aldh1L1 expressing cells.

Fate mapping indicates Aldh1L1 is expressed in postnatal neural stem cells in vivo

Using the same *Aldh1L1* BAC, we generated multiple lines of mice expressing the Cre recombinase (Tien et al. 2012). Crossing these to a reporter line revealed a robust labeling of astrocytes throughout the brain, as expected, as well as clear labeling of cells in the rostral migratory stream with the morphology of neuroblasts (Figure 4A). Likewise in the hippocampus, there was robust labeling in SGZ astrocytes, and sporadic labeling of neurons in the granule cell layer of the dentate gyrus, consistent with Cre activity in hippocampal neural stem cells as well (Figure 4B).

Outside of the neurogenic zones, we also saw sporadic labeling in cells with neuronal morphology (for example, in the upper layers of cortex). Double labeling with neuronal markers NeuN and NF200 confirms that *Aldh1L1* expressing cells can give rise to some cortical neurons (Figure 4C), likely due to the reported expression of *Aldh1L1* in some radial glia during development (Anthony and Heintz 2007). This same pattern of expression was reproduced to varying degrees across multiple lines generated by us, though we did note a surprising variability in neuronal expression even between littermates of the same line. Some neuronal labeling is evident as well in the *Aldh1L1* CRE line generated by GENSAT(www.gensat.org). In contrast, we did not see substantial overlap of *Aldh1L1* with the oligodendrocyte marker Mbp in the cortex suggesting *Aldh1L1* expressing cells do not give rise to this cell type in this tissue. In the spinal cord, there was some small percentage of Mbp cells labeled in the Cre lines, but no neurons, in line JD1884 (Tien et al. 2012).

Discussion

The utility of Aldh1L1 as a marker for astrocytes

Given the knowledge that a subset of astrocytes *in vivo* serve as neural stem cells, it is perhaps unsurprising that *Aldh1L1* is expressed in these cells as well. It is important to note that our studies here do not preclude the use of *Aldh1L1* as a marker of astrocytes in the mature brain. With both our transgenic lines and antibodies we saw robust and apparently complete labeling of astrocytes throughout the CNS. In the non-Cre lines, in all regions we saw perfect concordance of *Aldh1L1* BAC expression and Aldh1L1 protein, with the notable exception of some neurons in the anterior dorsal thalamus that were transgene positive, but antibody negative in multiple transgenic lines(*not shown*). However, genetic studies employing *Aldh1L1* driven Cre for astrocyte specific lineage studies will have to contend with the caveats presented here – existing *Aldh1L1* driven Cre lines will also recombine in post-natal neural stem cells and in some radial glial derived neurons, with variable penetrance. This does not preclude the use of these lines as astrocyte-specific in some situations – such as for Cre-responsive viral reagents delivered postnatally. It also suggests that the same BAC may be more effectively used in combination with inducible Cre constructs (such as Cre-ERT2) later in development to avoid labeling the early derived

neuronal populations, though even these may lead to recombination in *Aldh1L1* expressing cells in the periphery, such as in the liver or Schwann cells.

In vitro the expression of *Aldh1L1* does not particularly map to the mature astrocyte phenotype, with robust transgene expression in EGF/bFGF-containing neural stem cell culture conditions, and relatively poor expression in 'standard' serum containing astrocyte media. It would be interesting to determine how the transgene responds in new HBEGF culture conditions optimized to culture the more mature astrocyte (Foo et al. 2011).

Gene expression in forebrain astrocytes

One surprising facet of our analysis here was the robust differences in astrocyte gene expression between the forebrain (containing neural stem cells) and the cerebellum. As there were also a large number of genes found enriched in astrocytes of the cerebellum, the differences seen cannot be accounted for entirely by the presence of the neurogenic niche in the forebrain. It is possible that these are region specific factors, or potentially factors needed for the specialized giant astrocyte-like cell of the cerebellum: the Bergmann glia. These cells also express *Aldh1L1*, but have a distinct morphology and gene expression from other astrocyte-like cells (Doyle et al. 2008; Zhang and Barres 2010).

