Jagged1 signals in the postnatal subventricular zone are required for neural stem cell self-renewal

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Neural stem cells (NSCs) in the postnatal mammalian brain self-renew and are a source of neurons and glia. To date, little is known about the molecular and cellular mechanisms regulating the maintenance and differentiation of these multipotent progenitors. We show that Jagged1 is required by mitotic cells in the subventricular zone (SVZ) and stimulates self-renewal of multipotent epidermal growth factor-dependent NSCs. Jagged1-expressing cells line the adult SVZ and are juxtaposed to Notch1-expressing cells, some of which are putative NSCs. *In vitro*, endogenous Jagged1 acts through Notch1 to promote NSC maintenance and multipotency. *In vivo*, reducing Jagged1/Notch1 signaling decreases the number of proliferating cells in the SVZ. In addition, soluble Jagged1 promotes self-renewal and neurogenic potential of multipotent neural progenitors *in vitro*. Our findings suggest a central role for Jagged1 in the NSC niche in the SVZ for maintaining a population of NSCs in the postnatal brain.

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Introduction

Many adult mammalian tissues contain stem cells in niches with complex microenvironments that provide the appropriate determination and differentiation signals for the formation of new cells (van der Kooy and Weiss, 2000; Watt and Hogan, 2000; Blau *et al*, 2001; Chu and Gage, 2001; Price and Williams, 2001; Toma *et al*, 2001; Weissman *et al*, 2001; Wagers *et al*, 2002; Alvarez-Buylla and Lim, 2004). In the adult mammalian central nervous system (CNS), a population of neural stem cells (NSCs) resides in the periventricular area of the forebrain lateral ventricles (subventricular zone (SVZ)) (Reynolds and Weiss, 1992; Morshead *et al*, 1994).

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These NSCs display the principal characteristics of multipotency and self-renewal. In the rodent brain, SVZ is the source of neuroblasts that converge and migrate in chains from the lateral walls of the forebrain along defined 'glial tubes' to the olfactory bulb throughout life (Alvarez-Buylla and Temple, 1998). Upon reaching the olfactory bulb, the neuroblasts differentiate into granule and periglomerular interneurons.

In the SVZ, four cell types have been defined based on their morphology. Under the ependymal cells lining the lateral ventricle resides a population of periventricular astrocytes (B cells) (reviewed by Doetsch, 2003), and elegant use of transgenic and retroviral approaches suggests that some of these B cells have NSC properties (Doetsch *et al*, 1999). The B cells are proposed to undergo asymmetric cell divisions to generate transient amplifying cells (C cells). C cells proliferate rapidly, and undergo a limited number of cell cycles before generating neuroblasts (A cells) (reviewed by Doetsch, 2003). Although recent data indicate that glial cells both in rodents as well as in other species can have stem cell-like properties (Malatesta *et al*, 2000; Noctor *et al*, 2001), the identity of the mammalian SVZ stem cell remains to be determined unequivocally.

The identification and characterization of mammalian NSCs *in vivo* has proven to be problematic due to the lack of specific markers. The most commonly used method for identifying NSCs is their self-renewal capacity and multipotency in neurosphere cultures. Within the neurospheres, NSCs self-replicate, and can be induced to differentiate into neurons, astrocytes and oligodendrocytes. *In vitro*, postnatal SVZ-derived NSCs are dependent upon epidermal growth factor (EGF) (Morshead *et al*, 1994; Doetsch *et al*, 1999; Tropepe *et al*, 1999). Recent data imply that neurospheres can be derived from transient amplifying cells under the influence of EGF (Doetsch *et al*, 2002).

Notch signaling has been shown to be involved in the maintenance and differentiation of mammalian embryonic neural progenitors in the neural tube (de la Pompa *et al*, 1997; Hitoshi *et al*, 2002; Lutolf *et al*, 2002). Ectopic activation of Notch signaling in the neural tube results in expansion of neural progenitors at the expense of neurogenesis (Lardelli *et al*, 1996; Gaiano *et al*, 2000). However, little is known about the mechanisms that control NSC maintenance and differentiation in the postnatal brain. Notch1 is prominently expressed by putative NSCs in the neurogenic regions of the adult mouse brain (Stump *et al*, 2002; Irvin *et al*, 2004) and antisense experiments suggest a role for Notch1 in the maintenance of embryonic derived NSC (Chojnacki *et al*, 2003).

Jagged1 is a member of the Serrate/Jagged family of canonical Notch ligands (Lindsell *et al*, 1995), and is widely expressed during development and in the adult CNS (Lindsell *et al*, 1996; Stump *et al*, 2002; Irvin *et al*, 2004). Although data suggest that Jagged1/Notch signaling may regulate oligodendrocyte maturation and

myelination in the murine brain (Wang *et al*, 1998; Genoud *et al*, 2002), its role in the CNS is not clear due to the early embryonic lethality of Jagged1-deficient mice (Xue *et al*, 1999).

We have shown previously that Jagged1 is expressed in the neurogenic regions of the postnatal brain (Stump *et al*, 2002). Now we have addressed the role of Jagged1 signaling in the postnatal SVZ and NSC. We found that Jagged1 is expressed by cells in the SVZ and rostral migratory stream (RMS), and is required for maintained proliferation in both regions of the postnatal mouse brain. Additionally, we show that loss of Jagged1 function causes a loss of self-replicating NSCs *in vitro*. Finally, Jagged1 treatment of NSCs *in vitro* can substitute for mitogens such as EGF and increase the neurogenic potential. Our experiments identify Jagged1 as a key regulator of NSC self-renewal and differentiation potential within the postnatal brain.

