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The CSF-1 receptor ligands IL-34 and CSF-1 exhibit distinct developmental brain expression patterns and regulate neural progenitor cell maintenance and maturation

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Abstract

The CSF-1 receptor (CSF-1R) regulates CNS microglial development. However, the localization and developmental roles of this receptor and its ligands, IL-34 and CSF-1, in the brain are poorly understood. Here we show that compared to wild type mice, CSF-1R-deficient (*Csf1r−/−*) mice have smaller brains of greater mass. They further exhibit an expansion of lateral ventricle size, an atrophy of the olfactory bulb and a failure of midline crossing of callosal axons. In brain, IL-34 exhibited a broader regional expression than CSF-1, mostly without overlap. Expression of IL-34, CSF-1 and the CSF-1R were maximal during early postnatal development. However, in contrast to the expression of its ligands, CSF-1R expression was very low in adult brain. Postnatal neocortical expression showed that CSF-1 was expressed in layer VI, whereas IL-34 was expressed in the meninges and layers II–V. The broader expression of IL-34 is consistent with its previously implicated role in microglial development. The differential expression of CSF-1R ligands, with respect to CSF-1R expression, could reflect their CSF-1R-independent signaling. *Csf1r−/−* mice displayed increased proliferation and apoptosis of neocortical progenitors and reduced differentiation of specific excitatory neuronal subtypes. Indeed, addition of CSF-1 or IL-34 to microglia-free, CSF-1R-expressing dorsal forebrain clonal cultures, suppressed progenitor self-renewal and enhanced neuronal differentiation. Consistent with a neural developmental role for the CSF-1R, ablation of the *Csf1r* gene in Nestin-positive neural progenitors led to a smaller brain size, an expanded neural progenitor pool and elevated cellular apoptosis in cortical forebrain. Thus our results also indicate novel roles for the CSF-1R in the regulation of corticogenesis.

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Keywords

CSF-1; IL-34; neural stem cells; neurogenesis; nestin; cerebral cortex

Introduction

Cytokines that regulate hematolymphopoiesis and bone morphogenesis also play significant roles in nervous system development beyond those ascribed to classical neurotrophins and CNS mitogens (Bernd, 2008; Mehler and Kessler, 1997). The intimate involvement of these cytokines in developing and adult brain reflect their increasingly well recognized, complex and context-dependent roles in orchestrating diverse cellular and molecular CNS processes, including regional trophic signaling, neuronal and glial cell specification, neuroblast migration, neurite outgrowth, and neuronal and neural network homeostasis, plasticity and connectivity (Bauer et al., 2007; Mehler and Gokhan, 1999; Mehler et al., 1993; Temple, 2001).

Colony-stimulating factor-1 (CSF-1), also known as macrophage-CSF (M-CSF) is the primary growth factor for tissue macrophages and osteoclasts (Byrne et al., 1981; Guilbert and Stanley, 1980; Stanley et al., 1983; Tanaka et al., 1993; Tushinski et al., 1982). The effects of CSF-1 are mediated by the CSF-1 receptor tyrosine kinase (CSF-1R) (Dai et al., 2002; Pixley and Stanley, 2004; Sherr et al., 1985). The receptor is expressed on macrophages (Byrne et al., 1981), osteoclasts (Kodama et al., 1991) and Langerhans cells (Ginhoux et al., 2006), as well as cells of non-hematopoietic origin (Arceci et al., 1989; Huynh et al., 2009) and CNS microglia (Sawada et al., 1990; Suzumura et al., 1990). *Csf1*^{op}/*Csf1*^{op} or *Csf1*$^{op/op}$ mice are homozygous for *osteopetrotic* mutation, a naturally occurring recessive mutation due to single nucleotide insertion in exon 4 of *Csf1* gene leading to a frame-shift and a 63 amino acid long truncated, inactive protein (Marks and Lane, 1976; Wiktor-Jedrzejczak et al., 1990; Yoshida et al., 1990). These mice exhibit a pleiotrophic phenotype including toothlessness, skeletal defects, reduced body weight, deficits in tissue macrophages and osteoclasts, as well as male and female reproductive defects (Cecchini et al., 1994; Cohen et al., 1996; Kodama et al., 1991; Marks and Lane, 1976; Pollard et al., 1991; Wiktor-Jedrzejczak et al., 1990; Wiktor-Jedrzejczak et al., 1982; Yoshida et al., 1990). In contrast to *Csf1*$^{op/op}$ mice that exhibit ~30% reduction in microglia, microglia are absent in CSF-1R-deficient, *Csf1r*$^{-}$/*Csf1r*$^{-}$ (*Csf1r*−/−) mice (Dai et al., 2002; Erblich et al., 2011; Ginhoux et al., 2010), in agreement with the existence of a second CSF-1R ligand, interleukin-34 (IL-34) (Lin et al., 2008). Consistent with a role for IL-34 in brain, *IL34* mRNA is expressed earlier and at 10-fold higher levels than *Csf1* mRNA (Wei et al., 2010). Moreover, CSF-1 is required for maintenance of GABAergic cortical circuitry and promotion of neurite outgrowth (Michaelson et al., 1996), for control of neuroendocrine pathways in hypothalamus (Cohen et al., 2002) and for enhancing survival of a subset of adult neocortical neurons (Wang et al., 1999a), thereby suggesting that CSF-1R signaling has additional important trophic roles in neural cells.