In the forebrain data, our comparative analysis identified several factors known to be important in adult neurogenesis. This suggests that others genes from this analysis may be equally important for astrocytes in the neurogenic niche. For example, it is well known that Notch signaling is essential for maintenance of adult neurogenesis (Imayoshi and Kageyama 2011), and our data reflect a preponderance of Notch signaling in astrocytes of the forebrain. Yet, there is a puzzle to these data – deletion of the *Rbpj*, a key component of all intracellular Notch signaling cascades, totally ablates adult neural stem cell maintenance in the telencephalon (Imayoshi et al. 2010), yet triple knockouts of the downstream transcription factors *Hes1;Hes3;Hes5* do not recapitulate this phenotype in the telencephalon (Hatakeyama et al. 2004). Examination of our data suggests that *Hes7* may also be expressed in forebrain astrocytes, and could be compensating for this loss. To facilitate studies in this direction, we have provided a more comprehensive list of the output of this analysis as Supplemental Table 1. Other genes not before studies in this context, such as *Dbx2* or *Notch4* might be equally important for adult neurogenesis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The mouse lines utilized here were generated in the laboratory of Nathaniel Heintz with funding from the Adelson Medical Research Foundation and HHMI. We thank Juliet Zhang for technical assistance, David Rowitch, Hui-Hsin Tsai and Ben Barres for their assistance in the evaluation of Cre lines, Sergey Krupenko for *Aldh1L1* antibody, and Kelly Monk for advice. This work was supported by the NIH(1R21NS083052-01), and the Mallinckrodt foundation.

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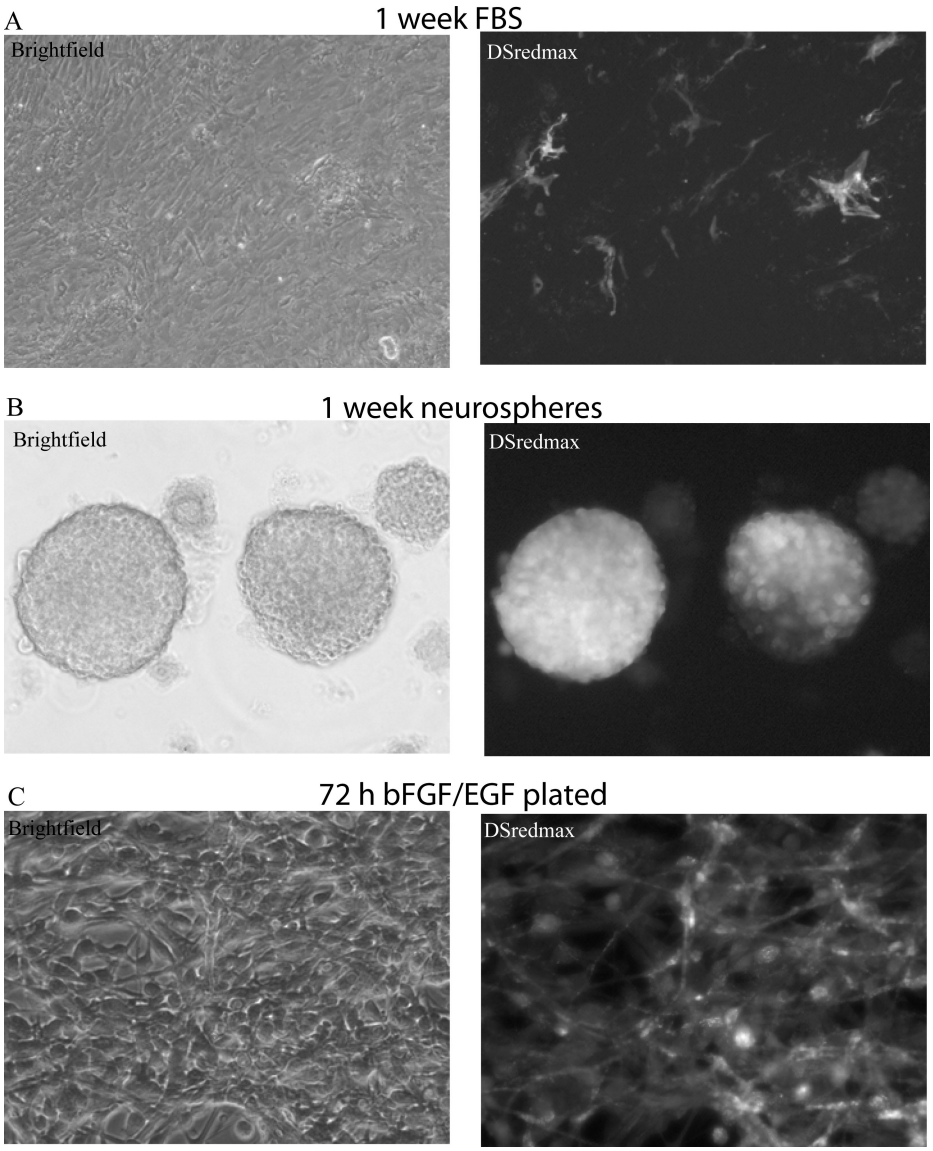