Results

Notch1 and Jagged1 are expressed in the SVZ

To address whether adult-type mammalian NSCs are regulated by Notch signaling, we analyzed the location of Notch1-expressing cells in the mouse SVZ. *In situ* mRNA hybridization (Supplementary Figure 1A), and immunohistochemistry (Supplementary Figure 1B) using an affinity-purified antibody raised against Notch1 (Stidworthy *et al*, 2004) revealed Notch1-expressing cells clustered in the SVZ under and within the ventricle lining. In addition, using affinity-purified antibodies, we identified Jagged1-expressing cells lining the lateral ventricle walls adjacent to the SVZ (Figure 1A). These Jagged1-expressing cells are located in and just under the ependymal cell layer (Figure 1B, arrows) and project processes into clusters of tightly packed cells within the SVZ (Figure 1B and H, arrowheads).

Figure 1 Notch1 and Jagged1 are expressed in the SVZ. (A) Jagged1 is expressed by cells lining the SVZ. (B) Jagged1-expressing cells are associated with the subependymal cells (arrows) and project processes into the SVZ (arrowheads and H). (C) GFAP immune-reactivity is localized to the subependymal cells lining the lateral ventricles (and arrows in G). (D) GFAP-expressing cells extend processes into the SVZ similar to Jagged1-expressing cells (arrowheads and in H). (E) Jagged1 (red) colocalizes with GFAP (green) in cells lining the SVZ (arrowhead) and adjacent to cells clustered close to the ventricular lining (nuclei stained with DAPI, arrows; blue). (F) Consecutive sagittal section to that in E stained with anti-Notch1 antibodies (arrows; brown). Tightly packed, Notch1-positive clusters of cells lie adjacent to the Jagged1/GFAP-positive cells (arrowhead). (G) Double immunostaining shows Jagged1 (red)/GFAP (green)-expressing cells lining the lateral ventricle (arrow) and projecting processes into the SVZ. (H) Higher magnification of the Jagged1 (red)/GFAP (green) double-positive processes (arrowheads) intermingled between clusters of tightly packed cells. (I) Notch1 and GFAP immunostaining on a section consecutive to that in G and H (arrow; ventricle lining). (J) Notch1-expressing cells (arrows) are clustered in the SVZ and adjacent to GFAP-positive cells (open arrows). (K) Notch1-positive cells (arrow) in the subependymal layer of the SVZ do not express Jagged1, whereas the ventricle lining (arrowhead) stains for both Notch1 and Jagged1. (L) Most Notch1-positive cells (arrow) in the caudal SVZ do not express Doublecortin (Dcx; green). However, some Notch1 is occasionally detected at low levels in a few Dcx-positive neuroblasts (arrowhead) under the ependymal lining (dotted line). (M) Dcx expressing neuroblasts in the medial SVZ and RMS express Notch1 (open arrow). (N) Jagged1 (arrow) does not overlap with Dcx expression in the SVZ or RMS. Scale bar in A = 500 µm for panels A and C, 50 µm for panels B, D, G, and I, 25 µm for E, F, H, and J, 15 µm for K, and 10 µm for M and N and 5 µm for L.

Immunostaining with anti-glial fibrillary acid protein (GFAP) antibodies identified astrocytes throughout the brain and lining the SVZ (Figure 1C and D, arrows) that project processes into the underlying neurogenic region (Figure 1D and H, arrowheads), similar to Jagged1-expressing cells. In addition, we observed Jagged1-, GFAP- and Notch1-expressing cells along the RMS (data not shown). Double immunofluorescence with anti-Jagged1 and anti-GFAP antibodies revealed coexpression in cells lining the SVZ (Figure 1E, arrowhead) and in processes projecting through clusters of tightly packed cells within the SVZ (Figure 1E, G and H). Immunostaining on consecutive 5- μ m paraffin sections showed that some of these clustered cells express Notch1 (Figure 1F, arrows). We then examined the relationship between Jagged1- and Notch1-expressing cells. We performed immunostaining on consecutive sections of adult mouse SVZ with antibodies to Jagged1, GFAP, and Notch1. We found Jagged1 to be prominently expressed by GFAP-positive cells in the SVZ (Figure 1H, arrowheads) and within the ventricular lining (Figure 1G, arrow). Notch1 is expressed by clustered cells (Figure 1I and J, arrows) that are adjacent to GFAP-positive and presumably Jagged1-expressing cells (Figure 1I and J, open arrows). Consecutive immunostaining for Jagged1 and Notch1 revealed that Notch1-expressing cells in the SVZ do not express detectable levels of Jagged1 (Figure 1K, arrow), whereas some coexpression is seen in the ventricle lining (Figure 1K, arrowhead).

We then addressed whether the clustered Notch1-expressing cells also expressed the neuroblast marker Doublecortin (Dcx). Immunostaining showed Dcx-expressing cells in the rostral SVZ and along the RMS (Supplementary Figure 1C–E). Therefore, we analyzed Dcx expression in the caudal SVZ, the region where the majority of the Notch1-positive cells are found. Most of the Notch1-positive cells in the subependymal region of the caudal SVZ were clustered near to, but did not express, Dcx (Figure 1L, arrow). Conversely, the majority of the Dcx-positive cells migrating under the ependymal lining did not express Notch1 (Figure 1L). Occasionally however, we did find a small number of Notch1-positive cells in the caudal SVZ that expressed low levels of Dcx (Figure 1L, arrowhead). Therefore, we addressed whether Notch1 expression in the proximal RMS overlaps with Dcx expression in migratory neuroblast. Indeed, in the proximal and medial RMS, many Notch1-positive cells coexpressed Dcx (Figure 1M, open arrow) although most of the Dcx-expressing cells in the distal RMS did not express Notch1 (Supplementary Figure 1D–F). Jagged1 expression never overlapped with Dcx, whether in the SVZ or RMS (Figure 1N, arrow).