In the present study, using CSF-1-reporter-LacZ mice coupled with immunochemistry, we demonstrate that CSF-1 and IL-34 exhibit a distinct regional and often complementary expression profiles, with a broader expression of IL-34. While CSF-1R expression decreases dramatically with development from neonatal to adult brain, IL-34 and to a lesser extent CSF-1 expression are maintained at higher levels. Focusing on the developing cortex, CSF-1 expression appears later than the CSF-1R and it is primarily localized to layer VI of neocortical neurons in the postnatal brain. In contrast, IL-34 is expressed by neocortical neurons in layers II–V, as well as by meningeal cells. We show that the CSF-1R is expressed by a small subset of Nestin-expressing dorsal forebrain progenitors and by

microglia. *Csf1r−/−* mice exhibited forebrain developmental abnormalities, including increased proliferation of neural progenitor cells. *In vitro* clonal expansion and differentiation experiments, using *Nestin*-positive forebrain progenitors, indicate that both CSF-1 and IL-34 suppress progenitor self-renewal. The *Csf1r−/−* abnormalities of smaller brain size, increased dorsal forebrain progenitor numbers and elevated cellular apoptosis are partially recapitulated in *Nestin-Cre/+; Csf1r*$^{flox/flox}$ (Li et al., 2006; Tronche et al., 1999) mice. Together, these experiments demonstrate a direct role for the CSF-1R in progenitor cell-mediated neural development and further suggest that CSF-1 and IL-34 have broader biological functions than previously thought.

Materials and Methods

Mice

The generation, maintenance and genotyping of the *TgN(Csf1-Z)7Ers/+ (TgZ7/+)* (Ryan et al., 2001), *Csf1*$^{op/op}$ and *Csf1r−/−* (Dai et al., 2002; Dai et al., 2004b) mice on the FVB/NJ background and the *Nestin-GFP* transgenic reporter (Mignone et al., 2004), *Nestin-Cre* (*Nes-Cre/+*) transgenic (Tronche et al., 1999) and the *Csf1r*$^{flox/flox}$ (*Csf1r*$^{fl/fl}$) (Li et al., 2006) mice on the C57BL/6 background were as described.

Brain weight measurement

After careful removal of the cranium, unperfused brains, including both olfactory bulbs (OB), were cut between pons and medulla. Brains were then removed from the skull sockets after severing the optic chiasma and weighed using a precision weighing balance.

Histology, Histochemistry and Immunohistochemistry (IHC)

Mice were perfused with 5 ml of cold PBS, followed by 15 ml of perfusate. For histological analysis, the perfused (4% paraformaldehyde (PFA) in PBS, pH 7.4) brains were sectioned (1–2 mm), fixed in perfusate (16 h, 4°C), embedded in paraffin and stained with hematoxylin and eosin. For whole mount X-gal staining, perfused (4% PFA) tissues were fixed in 4% PFA (30 min, 4°C), or in 2% PFA plus 0.01% glutaraldehyde, pH 7.4 (1h, 4°C), washed with PBS and incubated (16h, 32°C), on a rocker, in X-gal solution (Hennighausen et al., 1995). For X-gal staining of frozen sections, perfused (1.5% pre-chilled PFA, pH 7.4) brains were dissected, fixed in 1.5% PFA in 30% sucrose in PBS, pH 7.4 (1 h, 4°C), sliced into 1–2 mm thick sections, fixed (same fixative, 8–10 h), washed with PBS and embedded in OCT. Sections (10 μm) were stained with X-gal. For IHC, perfused (4% PFA) brains were sectioned (1–2 mm), fixed in perfusate (6 h, 4°C), incubated successively with 15%, then 30%, sucrose in PBS (6 h, 4°C, each), embedded in OCT and cut into 30μm sections. Images were captured by either an Olympus Bx51 upright fluorescent microscope with Olympus MicroSuite™- Five Biological Suite software, NY, USA, or by a Leica AOBS SP2 confocal microscope with Leica confocal software, Germany. Quantification of cell numbers following IHC was carried out either manually, or by using “Image J” software. Antibody dilution and isotype for IHC: Nestin, mouse IgG_1 (1:250, BD Biosciences); β-tub III, mouse IgG_{2b} (1:400, SIGMA); PDGFRα, polyclonal goat IgG (1:300, Chemicon); NG2, polyclonal rabbit IgG (1:300, Chemicon); O4, mouse IgM (1:300, Chemicon); S100 β, mouse IgG_1 (1:100, SIGMA); NeuN mouse IgG_1-Alexa fluor-488 (1:100, Chemicon); APC, mouse IgG_{2b} (1:100, Calbiochem); GFAP, mouse IgG_1 (1:400, SIGMA), or mouse IgG_{2b} (1:200, BD Pharmingen); CSF-1R (IK), goat IgG (1.25 μg/ml,(Wang et al., 1999b)); β galactosidase, Goat IgG (1:100, AbD Serotec); IL-34, polyclonal rabbit IgG (1.25 μg/ml, a gift from Five Prime Therapeutics, CA, USA); BrdU, mouse IgG_1 (1:100, Nova Castra); Iba1, polyclonal rabbit IgG (1:700, Wako Chemicals, USA); Pax6, polyclonal rabbit IgG (1:100, Milipore); Calbindin, polyclonal rabbit IgG (1:800, Milipore); Tbr1, polyclonal rabbit IgG (1:500, Abcam); CTIP2, rat IgG (1:500, Abcam); Satb2, mouse IgG_1 (1:500,

Abcam); Cux1, polyclonal rabbit IgG (1:100, Santa Cruz); Tbr2, polyclonal rabbit IgG (1:500, Abcam); MBP, mouse IgG_{2b} (1:250, R&D). Secondary antibodies used were conjugated to Fluorescein Isothiocyanate (FITC, 1:200), Tetramethyl Rhodamine Isothiocyanate (TRITC, 1:200) (Southern Biotechnology Associates, Inc., Al, USA), Alexa fluor-594 or Alexa fluor-647 (1:1500) (Molecular Probes, Invitrogen, oregon, USA).