Figure 1. *Aldh1L1* is robustly expressed in neural stem cell cultures

A) In cultures derived from a P7 mouse cortex from a mouse line, in which an *Aldh1L1* BAC promoter drives DSredmax, relatively few cells express DSredmax when plated for one week under standard astrocyte culture conditions. B) In contrast, in neurosphere culture conditions (with EGF and bFGF), many cells robustly express dsredmax. C) Plated cells from these neurospheres continue to show DSredmax expression in the presence of EGF and bFGF

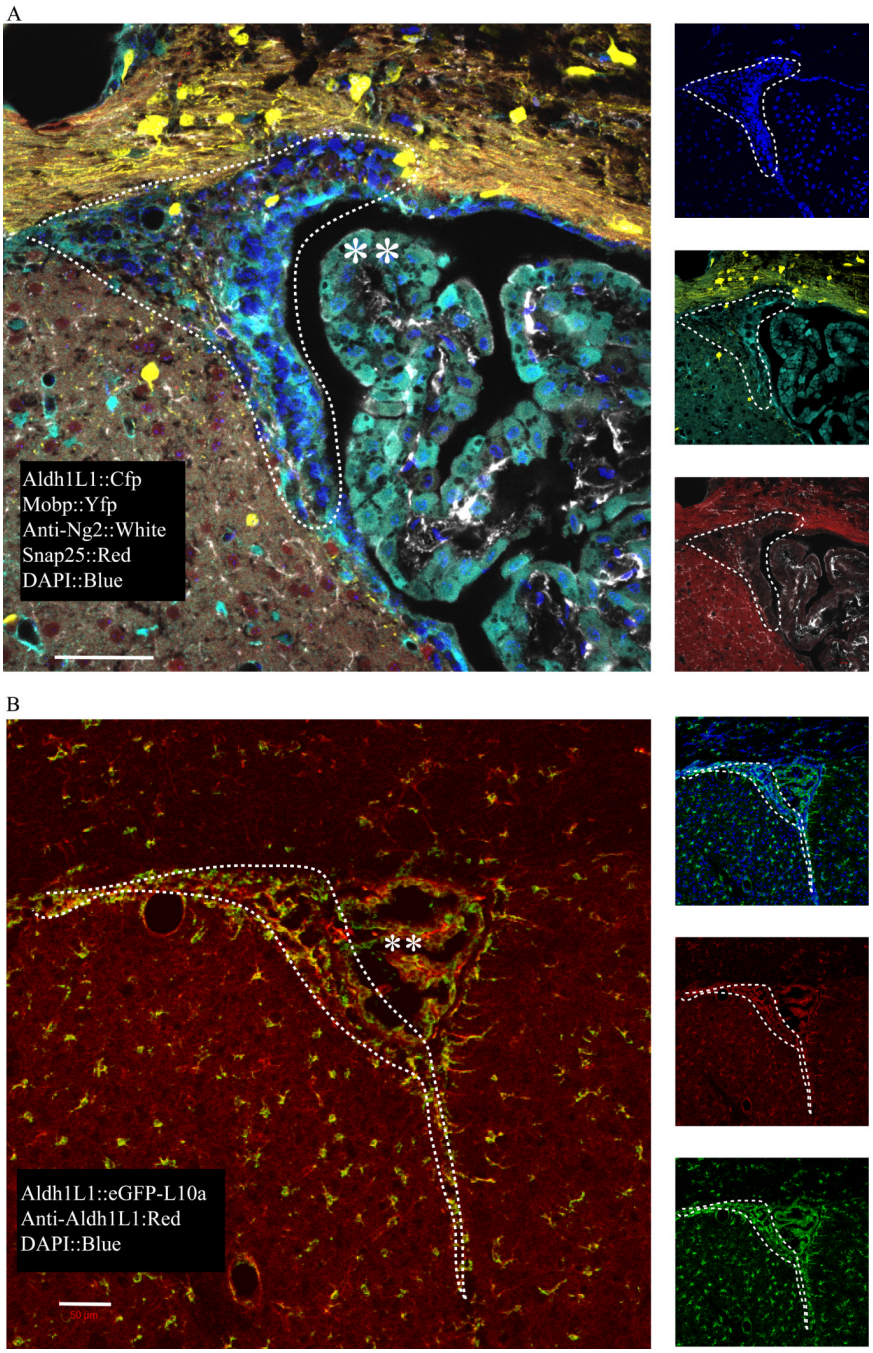
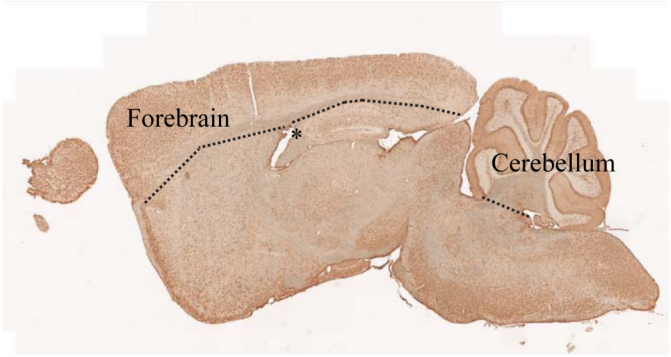