Compound reductions in Jagged1 and Notch1 results in reduced mitosis in the SVZ

To address the function of Jagged1 in the postnatal brain, we generated a conditional allele for *Jagged1* (Figure 2A). The first and second coding exons of the *Jagged1* gene were flanked with LoxP sequences (Supplementary data). Floxed *Jagged1* mice are indistinguishable from control littermates and show no anatomical defects (data not shown). A null allele for *Jagged1* was generated by breeding floxed *Jagged1* mice with CMV-Cre deleter mice. Mice hemizygous for the *Jagged1*-null allele were identified by PCR (Figure 2B) and showed no obvious anatomical defects in the CNS, whereas

mice homozygous for the *Jagged1*-null alleles died *in utero* as described previously (Xue *et al*, 1999).

Mammalian Notch signaling has been shown to be sensitive to gene dosage (McCright *et al*, 2002). Therefore, we reduced Jagged1 and Notch1 signaling *in vivo* by intercrossing *Jagged1* and *Notch1* hemizygous null mice to generate animals lacking one *Jagged1* and one *Notch1* allele (double hemizygotes). Hemizygous *Notch1*-null mice are viable and have no obvious defects (Radtke *et al*, 1999). Double-hemizygous mice were born but, in contrast to single-hemizygous mutants, the majority died within a few hours of birth and only 10% survived up to 8 days. We performed histological analyses and immunostaining on the brains of postnatal day four (P4) and P8 hemizygous mutant mice. At P4 the brains of double-hemizygous mutants and control wild-type (wt) mice were anatomically similar and histological stains did not reveal obvious abnormalities in the SVZ and RMS (data not shown). To address changes in mitotic cell activity, we performed antiphosphohistone III (PH3) staining and analyzed the proportion of cells in the M phase of the cell cycle within the SVZ (Figure 2C–F) and RMS (Figure 3A–D) of mutant and control mice.

At P4, double-hemizygous mice displayed a highly significant (Student's *t*-test $P < 0.001$) reduction in the proportion of M-phase cells in the SVZ compared to wt littermates and single-hemizygous mutants (Figure 2G). At P8, we observed a 60% reduction in PH3-positive cells in the SVZ of double-hemizygous mice compared to control mice (Figure 2H). In addition, the SVZ of P8 mutants was thinner, with a reduced cellularity (data not shown). A similar reduction in mitotic cells was also observed in the RMS of P4 double-hemizygous mice (Figure 3E). Hence, loss of function of one *Jagged1* and one *Notch1* allele results in a significant reduction in the proportion of SVZ cells in the M phase of cell cycle. These data indicate that reduced Jagged1/Notch1 signaling results in a perturbation of mitotic cells in the neurogenic SVZ of the postnatal brain.

Jagged1 and Notch1 are expressed by defined cells in neurospheres

Due to the absence of Cre-recombinase-expressing mouse lines for the specific and conditional inactivation of *Jagged1* from the adult SVZ *in vivo*, we turned to the well-established and versatile neurosphere cell culture system to address Jagged1 signaling in NSCs. We isolated NSCs from the forebrain of perinatal wt mice and maintained them in clonal spherogenic cultures supplemented with EGF to expand adult-type NSCs (Temple and Alvarez-Buylla, 1999). In order to focus on self-replicating spherogenic stem cells, we passaged these primary neurospheres and analyzed the expression of Jagged1 in the spheres formed 5 days later. Jagged1-expressing cells were found on the surface of neurospheres and many also expressed the intermediate filament protein Nestin (Figure 4A–C, arrows). When neurospheres were plated onto culture dishes, Jagged1 expression also colocalized with GFAP (Figure 4D and D, arrows), confirming the expression observed in the SVZ *in vivo* (Figure 1E, G and H). Whereas many Nestin-expressing cells also stained with the Jagged1 antibodies, only a subpopulation of the GFAP-positive cells expressed Jagged1, as *in vivo* (Figure 4E). In contrast, Notch1-expressing cells on the surface of

Figure 2 Jagged1 and Notch1 regulate proliferation in the SVZ. (A) Schematic representation of the *Jagged1* locus and targeting construct (see Supplementary data). (i–iii) A PGK-neo selection marker flanked by two *loxP* sites, as well as a third *loxP* site, were inserted as shown. Black boxes indicate exons 1 and 2, ATG the start codon, and the black triangles the *loxP* sites. (ii) Homologous recombinants were detected by PCR with primers P1–6, and by Southern blotting of genomic DNA using probe S1. (iii) The PGK-neo cassette was removed by transient Cre-recombinase expression in ES cells. (B) Floxed *Jagged1* animals were genotyped with primers P3 and P4 to detect floxed (300 bp) and wt loci (250 bp), and the *Jagged1*-null allele carrying mice with primers P1 and P4. (C, D) At P4, mice double hemizygous for *Jagged1* and *Notch1*-null alleles (d + / -) have a reduction in PH3-positive prophase cells (C—DAPI, D—PH3) in the SVZ (outlined with a dotted line) compared to wt littermates (E—DAPI, F—PH3). (G) Quantification revealed a highly significant (**Student's *t*-test $P < 0.001$), approximately 60% reduction in PH3-positive cells in the SVZ of double-hemizygous mutants ($n = 3$) compared to wt littermates ($n = 3$) at P4. (H) P8 double-hemizygous mutants showed >60% reduction in PH3-positive cells compared to wt littermates (**Student's *t*-test $P < 0.001$). Anterior–posterior (A–P) and dorsal–ventral (D–V) axis is shown for panels C–F.

Figure 3 Jagged1 and Notch1 regulate proliferation in the RMS. (A–D) P4 mice double hemizygous for *Jagged1* and *Notch1*-null alleles (d + / -) show a reduction of PH3-positive cells in the RMS (outlined with a dotted line) compared to wt (+ / +) littermates. (E) Quantification revealed a highly significant (**Student's *t*-test $P < 0.001$) reduction in proliferating cells along the RMS of double-hemizygous mutants compared to wt control littermates at P4.