BrdU incorporation study

Postnatal 20-day old (P20) wild type (*Wt*), *Csf1*$^{op/op}$ and *Csf1r−/−* mice were injected intraperitoneally with 100μl of 20 mg/ml BrdU in 154 mM NaCl, 7 mM NaOH and sacrificed 2 h later. For IHC, brain sections were pretreated with 2N HCl (1 h, 37°C) neutralized with 0.1 M sodium borate solution, pH 8.5 for 10 min, quenched with 0.1% sodium borohydride for 10 min at room temperature and then incubated with anti-BrdU antibody.

Neurosphere culture and clonal analysis

Neurospheres (progenitor clones) were generated from cortical SVZ of P2 FVB *Wt* mice (Charles River), as described previously (Zhu et al., 1999). Cells were plated at a density of 30,000 cells per well (3 ml of culture media per well) and maintained in the presence of 10 ng/ml of EGF (R&D, MN, USA) for 7 DIV in the presence or absence of 60 ng/ml of recombinant human CSF-1 (rhCSF-1, a gift from Chiron Corporation, Emeryville, CA). CSF-1 was added on the first and fourth DIV. Secondary clones were obtained by dissociation of the primary clones at 7 DIV that were propagated under similar conditions. To study the role of CSF-1 on progenitor differentiation, primary and secondary clones, at 7 DIV, were plated onto poly-D-lysine coated-coverslips in the presence of 1 ng/ml of recombinant human bFGF (BD Biosciences, MA, USA) and 2 μg/ml of laminin (BD Biosciences, MA, USA). The adherent NPC clones were fixed at 4 days and 7 days with 4% PFA, pH 7.4 (20 min, 37°C) and studied for the expression of specific lineage markers by immunofluorescence. To study the effects of CSF-1 and IL-34 on purified progenitors, P2 cortical SVZ-derived NPC clonal cultures from pooled *Wt*, *Nestin-GFP/+* and *Nestin-GFP/Nestin-GFP* brains were established and maintained in the presence of 10 ng/ml of EGF for 3 DIV, as described above. Cortical progenitor clones thus obtained were dissociated, sorted for GFP positivity using DakoMoflo XDP and 20,000 GFP+ cells were plated at 3 ml per well in 6–well tissue culture plates, in the presence of 80 ng/ml of either rhCSF-1 or mouse IL-34 (R&D, MN, USA), or both. CSF-1 and IL-34 were added on the first and fourth DIV. Secondary clones were generated as described above. To study the role of CSF-1 and IL-34 in progenitor differentiation, primary and secondary clones, at 7 DIV, were treated as described. Antibody dilution and isotype for immunocytochemistry: β tub-III, mouse IgG_{2b} (1:700, SIGMA); O4, mouse IgM (1:700, SIGMA); GFAP, mouse IgG_1 (1:700, SIGMA), CSF-1R (IK), goat IgG (1 μg/ml, (Wang et al., 1999b)), F4/80, rat IgG_{2b} (1:200, (Hume et al., 1983)). Secondary antibodies used were conjugated to TRITC (1:200) (Southern Biotechnology Associates, Inc., Al, USA), Alexa fluor-350, Alexa fluor-594, or Alexa fluor-647 (1:1500) (Molecular Probes, Invitrogen, Oregon, USA). The number and the area of the progenitor clones were measured using the Olympus MicroSuite™- Five Biological Suite software, NY, USA.

RNA isolation and RT-PCR

RNA from FACS purified GFP+ fraction derived from cortical progenitor clones obtained from P2 *Wt, Nestin-GFP/+* and *Nestin-GFP/Nestin-GFP* pups, following incubation with 10 ng/ml of EGF for 3 DIV and control RNA from L-cells (Stanley and Heard, 1977) and BAC1.2F5 macrophages (Morgan et al., 1987) was isolated using TRIzol reagent (Invitrogen, CA, USA) and RNeasy mini kit (QIAGEN, Maryland, USA) following manufacturer's instructions. Reverse transcription (RT)-PCR reactions were carried out

using SuperScript III One-step RT-PCR system with Platinum Taq DNA Polymerase (Invitrogen, CA, USA). Primers for RT-PCR:

CSF-1R: (5′-TGCTGGCCACAGTTTGGCATG-3′ and

5′-CTTTGACATACAAGTGGATGGT-3′; 279bp),

F4/80: (5′-ATGTGGGGCTTTTGGCTGCT-3′ and

′-TGAGTCACTTTGAAGACATT-3′; 270bp),

β-actin: (5′-CGTGGGCCGCCCTAGGCACCA-3′ and

5′-TTGGCCTTAGGGTTCAGGGGGG-3′; 243 bp).

IL-34 staining of 293T cells and F4/80 staining of BAC1.2F5 cells

293T cells either untransfected or transfected with mouse IL-34 cDNA were cultured in DMEM (GIBCO, NY, USA) with 10% fetal calf serum (FCS) in the absence or presence of 5 μg/ml of puromycin respectively for 20 hours on the poly-D-lysine-coated coverslips. They were fixed with 4% PFA, pH 7.4 (20 min, 37°C) and stained with either purified rabbit IgG or affinity-purified rabbit anti-IL-34 antibody (1 μg/ml, a gift from Five Prime Therapeutics, CA, USA). BAC1.2F5 cells were similarly cultured in α MEM with 10% FCS in the presence of 36 ng/ml of CSF-1 for 16 hours, fixed and stained with either rat IgG or, anti-F4/80 monoclonal antibody (1:200, (Hume et al., 1983)).

Statistical Analysis

Data were expressed as means ± standard deviations (SD) or means ± standard error of means (SEM). Student's *t* test (2 tailed) was used to test significance. Differences were considered statistically significant if comparison of data sets yielded P values of ≤0.05.