Figure 2. Aldh1L1 is expressed in neurogenic regions *in vivo*

A) In mouse line Prism JD1849, in which an *Aldh1L1* BAC promoter drives expression of Cerulean, expression is seen in parenchemal astrocytes, as expected, but also in the neurogenic SVZ region (dotted lines) adjacent to lateral ventricles, and choroid plexus (**). B) In bacTRAP mouse line ALdh1L1 JD130, protein (red) labeling is consistent with Aldh1L1 BAC Egfp-L10a expression in all regions, including SVZa (dashed line). *All scale bars = 50 micron*

A



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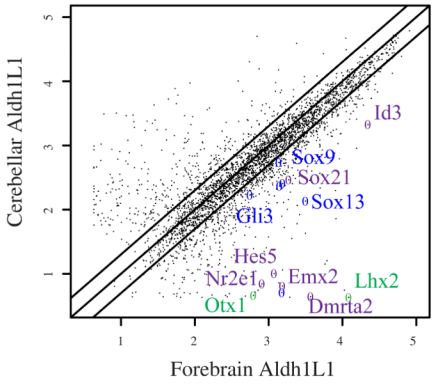

C

Entrez	Symbol	Description	logFC	adj.P.Val	pSI
242620	Dmrta2	doublesex and mab-3 related transcription factor like family A2	9.72	1.56E-07	4.81E-06
13797	Emx2	empty spiracles homolog 2 (Drosophila)	8.11	5.53E-06	4.81E-06
14634	Gli3	GLI-Kruppel family member GLI3	1.76	8.03E-05	2.89E-05
15208	Hes5	hairy and enhancer of split 5 (Drosophila)	6.71	1.57E-05	2.89E-05
15214	Hey2	hairy/enhancer-of-split related with YRPW motif 2	2.62	2.01E-05	0.0036228
15901	Id1	inhibitor of DNA binding 1	2.50	0.0015976	0.0008612
15903	Id3	inhibitor of DNA binding 3	3.44	1.29E-05	4.81E-06
16870	Lhx2	LIM homeobox protein 2	11.46	1.56E-07	9.62E-05
21907	Nr2e1	nuclear receptor subfamily 2, group E, member 1	6.91	9.74E-07	4.81E-06
18423	Otx1	orthodenticle homolog 1 (Drosophila)	6.87	5.53E-06	0.0002502
223227	Sox21	SRY-box containing gene 21	2.89	0.0001336	9.62E-06
20682	Sox9	SRY-box containing gene 9	1.25	0.0009028	0.0036565
223843	Dbx2	developing brain homeobox 2	3.40	0.0002899	4.81E-06
20668	Sox13	SRY-box containing gene 13	1.19	0.0176994	0.0039764