Figure 4 Jagged1 and Notch1 are expressed by neural progenitors. (A) Immunostaining showing Jagged1-expressing cells (arrow) on the surface of intact neurospheres. (B) Coimmunostaining with antibodies against the progenitor cell marker Nestin. (C) Enlarged overlay of Jagged1 and Nestin immunostaining indicating coexpression by a subpopulation of cells (arrow). (D) Acutely plated neurospheres immunostained with anti-Jagged1 and anti-Nestin antibodies showing coexpression by a subpopulation of cells (yellow; arrows), other Nestin-positive cells do not express Jagged1 (arrowhead). (E) Jagged1 and GFAP are coexpressed by many astroglia (yellow; arrows), but not all GFAP-positive cells express Jagged1 (arrowhead). (F) Confocal microscopic analysis of Notch1 expression by cells on the surface of most neurospheres (arrows). (G–G'') Cryosections (8 μ m) double immunostained for Notch1 and Nestin. (G) Notch1-expressing cells (arrow) are restricted to the surface of the neurosphere, as are the majority of the Nestin-expressing progenitors (arrow; G'). (G'') All Notch1-expressing cells express Nestin (arrow). (H) Confocal 3D reconstruction of Notch1 and GFAP double-immunostained spheres. The inset shows a single optical section and the absence of Notch1 and GFAP costaining.

neurospheres expressed the progenitor cell marker Nestin, but not detectable levels of GFAP (Figure 3F–H, arrows).

Ablation of Jagged1 blocks NSC self-renewal in vitro

To analyze the function of Jagged1 in EGF-dependent NSCs, we inactivated the *Jagged1* gene using an Adenoviral-Cre vector (Adeno-Cre; Kalamarides *et al*, 2002) in dissociated NSCs isolated from postnatal floxed *Jagged1* animals carrying the Cre-reporter *Rosa26R* (Figure 5A and B). The constitutive expression of β -galactosidase from the *Rosa26R* allele after Cre activity allowed us to address recombination efficiency (Figure 5C). Recombined floxed *Jagged1* NSCs, from here on referred to as Jagged1-deficient, formed secondary neurospheres at a rate similar to that seen for control-infected NSCs isolated from *Rosa26R* mice (Figure 5D). The Jagged1-deficient neurospheres were indistinguishable from infected control spheres in terms of size and morphology (data not shown). RT-PCR analysis of the infected Jagged1-deficient neurospheres showed a lack of Jagged1 mRNA 5 days post-infection, confirming gene ablation (Figure 5F). Other components of the canonical Notch signaling cascade were still present (Figure 5F), and quantitative analysis using real-time

PCR showed a downregulation of Hes1 and Hes5 to 40.3 ± 2.8 and $2.0 \pm 1.2\%$ of levels in Adeno-Cre-infected *Rosa26R* cells, respectively (Figure 5H and Supplementary Figure 3).

To test whether Jagged1-deficient cells retain their self-renewing capacity and can be maintained in the spherogenic assay, we passaged the neurospheres 5 days post-infection (Figure 5A). The number of secondary spheres formed from each post-infection neurosphere is a direct reflection of the number of self-replicating stem cells. As expected, Adeno-Cre-infected *Rosa26R* control cells retained their self-renewal capacity with an average of 7.6 ± 0.16 spherogenic stem cells per infected neurosphere. By contrast, the Jagged1-deficient NSCs showed a striking reduction in their self-replicating ability, with an average of only 1.7 ± 0.53 stem cells present in the neurospheres 5 days post-ablation (Figure 5E). Hence, Jagged1-deficient neurospheres contained only 1/5 of the normal number of stem cells.

RT-PCR analysis of the secondary post-infection neurospheres demonstrated complete ablation of *Jagged1* from the infected floxed *Jagged1* cells, excluding the possibility that the remaining spherogenic cells had failed to recombine the *Jagged1* loci (Figure 5G). Residual Jagged1 protein or

Figure 5 Jagged1 is required for NSC self-renewal. (A) Schematic representation of the experimental approach. Primary EGF-dependent neurospheres were dissociated (1) and infected at a multiplicity of infection of 50 with Adeno-Cre virus (2). Infected NSCs were cultured for 5 days post-infection (3) and used in differentiation assays for RNA isolation, or were dissociated (4) and cultured under spherogenic conditions for a further 5 days (5). (B) Conditional *Jagged1* gene rearrangement induced by infection of dissociated, floxed *Jagged1* NSCs *in vitro* with an Adeno-Cre virus. The *Rosa26R* Cre-reporter allele was included to follow recombination efficiency by monitoring the constitutive expression of β -galactosidase following Cre recombinase activity. (C) X-gal staining for the *Rosa26R* Cre-reporter allele (R26R) β -galactosidase activity showed 95–99% recombination efficiency in floxed *Jagged1* as well as in control cells carrying only the *Rosa26R* reporter. (D) Quantification of neurospheres generated from 25 000 dissociated primary neurosphere cells. *Jagged1*-ablated NSCs retain their sphere-forming potential after infection comparable to wt cells. (E) Dissociation and secondary neurosphere formation revealed that post-ablation *Jagged1*-deficient NSCs lose their self-renewal capacity. Graph depicts the number of sphere-forming cells retained in the Adeno-Cre-infected neurospheres post-ablation (**Student's *t*-test $P < 0.001$). (F) RT-PCR analysis of canonical Notch signaling components after *Jagged1* ablation. (G) RT-PCR analysis of secondary spheres post-ablation confirmed the loss of *Jagged1* and *Notch1* mRNA. (H) Quantitative real-time PCR analysis showing downregulation of the *Notch1* and its targets *Hes1* and *Hes5*. GAPDH mRNA was used to standardize the cDNA in the reactions. The results shown in (F), (G), and (H) are averages of two representative cDNA preparations from independent samples and experiments. Scale bar in (B) = 100 μ m.

molecular compensation may explain the persistence of some self-renewing cells in the *Jagged1*-deficient neurospheres, although we could not detect *Jagged1* protein on recombined cells (Figure 6A–D, red). Conversely, it is possible that *Jagged1* ablation results in NSCs forming transient intermediates with a limited spherogenic capacity.