Results

Brain developmental abnormalities in *Csf1*$^{op/op}$ and *Csf1r*−/− mice

In contrast to the 40% survival rate of *Csf1*$^{op/op}$ FVB/NJ mice, *Csf1r*−/− FVB/NJ mice did not survive beyond one month of age (Dai et al., 2004a). Comparative analysis of P20 *Csf1*$^{op/op}$ and *Csf1r*−/− FVB/NJ mice revealed that, while both mutant brains are smaller in size (Fig. 1A), with a greater brain mass (Fig. 1D), than wild type brains, *Csf1r*−/− brains were smaller than *Csf1*$^{op/op}$ brains. The *Csf1r*−/− mice also displayed atrophy of the olfactory bulb (OB) (Fig. 1A, red arrows) and an expansion in the size of lateral ventricles (Fig. 1B, asterisk). Seventy-five percent of the *Csf1r*−/− mice had a thinner neocortex than wild type mice, with a general reduction in the thickness of all cortical layers (Fig. 1C). In contrast, *Csf1*$^{op/op}$ brains exhibited greater neocortical thickness than wild type brains (Fig. 1C). These observations suggest important roles for the CSF-1R in normal CNS homeostasis.

Temporal expression of IL-34, CSF-1 and the CSF-1R in neocortex

To visualize the expression profile of CSF-1 in developing brain, we employed a transgenic mouse *Csf1-LacZ* reporter line (*TgZ7*) that expresses a nuclear-localized β-galactosidase, under the control of the *Csf1* promoter and first intron (Fig. S1A) in a cellular pattern recapitulating expression of the endogenous *Csf1* gene (Ryan et al., 2001). β-galactosidase was detected by X-gal staining (Figs. S1B,C) and by immunochemistry employing an anti-β-galactosidase antibody. IL-34 and CSF-1R expression were detected using affinity-purified, specific polyclonal antibodies (Figs. S2, S3). Our initial analysis of CSF-1 reporter mice revealed that the *Csf1* promoter is active in the developing telencephalon and

cerebellum (Figs. S1 B,C). We further compared the temporal expression profiles of CSF-1R signaling molecules in the cortical forebrain by immunostaining of *TgZ7* brain sections with anti-β-galactosidase, anti-IL-34 and anti-CSF-1R antibodies.

CSF-1R is expressed on microglia-like cells in E11.5 brain (Ginhoux et al., 2010). In addition, *IL34* mRNA was present in the E11.5 telencephalon. However, we failed to detect any significant expression of either IL-34 (data not shown), or CSF-1 reporter protein at this stage (Fig. S1B). By E15.5, CSF-1 reporter expression appeared within the SVZ/VZ with IL-34 expression within the marginal zone and the cortical plate (Fig. 2A). At this stage, CSF-1R expression was observed throughout the dorsal telencephalon, including within the SVZ/VZ (Fig. 2A). Within the P2 neocortex, IL-34 was predominantly expressed in layer V and the meninges (Fig. 2A), consistent with the presence of microglia in the latter region (Boya et al., 1979; Perry et al., 1985). IL-34 expression was not detected on Cajal-Retzius cells (data not shown). In contrast, CSF-1 expression was detected solely in layer VI (Figs. S1B,2A). At P20, the expression of CSF-1 was similar, while IL-34 expression expanded into the upper layers (II–IV) (Figs. S1B, 2A). Interestingly, robust CSF-1R expression was observed in the P2 SVZ and in the meninges (Fig. 2A) and expression was reduced in the P20 generative zones with enhanced expression in the upper cortical layers (Fig. 2A).

Our expression analysis revealed that CSF-1R and its signaling ligands are maximally expressed during postnatal development in the cerebral cortex. To further identify the neural cell subtypes expressing these signaling proteins, sections of postnatal *TgZ7* cortices were stained for each of the CSF-1R signaling factors in combination with antibodies against different neural lineage markers. Within the P2 cortex, while CSF-1 reporter expression exhibited exclusive overlap with Tbr1 (Hevner et al., 2001), a marker present solely in mature neurons of layer VI, IL-34 was expressed on CTIP2 immunoreactive (Chen et al., 2008) neurons of layer V (Fig. 2B).

Interestingly, CSF-1R was expressed on a small subset of neural progenitors (Nestin+ cells) and some immature neurons (β-tubIII+ and NeuN-cells) (Fig. S4). Within the P20 cortex, while CSF-1 expression persisted on layer VI mature neurons, IL-34 expression was observed on Satb2 immunoreactive (Britanova et al., 2008) mature neurons of layers II–IV (Fig. 2B). At P20, CSF-1R expression was primarily restricted to microglia (Ginhoux et al., 2010).

Together, the co-localization studies and the temporal expression analysis indicate that IL-34 and CSF-1 exhibit complementary expression profiles in the developing cortex. While both ligands are expressed by a specific subset of mature cortical neurons, the CSF-1R, in addition to its expression on microglia, is also expressed on a small subset of dorsal forebrain progenitors.

CSF-1R suppresses the expansion of dorsal forebrain progenitors

To examine the role of CSF-1R signaling in neural progenitor expansion *in vivo*, we first performed immunofluorescence microscopy of the embryonic and postnatal neocortex of *Wt*, *Csf1*$^{op/op}$ and *Csf1r−/−* mice using neural progenitor and cellular proliferation markers (Figs. 3A–3C). There was an increase in the number of radial glia (Nestin+ and Pax6+) and basal (Tbr2+) progenitors in the E15.5 *Csf1r−/−* cortex (Fig. 3A). Within the generative zone of the lateral ventricle, from *Wt* through *Csf1*$^{op/op}$ to *Csf1r−/−* P20 brains, there was a graded increase in the number of neural progenitors (Nestin+ GFAP+) (Fig. 3B). In addition, BrdU pulse-labeling indicated that there was a graded enhancement of cellular proliferation in this region of the mutant brains (Fig. 3C).