Figure 3. Forebrain astrocytes express neural stem cell genes *in vivo*

A) Sagittal section of adult mouse brain from TRAP line *Aldh1L1* JD130 stained with anti-GFP antibody shows *Aldh1L1* BAC activity throughout the brain. Forebrain/cortical dissection included the corpus callosum and ventricular zone of the lateral ventricles. B) Scatterplot of microarray data(*log scale*) shows robust enrichment of known factors involved in regulation of neurogenesis (purple) or forebrain development (green), as well as new candidate factors identified here (blue). Black lines are 0.5, 1, and 2 fold. Median of three experiments, showing only those genes scored as expressed (see methods). C) Table highlighting several of the transcription factors enriched at least two fold in forebrain astrocytes. Complete analyzed data are available in Supplemental Table 1. logFC: log base 2 of the ratio of forebrain to cerebellar astrocyte gene expression. Adj.P.Val: B-H corrected p-value comparing forebrain to cerebellar astrocytes. pSI: Specificity index statistic for the relative enrichment of the transcript in forebrain neurons compared to all other neural cell types collected.

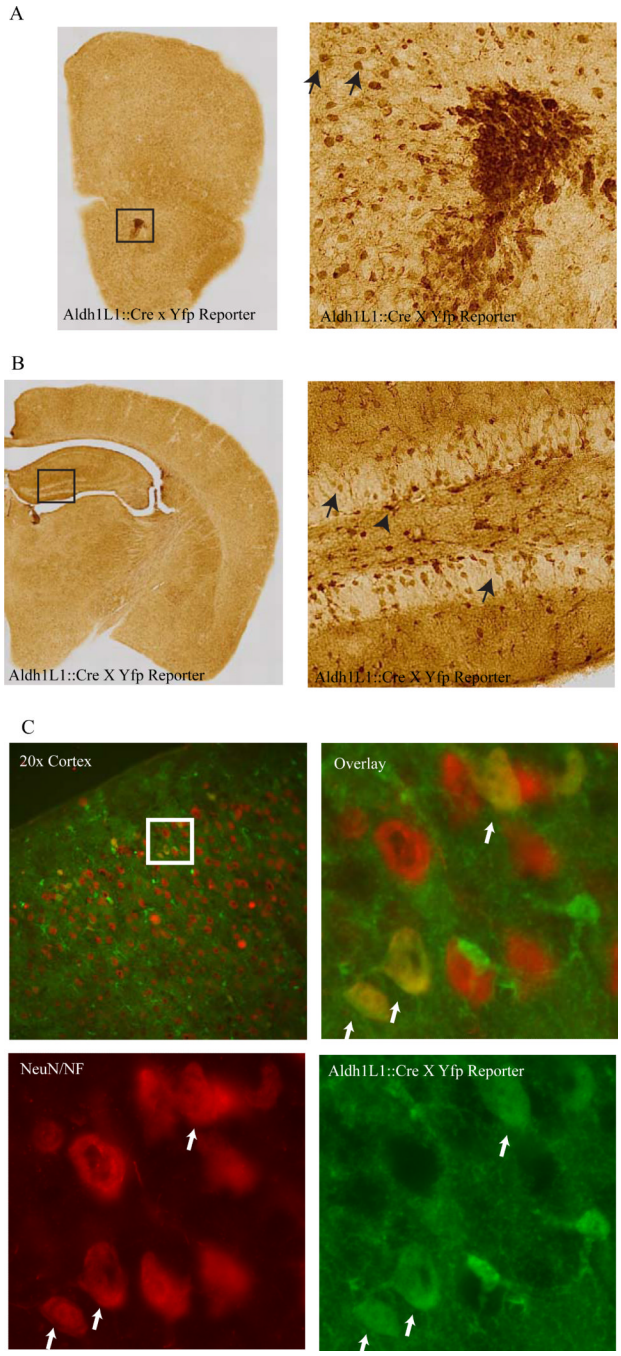

Figure 4. Lineage tracing with Aldh1L1 Cre mice labels neurons

A) Anti-GFP antibody staining of mouse forebrain from and Aldh1L1 Cre line JD1884, crossed to a YFP reporter mouse, shows expression in glia throughout the parenchema, and in presumptive neuroblasts of the rostral migratory stream (box, right panel) and adjacent neurons in the olfactory bulb. B) In the neurogenic dentate gyrus (box, right panel), GFP is seen in glial cells in the SGZ (arrowhead) and neurons of the granule cell layer (arrows). C) Immunofluorescence for YFP (green) and a mix of neuronal markers (NeuN and NF200, red), reveals some upper layer cortical neurons also derive from *Aldh1L1* expressing cells (white arrows).