The remaining *Jagged1*-deficient secondary neurospheres also showed a major reduction in the expression of *Hes5* compared to infected control spheres (*Jagged1*-ablated 2°; Figure 5H and Supplementary Figure 3A). Surprisingly, a dramatic reduction in *Notch1* mRNA levels was observed in the spheres formed after *Jagged1* ablation (Figure 5F, G and H), whereas mRNA levels of other members of the Notch family were not affected (Supplementary Figure 2A). In addition, semiquantitative PCR analysis showed a slight increase in the expression of the proneural genes *Mash1* and *Math1* following *Jagged1* ablation, but not of the later neuronal marker *NeuroD*, the glial marker *GFAP*, and the progenitor cell marker *Nestin* (Supplementary Figure 2A). The remaining secondary *Jagged1*-deficient neurospheres could not be maintained by further dissociation, indicating that the loss of *Jagged1* leads, over time, to a complete loss of self-replicating NSCs.

Jagged1* deficiency does not affect differentiation potential *in vitro

To address whether loss of *Jagged1* affects not only the self-renewing potential but also the lineage determination of NSCs, we induce differentiation under clonal conditions. *Jagged1*-deficient neurospheres plated as effectively as control neurospheres and spread onto the coated dishes over the 6-day period. After differentiation, the cells were fixed and immunostained. *Jagged1* protein was not detectable on the *Jagged1*-ablated spheres (Figure 6A–D). Surprisingly, *Jagged1*-deficient neurospheres formed neurons, astroglia, and oligodendroglia as efficiently as Adeno-Cre-infected *Rosa26R* neurospheres (Figure 6E). Hence, although *Jagged1* regulates stem cell maintenance, loss of function does not directly prevent NSCs from adopting specific neural cell fates *in vitro*.

***Jagged1* functions through *Notch1* to maintain NSC self-renewal**

Our *in vivo* and *in vitro* data indicate that *Jagged1* is required for NSC maintenance through the activation of *Notch1* (Figures 2 and 3). To address this, we performed an Adeno-Cre-mediated conditional gene inactivation of *Notch1* from

Figure 6 Jagged1 regulates self-renewal, but not cell lineage commitment. Jagged1-deficient neurospheres (Δ/Δ) plated at clonal density give rise to clones containing (A) neurofilament (NF160)-positive neurons, (B) GFAP-positive astroglia, (C) O4-positive oligodendrocytes and (D) Nestin-positive progenitors (green A–D). Jagged1 protein was not detected on ablated cells (red; A–D). (E) Quantification of the percentage of clones containing lineage-specific marker-positive cells in Jagged1-deficient and control Adeno-Cre-infected cells. Scale bar in (A) = 10 μ m for panels A–D.

NSCs *in vitro*, using cells isolated from floxed *Notch1* mice also carrying the *Rosa26R* allele (Figure 7A) (Radtke *et al*, 1999; Lutolf *et al*, 2002). Comparable to the observations made following *Jagged1* ablation, specific loss of Notch1 function did not prevent sphere formation 5 days post-infection with Adeno-Cre (Figure 7C). However, Notch1 could no longer be detected at the protein or mRNA levels (Figure 7B and E). We observed a 93% reduction in Notch1 mRNA levels by Affymetrix microarray analysis of RNA levels in the Notch1-deficient spheres compared to infected *Rosa26R* spheres. However, dissociation of the Notch1-deficient neurospheres revealed a dramatic 250-fold reduction in self-replicating NSCs (8.1 ± 1.1 stem cells/sphere for control compared to 0.04 ± 0.01 in Notch1-deficient spheres: Figure 7D).

Analysis of the canonical Notch pathway components after *Notch1* ablation showed a reduction in *Hes* gene expression, particularly *Hes5* (Figure 7E), which, by Affymetrix microarray analysis, was downregulated to 20% of the level seen in Adeno-Cre-infected *Rosa26R* cells (data not shown). The remaining *Hes* mRNA could be due to RNA stability or residual Notch1 protein. However, we were unable to analyze changes in gene expression in secondary infected neurospheres, since Notch1-deficient neurospheres could not be dissociated and maintained. Although ablation of either *Notch1* or *Jagged1* resulted in a drastic loss of NSCs in the neurosphere assay, the expression levels of Notch2 and Notch3 were not significantly affected by ablation of either

Figure 7 Notch1 mediates the Jagged1 signal for NSC self-renewal. (A) Floxed *Notch1* alleles were generated by insertion of *loxP* sites (triangle) upstream of the first coding exon of the gene. Following Cre-mediated recombination, the first exon and part of the putative promoter of the *Notch1* are ablated to generate a null allele (Radtke *et al*, 1999). The *Rosa26R* Cre-reporter allele was included in the analysis for lineage tracing. (B) Adeno-Cre infection of dissociated NSCs isolated from floxed *Notch1* mice results in a complete loss of Notch1 protein expression after 5 days. (C) *Notch1*-deficient NSCs form neurospheres at the same rate as infected control cells. (D) Dissociation of the neurospheres 5 days post-infection showed an almost complete loss of spherogenic cells in the *Notch1*-ablated population (**Student's *t*-test $P < 0.001$). (E) RT-PCR confirmed a dramatic reduction in the levels of *Notch1* mRNA in the *Notch1*-ablated, compared to infected, control neurospheres (R26R) after 5 days, and moderate reductions in *Hes1* and *Hes5* expression. (E) shows a representative series of results using two RNA samples from independent experiments. GAPDH mRNA was used to standardize the cDNA in the reactions. Scale bar in (B) = 50 μ m.

gene, nor were the levels of the ligands Dll1, Dll2, Dll3, and Jagged2, indicating a lack of compensatory signaling (Supplementary Figure 2A and data not shown). In addition, expression levels of the proneural genes *Mash1* and *Math1* and the early neuronal marker *NeuroD* were not affected (Supplementary Figure 2A and Affymetrix microarray; to be presented elsewhere). Hence, Jagged1 and Notch1 are required by NSCs for self-maintenance.