To further investigate a possible inhibitory role of CSF-1R signaling in progenitor proliferation, we utilized *in vitro* clonal expansion assays. Initial neural differentiation paradigms that employ EGF-responsive neural progenitor clones derived from the P2 cortical SVZ of *Wt* mice revealed the presence of contaminating F4/80+ microglia (Hume et al., 1983) in these cultures (Fig. S10C). To generate microglia-free clones, we isolated EGF-responsive cortical SVZ neural progenitors from P2 *Nestin-GFP* mice (Mignone et al., 2004) (Fig. 3D). We expanded these cells in the presence of EGF for 3 days *in vitro* (DIV) and dissociated the resulting small EGF-responsive clones for FACS-purification of GFP+ cells. We then verified that the pooled GFP+ cells express mRNA for the CSF-1R, but not for F4/80 (Fig. 3E). *In vitro* clonal expansion analysis of these purified *Nestin-GFP* P2 cortical SVZ-derived neural progenitor cells (Fig. S5A) demonstrated that either CSF-1 or IL-34 was sufficient to inhibit the self-renewal (Fig. 3F), but not to promote the proliferation (Fig. 3G) of these neural progenitors. Together, our *in vivo* and *in vitro* results strongly suggest a direct role for CSF-1R-signaling in the suppression of dorsal forebrain progenitor expansion.

CSF-1R enhances neuronal differentiation of dorsal forebrain progenitors

To examine the role of CSF-1R signaling in neuronal differentiation *in vivo*, we performed immunofluorescence microscopy of the embryonic and postnatal neocortex of *Wt*, $Csf1^{op/op}$ and *Csf1r−/−* mice using markers that permitted analysis of the laminar distribution of mature excitatory neuronal subtypes (Figs. 4A,B). At E15.5, the *Csf1r−/−* cortex displayed a reduction in the number of Tbr1+ and CTIP2+ lower layer and Cux1+ upper layer neurons, while Satb2+ neurons in layers II–IV were increased (Fig. 4A). In P20, we observed a consistent reduction in CTIP2+ as well as Cux1+ neuronal subtypes in both $Csf1^{op/op}$ and *Csf1r−/−* mice and a reduction of Tbr1+ neurons only in $Csf1^{op/op}$ mice (Fig. 4B). Although Nissl staining of neocortex showed an apparently normal distribution of neurons in both mutants (Fig. 1C), excitatory neuronal subtype analyses revealed loss of layer-specific neuronal subtypes in both $Csf1^{op/op}$ and *Csf1r−/−*mice.

To further assess the role of CSF-1R signaling in neuronal differentiation, we utilized *in vitro* clonal differentiation assays using *Nestin-GFP* mice. Indeed, adherent GFP+ neural progenitor clones continued to express the CSF-1R in the absence of significant microglial contamination (Fig. 4C). The CSF-1R was expressed by protoplasmic astrocytes (Doetsch et al., 1999; Miller and Raff, 1984) and by neurons, but not by oligodendrocytes (Fig. 4C). *In vitro* clonal differentiation analysis further demonstrated that either CSF-1 or IL-34 facilitated the generation of bipotent neuronal/astroglial clones at the expense of unipotent astroglial clones (Figs. 4D, S5B). Co-application of CSF-1 and IL-34 resulted in a further increase in the generation of neuron-containing clones without corresponding changes in the generation of astrocyte-containing clones, independent of overall clonal lineage composition (Fig. 4D). Together, these *in vivo* and *in vitro* results indicate a direct role for CSF-1R-signaling in the enhancement of neuronal differentiation of dorsal forebrain progenitors.

CSF-1R promotes survival of neural progenitors and committed precursors

We next studied whether CSF-1R-signaling was associated with regulation of survival of distinct cortical cells. We examined the *Wt* and the mutant brains for active caspase-3 expression and TUNEL activity. Our results revealed an increase in the number of apoptotic cells in the E15.5 (Fig. 5A) and P20 (Fig. 5B) *Csf1r−/−* cortex and that the rate of apoptosis in *Csf1r−/−* cortex was comparable between these two developmental stages (Figs. 5A,B). Suggestive of a significant pro-survival role for IL-34, $Csf1^{op/op}$ cortex compared to *Csf1r−/−* cortex had significantly fewer apoptotic cells (Fig. 5B). Interestingly, the majority of the apoptotic cells in *Csf1r−/−* cortex resided in the SVZ/VZ at E15.5 and in the anterior SVZ at P20. To further determine the identities of apoptotic cells in the *Csf1r−/−* cortex, we employed immunofluorescence microscopy of P20 brain sections using active caspase-3 and

various neural developmental markers. Within the anterior SVZ, there was preferential co-localization of active capase-3 staining with markers of neural progenitors (Nestin, GFAP), significant association with neuronal and glial cell precursors (β-tub III, S100β, PDGFRα, O4), but absence of co-localization with markers of post-mitotic neurons (NeuN) and mature oligodendrocytes (APC) (Table 1). These findings suggest that CSF-1R signaling either directly promotes the survival of forebrain neural progenitors, or limits the differentiation potential of specific neuronal and glial lineages, the failure of which in the *Csf1r−/−* brains, would lead to their surplus production and subsequent pruning by a CSF-1R-independent mechanism. In both *Csf1*$^{op/op}$ and *Csf1r−/−* mice, we also observed the presence of apoptosis in the granule cell layers of the OB and the DG of the hippocampus, two areas associated with adult neurogenesis (Fig. S6). In both mouse mutants, the numbers of apoptotic cells observed in the OB were similar and significantly higher than in the SVZ and DG regions, reflecting a predominant pro-survival role for CSF-1 acting through the CSF-1R in OB.

The CSF-1R mediates crossing of axons through the midline in the corpus callosum

An important aspect of cortical development is the midline crossing of the corpus callosum. This requires an orchestrated development of radial glia that subsequently express GFAP and populate the cortical midline and indusium griseum (IG) by E14.5, prior to axonal routing (Kriegstein and Gotz, 2003; Shu et al., 2003; Shu and Richards, 2001; Silver et al., 1993). Our initial observations showed that the forebrain callosal commissures failed to cross and instead formed Probst bundles near the midline of 80% (n=9) of *Csf1r−/−* P20 forebrain cortex (Fig. 6A). In the E15.5 *Csf1r−/−* forebrain, there was increased apoptosis of GFAP+ cells with normal survival of neurons (Fig. 6B), suggesting that CSF-1R-mediated survival and development of GFAP+ cells could ensure a normal midline crossing of the axons. In addition to microglia (Fig. 6C, upper middle panel and box 1), a subset of these GFAP+ cells (Fig. 6C, upper right panel and box 2), also express the CSF-1R. Interestingly, commissural development occurs normally in 78.8% (n=7) of age-matched *Csf1*$^{op/op}$ brains (data not shown). This suggests a primary role of IL-34 in regulating this process. Consistent with this, IL-34 expression was observed in the IG, as well as along the dorsal midline of the E15.5 cortex (Fig. 6C, upper left panel). These observations demonstrate that CSF-1R-signaling facilitates midline crossing of the corpus callosum, in a non-cell autonomous manner, that is likely to be predominantly regulated by IL-34-dependent survival and development of GFAP+ cell populations at the dorsal midline of the embryonic brain.

The neonatal lethality and the forebrain phenotypes of *Csf1*$^{op/op}$ and *Csf1r −/−* mice are partially recapitulated in *Nes-Cre/+; Csf1r*$^{flox/flox}$ mice

To further assess its neural role, we selectively ablated the CSF-1R in Nestin-positive neural progenitors by mating *Csf1r*$^{flox/flox}$ (*Csf1r*$^{fl/fl}$) mice (Li et al., 2006), with mice expressing the Cre transgene driven by the rat *Nestin* promoter and 2nd intron (*Nes-Cre/+*) (Tronche et al., 1999). These crosses were on the C57BL/6 background. On this background, the *Csf1*$^{op/op}$ and *Csf1r−/−* phenotypes are more severe than on the FVB/NJ background. Both mutant progeny die between E18.5 and P2 and heterozygous *Csf1r+/−* progeny also have reduced survival (S.N., X-M.D. and E.R.S., unpublished observations). Progeny of matings of *Nes-Cre/+; Csf1r*$^{fl/+}$ with *Csf1r*$^{fl/fl}$ mice yielded normal Mendelian genetic ratios at E18.5, but significantly lower than expected numbers of *Nes-Cre/+; Csf1r*$^{fl/fl}$ and *Nes-Cre/+; Csf1r*$^{fl/+}$ progeny at P20 (Table 2).

Compared with *Csf1r*$^{fl/fl}$ mice, the majority of the *Nes-Cre/+; Csf1r*$^{fl/fl}$ mice that survived to P20 had a smaller brain size, but a similar brain mass (Fig. 7). They also possessed smaller forebrains (Fig. 7B), but exhibited variable neocortical thickness (data not shown). No abnormalities were observed in the midline crossing of the corpus callosum, in the size of

the ventricles (Fig. 7B) or the OB (Fig. 7A) (n=5). However, *Nes-Cre/+; Csf1r*$^{fl/fl}$ mice displayed an increase in the size of the forebrain progenitor pool (Fig. 8A) and elevated levels of forebrain cellular apoptosis (Fig. 8B). Thus the neonatal lethality, smaller brain size, enhanced forebrain progenitor proliferation and forebrain cellular apoptosis phenotypes of the *Nes-Cre/+; Csf1r*$^{fl/fl}$ mice partially recapitulated those of the *Csf1r−/−* and *Csf1*$^{op/op}$ mice (Table 3). However, in *Nes-Cre/+; Csf1r*$^{fl/fl}$ mice, some specific *Csf1r−/−* abnormalities (atrophy of the OB, enlarged lateral ventricle size and the failure of midline crossing of callosal axons) were not observed, while others (altered cortical thickness and impaired neuronal differentiation) were only partially recapitulated (Table 3).

Predominant expression of IL-34, reduced expression of CSF-1 and minimal expression of the CSF-1R in adult brain

We have previously shown that mRNAs for both *IL34* and *Csf1* are expressed in adult brain and that the expression of IL34 is approximately 10 times higher than CSF-1 expression (Wei et al., 2010). Interestingly, expression of Csf1r mRNA (Fig. 9A) and protein (Fig. 9B) were minimal in various adult (P60) brain regions, although transcript and protein expression were at significant levels during early postnatal (P8) development, in agreement with the previous observations (Michaelson et al., 1996). In the absence of CSF-1 reporter expression, IL-34 was expressed and occasionally co-expressed with the CSF-1R in various early postnatal (P8) forebrain regions (Fig. S7). In contrast, IL-34 protein was expressed in various adult brain regions (Fig. 9C), where we failed to detect significant expression of the CSF-1R (Fig. 9B). In addition, the CSF-1 reporter protein was not detected in most adult brain regions where IL-34 was expressed, except in the RMS and hippocampus (Fig. 9C). The persistently high levels of IL-34 in different areas of adult brain, in the absence of CSF-1R expression, suggest the existence of additional IL-34 receptors.