Notch1 deficiency does not affect differentiation potential *in vitro*

We found that loss of Jagged1 did not affect the differentiation potential of NSCs *in vitro* (Figure 6). Therefore, we analyzed the potential of Notch1-deficient neurospheres. Clonal analysis showed that Notch1-deficient neurospheres formed neurons, astroglia, and oligodendroglia similar to

Adeno-Cre-infected control *Rosa26R* neurospheres, although Notch1 protein was no longer detectable (Figure 8A–E). Both Jagged1- and Notch1-deficient cultures contained Nestin-positive clones, although their number was slightly reduced in the Notch1-deficient cultures (Figure 8D and E).

NSCs of the adult SVZ are dependent on Jagged1 and Notch1

Our *in vitro* and *in vivo* analyses were based on NSCs isolated from the perinatal mouse brain and the SVZ of mutant mice shortly after birth. Due to the postnatal lethality observed in double-hemizygous Jagged1/Notch1 mice, we addressed whether NSCs isolated from the SVZ of adult mice are also dependent upon Jagged1 and Notch1. We generated neurospheres from microdissected SVZs of adult floxed *Jagged1* and floxed *Notch1* mice and *Rosa26R* mice. Although the number of neurospheres generated from adults is considerably lower than from perinatal mice, we were able to analyze the NSCs for Jagged1 and Notch1 dependency.

We repeated the spherogenic assays and performed quantitative PCR analysis of gene regulation with NSCs isolated from three individual adult mice of each genotype. In complete agreement with the findings for EGF-dependent cells from perinatal mice, neither ablation of *Jagged1* ($105.9 \pm 7.0\%$ of control) nor *Notch1* ($96.5 \pm 2.9\%$ of control) affected sphere formation 5 days post-infection (Figure 9A).

Figure 8 *Notch1* ablation does not alter cell lineage commitment. Notch1-deficient NSCs retain the ability to generate (A) neurofilament160-positive neurons, (B) GFAP-positive astrocytes, (C) O4-positive oligodendroglia, and (D) Nestin-positive progenitor cells. Notch1 protein was not detected on the ablated cells (green; A–D). (E) Quantification of clones containing lineage-specific marker-positive cells showed a significant reduction (*Student's *t*-test $P < 0.05$) only in the number of Nestin-containing clones in the *Notch1*-ablated population compared to infected control cells. Scale bar in (A) = 20 μ m for panels A–D.

Figure 9 *Jagged1* and *Notch1* are required by adult-derived NSCs. (A) Adult Jagged1-deficient (*Jag1* Δ/Δ ; $105.9 \pm 7.0\%$) and Notch1-deficient (*Notch1* Δ/Δ ; $96.5 \pm 2.9\%$) NSCs formed neurospheres as efficiently as Adeno-Cre-infected *Rosa26R* (wt) NSCs 5 days post-ablation. (B) Adult Jagged1-deficient (*Jag1* Δ/Δ ; $42.6 \pm 6.8\%$) and Notch1-deficient (*Notch1* Δ/Δ ; $4.6 \pm 2.1\%$) NSCs have a highly reduced self-renewal capacity. Graph depicts the relative number of spheres formed from *Jagged1*- and *Notch1*-ablated NSCs compared to Adeno-Cre-infected neurospheres (**Student's *t*-test $P < 0.001$). Each experiment was repeated in triplicate with three independent cultures. (C) Graphic representation and numerical values of real-time quantitative RT-PCR analysis of canonical Notch signaling components after *Jagged1* ablation compared to control-infected *Rosa26R* neurospheres. (D) Real-time PCR analysis of Notch1 and its targets after *Notch1* ablation compared to control-infected *Rosa26R* neurospheres. The values in (C) and (D) are averages of three individual samples performed in duplicate. The cDNA levels were standardized using GAPDH quantitative PCR.

However, as seen previously with perinatal-derived cells, Jagged1-deficient adult neurospheres showed a highly significant reduction in the number of secondary neurospheres ($42.6 \pm 6.8\%$ of control; Figure 9B). Furthermore, and also in agreement with the perinatal cell data, Notch1-deficient adult neurospheres had almost completely lost self-renewing, spherogenic NSCs ($4.6 \pm 2.1\%$ of control; Figure 9B).

We then addressed whether Notch signaling was affected in these adult NSCs by real-time PCR. At 5 days post-Jagged1 ablation, we found an almost complete loss of Jagged1 mRNA ($2.1 \pm 0.1\%$ of control; Figure 9C). Interestingly, Notch1 mRNA was completely lost, and Hes1 (down to $26.9 \pm 1.0\%$) and Hes5 (down to $2.6 \pm 0.9\%$) mRNA levels were highly reduced in adult Jagged1-deficient spheres compared to control-infected adult Rosa26R spheres (Figure 9C). Similarly, real-time PCR confirmed the ablation of Notch1 from infected adult floxed Notch1 NSCs 5 days post-infection, and a concomitant reduction in Hes1 (down to $9.6 \pm 1.9\%$ of control) and Hes5 (down to $0.2 \pm 0.1\%$ of control) expression (Figure 9D). These results are comparable to the changes in the expression of Notch target genes observed after Jagged1 and Notch1 ablation from perinatal NSCs, supporting the view that EGF-dependent stem cells isolated from perinatal and adult brains have similar requirements for Jagged1 and Notch1.