Discussion

Requirement of CSF-1R signaling in neural development

Comparative histological examination of brain regions in *Csf1r−/−* mice at P20, just prior to their demise, revealed relatively normal gross developmental anatomy. However, the reduced size and the greater mass of *Csf1r−/−* brains, the reduced thickness of the neocortex, the midline crossing defect in the corpus callosum, the atrophy of the OB and the expansion of the area of the lateral ventricles, suggest that CSF-1R signaling is essential for the developmental specification and early maturation of these brain regions. Consistent with our observations, olfactory deficits and hydrocephaly have recently been reported in *Csf1*$^{op/op}$ and *Csf1r−/−* mice respectively (Erblich et al., 2011). The early perinatal lethality and some of the *Csf1r−/−* abnormalities were observed in *Nes-Cre/+; Csf1r*$^{fl/fl}$ mice (Table 3), demonstrating that CSF-1R-signaling in neural progenitors partially contributes to CSF-1R-mediated neural developmental effects. Consistent with a direct action of CSF-1R ligands on neural progenitors, *in vitro* clonal expansion and differentiation assays, performed using early postnatal dorsal forebrain progenitors derived from *Nestin-GFP* mice, revealed suppressed progenitor self-renewal and enhanced neurogenesis mediated by each ligand, in the absence of the ancillary effects of microglia. Supporting these *in vitro* results, our *in vivo* observations of *Csf1*$^{op/op}$ and *Csf1r−/−* mice suggest that the size of postnatal cortical progenitor pools is regulated, in part, through the developmental effects of CSF-1R signaling on inhibition of neural progenitor cell proliferation and promotion of regional neurogenesis (Fig. S8B). Consistent with our results, expression of the CSF-1R has been previously reported on a subset of cortical, hippocampal, cerebellar and brain stem neurons (Wang et al., 1999a), including cerebellar Purkinje cells (Murase and Hayashi, 1998). In contrast, Erblich et al (2011) concluded, on the basis of studies using the transgenic CSF-1R reporter (*CSF-1R-EGFP* MacGreen (Sasmono et al., 2003)) mice, that the CSF-1R is solely

expressed by microglia. However, our anti-CSF-1R antibody that was formally demonstrated to be sensitive and highly specific, revealed CSF-1R expression in both microglial and neural cell types.

Our failure to completely recapitulate the *Csf1r−/−* mouse phenotype in the *Nes-Cre/+; Csf1r$^{fl/fl}$* mice (Table 3), that exhibit normal microglial development (Fig. S9), together with the evidence for a microglia-dependent role of the CSF-1R in oligodendroglial differentiation (Fig. S10), indicates that CSF-1R-signaling in microglia is required for normal CNS development. Indeed, it has been previously suggested that paracrine signaling originating from CSF-1R-expressing microglia is involved in the mediation of normal neural developmental processes (Papavasiliou et al., 1997).

Both *Csf1$^{op/op}$* and *Csf1r−/−* mice exhibit a failure of microglial homeostasis to different degrees (a 20% decrease in *Csf1$^{op/op}$* and a 100% decrease in *Csf1r−/−*), as well as subcortical oligodendrocyte and neocortical neuronal differentiation failures in both mutants. These cellular deficits could explain the smaller brains of these mutants. Although it is not clear why the brain mass of both mutants is increased, it may in part be explained by the reduced clearance of cell corpses due to the microglial deficiency.

Our observation of the increased thickness of the neocortex of *Csf1$^{op/op}$* mice and its decreased thickness in *Csf1r−/−* mice is surprising in view of the significantly stronger effect of CSF-1R deficiency in increasing the number of neural progenitors. It is possible that this difference is explained by the effects of microglia in the *Csf1$^{op/op}$* mice (absent in *Csf1r−/−* mice), despite our failure to observe greater excitatory neuronal differentiation in these mice compared with *Csf1r−/−* mice. Alternatively, CSF-1 could also act through another receptor in neural cells with actions opposing CSF-1R-mediated effects.

Distinct regional and cellular expression profiles of CSF-1 and IL-34

Analysis of the CSF-1 reporter mice showed that CSF-1 was predominantly restricted to mature neurons of the dorsal forebrain (Fig. 2B) and cerebellum (Fig. S11C) and that the CSF-1R is expressed by a small subset of forebrain progenitors (Fig. S4) and neurons of the cerebellar molecular layer and Purkinje cells, as well as microglia (Fig. S11C). Compared with CSF-1, IL-34 was similarly expressed by mature neurons, but located within distinct cortical laminae, as well as in meningeal cells. While IL-34 expression was limited to specific forebrain structures (neocortex, OB and striatum) (Figs. 2, 9C), CSF-1 expression was also observed in hindbrain regions (cerebellum) (Fig. S1B,C) and in the spinal cord (Wei et al., 2010). During embryogenesis, cerebellar CSF-1 expression was localized to the rhombic lip (Fig. S11B), the region that gives rise to excitatory glutamatergic and granule neurons (Carletti and Rossi, 2008; Hatten et al., 1997). During postnatal development, CSF-1 was expressed in layer VI of the neocortex, where excitatory neurons reside, whereas IL-34 was expressed in layers II–V of the neocortex, where both excitatory as well as inhibitory neurons and mature glial cell subtypes are found (Nadarajah et al., 2003), as well as the meninges. Together, these data suggest that CSF-1 and IL-34 have complementary and non-redundant functions in developing and adult CNS.