Jagged1 activity can replace growth factors to maintain adult-type NSCs in vitro

Our ablation experiments suggested that Jagged1 is required for NSC maintenance *in vivo* and *in vitro*; therefore, we addressed whether activation of Notch signaling is sufficient to increase NSC numbers. We treated wt EGF-dependent NSC cultures for 5 days with soluble Jagged1-Fc fusion protein (Figure 10A) (Shimizu *et al*, 1999) in EGF-containing, defined neurosphere medium. Subsequently, the number of self-replicating spherogenic cells in the cultures was assessed by dissociation. In the presence of EGF, Jagged1-Fc did not induce a significant increase in the number of NSCs in the cultures (data not shown). We then assessed whether Jagged1 could maintain neurosphere-forming cells in the absence of EGF. NSCs were dissociated and cultured in neurosphere medium containing increasing concentrations of Jagged1-Fc, but lacking EGF. Cells cultured in medium lacking both EGF and Jagged1-Fc (see Supplementary data) generated only few neurospheres (Figure 10B). However, Jagged1-Fc treatment resulted in a dose-dependent rescue of spherogenic cells almost to the levels seen in EGF-containing medium (Figure 10B). Hence, Jagged1 is able and sufficient to maintain NSCs in the absence of EGF.

Jagged1 treatment increases the neurogenic potential of NSCs in vitro

Increased Notch activity has been shown to induce gliogenesis in the nervous system (Furukawa *et al*, 2000; Gaiano *et al*, 2000; Morrison *et al*, 2000; Scheer *et al*, 2001). Thus, we assessed the multipotency of Jagged1-Fc-treated NSCs. Neurospheres treated for 5 days with soluble Jagged1-Fc were plated at clonal density on substrate-coated dishes to induce differentiation. Jagged1-Fc-treated NSCs retained the ability to generate neurons, astrocytes, and oligodendroglia, indicating multipotency and that activation of endogenous

Figure 10 Jagged1 can maintain NSC self-renewal *in vitro* in the absence of trophic factors and increases the neurogenic potential. (A) NSCs dissociated from primary wt neurospheres were treated with increasing concentrations of recombinant Jagged1 extracellular domain-human IgFc fusion protein. DSL—delta/serrate/lag domain, EGF—epidermal growth factor repeats, CR—cysteine-rich domain. (B) Quantification of sphere formation revealed a highly significant, dose-dependent increase in the number of spheres rescued by Jagged1-Fc treatment compared to cultures treated with mock medium (see Supplementary data) and cells grown in neurosphere medium containing 10 ng/ml EGF or lacking EGF (C) (**Student's *t*-test $P < 0.001$). Jagged1-Fc containing neurosphere medium lacking EGF was diluted 1:50, 1:10, 1:4, and 1:1 with neurosphere medium lacking EGF. (C–F) Jagged1-Fc-treated NSCs remain multipotent and can differentiate into (C) neurons (neurofilament160), (D) astrocytes (GFAP), (E) oligodendrocytes (O4), and remain as Nestin-positive progenitors (F). (G) Treatment of NSCs with soluble Jagged1 during neurosphere formation results in a highly significant (**Student's *t*-test $P < 0.001$) increase in neurons compared to EGF-treated cultures upon differentiation. EGF treatment reduced the Jagged1 effect on neurogenic potential. Scale bar in (C) = 10 μ m for panels C–F.

Notch signaling by Jagged1 does not result in lineage commitment (Figure 10C–F).

Although there were no obvious differences between the differentiation potential of EGF and Jagged1-Fc-treated neurospheres on a clonal basis, clones derived from NSCs grown in the presence of exogenous Jagged1 seemed to

contain more neurons. Hence, we quantified the proportion of β -TubulinIII/neurofilament 160-positive cells in the differentiation assays. In three independent experiments, the proportion of neurons generated upon differentiation increased three-fold (Student's *t*-test, $P < 0.001$) as a result of Jagged1-Fc treatment during sphere formation (Figure 10G). Interestingly, the increased neurogenic potential caused by Jagged1 treatment was abolished by the presence of EGF in the medium (Figure 10G).

Discussion

We show that active Jagged1 is required for the maintenance of multipotent adult-type NSCs *in vitro* and mitotic cells in the postnatal SVZ. This is the first demonstration of a function for Jagged1 in maintaining progenitors of the CNS, which we propose is through activation of Notch1. Reduced Jagged1/Notch1 signaling results in a reduction in proliferating cells in the neurogenic SVZ and RMS. Due to the absence of specific markers for NSCs *in vivo*, we could not exclude that not only NSCs but also transient amplifying cells and neuroblasts are affected by the reduction in Jagged1 and Notch1. However, using self-replication and sphere-forming potential as a reporter for NSCs *in vitro*, we can clearly show that Jagged1 is required for maintaining EGF-dependent NSCs isolated from perinatal and adult mice. Thus, we believe that the effects of reducing Jagged1/Notch1 signaling *in vivo* are due, at least in part, to a reduction in NSC numbers.

Notch signaling is activated by ligands of the Dll and Jagged families, as well as by noncanonical ligands such as Contactin and NB-3 (Hu *et al*, 2003; Cui *et al*, 2004). Although multiple putative ligands are present in the neurosphere cultures, Jagged1 is the only ligand required for multipotency and EGF-dependent NSC maintenance *in vitro*, in agreement with its localization in the SVZ *in vivo*. This is also supported by previous *in vitro* data reporting that an absence of the Notch ligand Dll1 does not affect neurosphere formation and NSC maintenance, but reduces oligodendrocyte and astrocyte differentiation (Grandbarbe *et al*, 2003). In contrast, we show that the loss of Jagged1 and Notch1 does not significantly alter neurogenic or gliogenic differentiation of NSC. These differences may be due to the source of EGF-dependent NSCs as Grandbarbe *et al* isolated embryonic-derived NSCs and we isolated NSCs from postnatal and adult brain.

The mechanism by which Jagged1 and Dll1 induce different effects on EGF-dependent progenitor cells is unclear. Changes in the O-glycosylation of Notch molecules by glucosaminyltransferases of the Fringe family modulate signaling responses to different classes of ligand (Fleming *et al*, 1997; Bruckner *et al*, 2000; Hicks *et al*, 2000). Thus, individual Notch ligands may act through specific receptors at different stages to regulate maintenance and differentiation. Furthermore, as loss of Notch1 affected predominantly maintenance of self-renewal and not lineage determination, one might speculate that Dll acts through another mechanism or Notch family member to regulate cell fate.

Although Notch1 ablation *in vitro* resulted in a reduction in Nestin-expressing clones, which is in agreement with a role for Notch1 in maintaining progenitors, Jagged1 ablation did not. It is possible that Notch1 plays a role in Nestin-expressing transient intermediate cells. Thus, loss of Notch1 may have resulted in the differentiation of both NSCs and

transient amplifying cells. In addition, loss of Jagged1 had a less pronounced effect on the loss of NSCs compared to ablation of Notch1, which may also reflect the maintenance of Nestin expression.

We show that endogenous Notch1 is not required for the formation of any neural lineage from NSCs (Figure 8). Furthermore, we show that the activation of endogenous Notch signaling by treatment with Jagged1 promotes multipotency rather than gliogenesis, resulting in increased neuronal differentiation potential (Figure 10). Our data and those of others fit with a model whereby Notch1 maintains NSCs in an undifferentiated and competent state to respond, potentially in combination with Notch signaling, to additional differentiation cues. Hence, we propose that direct activation of Notch signaling by Jagged1 does not result in glial differentiation but, in combination with other signals such as EGF or cytokine signaling downstream of the gp130 receptor, may potentiate gliogenesis (Chojnacki *et al*, 2003; Kamakura *et al*, 2004). This is supported indirectly by our observation that Jagged1-induced neurogenic potential is abolished in the presence of EGF. Similarly, ectopic activation of Notch signaling by intracellular domain overexpression may induce targets downstream of multiple Notch pathways, including those activated by Dll1.

The signals that modulate NSC self-replication and differentiation are likely to be encoded in the SVZ niche. Recent evidence suggests that blood vessel endothelium may constitute at least part of the NSC niche both in the embryo and adult (Shen *et al*, 2004). We find that Notch1-expressing progenitors are in close contact with Jagged1-positive cells within the neurogenic regions of the adult brain and that both Notch1 and Jagged1 are required for NSC maintenance. The Jagged1-expressing cells in the SVZ of adult mice also express GFAP and are located in a position consistent with them being SVZ astrocytes. Hence, both endothelial cells and SVZ astrocytes may provide Notch1 ligands to NSCs in the SVZ. It is interesting to note that, *in vitro*, endothelial cells induce expression of the Notch target Hes1 but not Hes5 (Shen *et al*, 2004). Ablation of Jagged1, on the other hand, results in reductions in Hes1, but predominantly Hes5 expression. Hence, it remains to be shown whether there are two niches in the adult SVZ, potentially for two populations of NSCs.

Jagged1 expression by a subpopulation of astroglia in neurospheres and in the adult SVZ is intriguing, based on the accumulating data that some SVZ astrocytes (B cells) have stem cell-like properties (reviewed by Doetsch, 2003). Although some of these data may be explained by GFAP-positive cells in the niche, providing signals to the NSC (Song *et al*, 2002), it is likely that multipotent progenitors in the SVZ activate, at some point, the GFAP promoter. Therefore, it remains possible that Jagged1 expressed by SVZ astrocytes signals to neighboring cells and promotes the multipotent NSC state. Thus, some SVZ astrocytes may represent the niche and others the multipotent progenitors. Notch1-expressing cells in the SVZ and in neurospheres do not express high levels of GFAP, and most are found in the caudal SVZ, where they do not express the neuroblast marker Dcx. Conversely, the distal RMS is comprised mainly of Dcx-positive cells, suggesting that the Notch-dependent NSCs are mainly located in the caudal SVZ, where few Dcx cells are found. Interestingly, neuroblasts in the medial RMS do

express Notch1, suggesting a potential function outside the neural progenitors.

We propose that Jagged1-expressing cells form the niche for a population of NSCs in the SVZ. Mechanistically, Jagged1 likely activates Notch1 on NSCs, preventing differentiation and, in a positive feedback loop, increasing Notch1 gene transcription. Our findings demonstrate that Jagged1 forms an important part of the stem cell maintenance pathway in the postnatal brain and acts through Notch1, whereas other ligands and Notchs do not seem to play a major role in maintenance.

Materials and methods

Production of 'floxed' Jagged1 locus

The floxed Jagged1 allele was generated by inserting *loxP* sites into the *Jagged1* gene, flanking the first and second coding exons and the sequences between (for detailed description, see Supplementary data).

Isolation of NSCs and culture of neurospheres

NSCs were isolated from early postnatal mice and cultured in defined medium supplemented with B27 and EGF (for detailed description, see Supplementary data). Quantification, immunofluorescence and cell fate analysis are described in detail in the Supplementary data.

RNA isolation and RT-PCR analysis

Total RNA was isolated from neurosphere cultures using Trizol Reagent (Roche) and purified using RNeasy extraction kit according to the manufacturer's instructions (Qiagen). RT-PCR reactions were performed using gene-specific primers and conditions described in the Supplementary data.

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Antibodies and immunostaining of cells and sections

Cells were fixed and processed for immunostaining as described in Supplementary data. Brain sections for immunostaining were either fresh frozen or paraffin embedded. Details of the staining procedure and antibodies are given in Supplementary data.

Soluble Jagged1-Fc treatment of NSCs

Soluble Jagged1-Fc was generated as described previously (Shimizu *et al*, 1999). Neurospheres were dissociated and cultured in defined medium supplemented with Jagged1-Fc in the presence or absence of EGF. Subsequently, the number of neurospheres generated in the cultures, the differentiation potential and the maintenance of self-replicating spherogenic cells were analyzed (see Supplementary data).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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