The complementary expression profiles of IL-34 and CSF-1 are consistent with the requirement of CSF-1R signaling for the development and maintenance of microglia

Microglia are macrophages that are broadly distributed throughout the central nervous system, constitute a first line of immunological defense and are activated in response to inflammation (Altman, 1994; Dickson et al., 1993). Recently, we showed that adult microglia derive predominantly from primitive extra-embryonic progenitors that are recruited to the brain primordia after development of the embryonic circulation at E9.5

(Ginhoux et al., 2010). In that study, we confirmed the partial reduction in the number of microglia (~20%) in *Csf1*$^{op/op}$ brains (Kondo et al., 2007; Wegiel et al., 1998) and showed that *Csf1r*−/− brains are completely devoid of microglia. These findings, coupled with our current and earlier (Wei et al., 2010) observations of the complementary and broad expression of IL-34 and CSF-1, are consistent with regulation of microglia by both factors, as their expression profiles are maintained in the adult brain. Interestingly, there was a higher frequency of amoeboid microglia in *Wt* compared with *Csf1*$^{op/op}$ brains (Fig. S12), suggesting that CSF-1 may be important for microglial activation. Microglial activation has been implicated in neurodegenerative diseases (Paresce et al., 1996). In addition, the phagocytic activity of microglia has been implicated in the removal of apoptotic cells during development as well as in disease states (Magnus et al., 2002; Takahashi et al., 2005) and the absence of microglia may, in part, contribute to the increase in apoptotic cells we observed in *Csf1r*−/− brains in the present study. How such CSF-1R signaling in microglia might contribute to paracrine regulation of normal brain development is not presently clear. However, microglia-derived soluble factors, including IL-1, IL-6 and insulin-like growth factor-1, have been reported to modulate the survival and behavior of diverse neural cell types (Choi et al., 2008; Lalancette-Hebert et al., 2007; Nakanishi et al., 2007).

CSF-1R-independent roles of IL-34 and CSF-1

Consistent with the previous demonstration that *IL34* mRNA is expressed at higher levels during brain development than either *Csf1* or *Csf1r* mRNA (Wei et al., 2010), we have shown that IL-34 protein is persistently and broadly expressed during development and in the adult brain. IL-34 and CSF-1 display infrequent co-expression patterns in adult brain, whereas CSF-1R expression is markedly reduced (Fig.S8A). Also, as mentioned above, our analyses of the mutant brains showed that the absence of CSF-1 results in larger forebrain size with increased cortical thickness, abnormalities distinct from CSF-1R-deficiencies. Thus the differential pattern of expression of the CSF-1R and its ligands, together with the unique *Csf1*$^{op/op}$ phenotype, could reflect the use of additional receptors by IL-34 and CSF-1.

CSF-1R signaling in neural progenitors

In hematopoiesis, CSF-1R signaling instructs macrophage lineage choice (Rieger et al., 2009) and regulates mononuclear phagocyte survival, proliferation and differentiation (reviewed in (Pixley and Stanley, 2004) and may stimulate myeloid lineage commitment (Sarrazin et al., 2009; Stanley, 2009). CSF-1R-mediated processes affecting developing and possibly mature forebrain neural progenitor pools involve inhibition of cellular expansion, while ensuring proper selective neuronal and glial cell differentiation. Thus CSF-1R-mediated differentiation of neural progenitor cells appears to be somewhat analogous to CSF-1R action on granulocyte/macrophage progenitors (Rieger et al., 2009), where CSF-1 commits cells to a macrophage fate. Moreover, while this lineage commitment results in less proliferation during myeloid expansion, the inhibition of more differentiated myeloid cell proliferation by CSF-1 has not been reported. However, CSF-1R signaling has been shown to induce G1 cell cycle arrest by the coordinate up-regulation of the cyclin-dependent kinase inhibitor, p21$^{cip1/waf1}$ and cyclin D1 in MCF-7 human breast cancer cells (Lee et al., 1999). Furthermore, c-myb, a *Csf1r* transcriptional repressor (Reddy et al., 1994), facilitates neural progenitor proliferation by up regulation of the transcription factors, Pax-6 and Sox-2 (Malaterre et al., 2008). As Pax-6 and Sox-2 assist in maintaining neural progenitor cell pools by inhibiting progenitor differentiation (Estivill-Torrus et al., 2002; Graham et al., 2003), it is possible that these transcription factors are targets of CSF-1R signaling.

Conclusion

Previous studies have implicated CSF-1 in the regulation and maintenance of microglia (Sawada et al., 1990; Suzumura et al., 1990) and suggested that CSF-1 might directly regulate neural cells (Michaelson et al., 1996; Wang et al., 1999a). We have recently shown that microglia are established in early embryogenesis and that their lineage elaboration and maintenance are absolutely dependent on CSF-1R signaling (Ginhoux et al., 2010). Together with our present finding of the broad and complementary regional and local expression profiles of IL-34 and CSF-1 and our earlier demonstration of high levels of expression of IL34 mRNA in developing and adult brain (Wei et al., 2010), these studies implicate IL-34, as well as CSF-1 in CNS microglia regulation. In contrast, the focus of the present study has been on the nature and significance of expression of the CSF-1R on neural progenitors. We have shown that the CSF-1R, via the action of its two known ligands, plays a very significant and direct role in the regulation of progenitor cell proliferation and differentiation. That the direct regulation of neural progenitors through the CSF-1R is biologically significant has been demonstrated by the perinatal lethality of the *Nes-Cre/+; Csf1r*$^{fl/fl}$ mice, which phenocopies the perinatal death of *Csf1r−/−* mice. Similar to its different roles in the commitment and differentiation to the myeloid lineage and macrophages, CSF-1R signaling is also involved in regulation of the proliferation, differentiation and survival of neural progenitor cells. Relevant to our study, dominant point mutations in the *Csf1r* gene have recently been shown to cause hereditary diffuse leukoencephalopathy, a disease primarily affecting the subcortical white matter tracts (Rademakers et al., 2011).

Among many remaining questions to be addressed are: How do the non-overlapping regional expression profiles of IL-34 and CSF-1 contribute differentially to neural development? Do IL-34 and CSF-1 instruct lineage commitment, as observed for CSF-1 action on bipotential granulocyte/macrophage progenitors? Is IL-34 required for the generation and maintenance of a majority of the CNS microglia? What is the nature of CSF-1 action on microglia in brain? How do these cytokines contribute to regulation of migration of microglial progenitors from the yolk sac to the primordial brain and of neural progenitors and neurons in the developing cerebral cortex and RMS? Irrespective of the answers to these questions, the present study demonstrates that CSF-1 and IL-34 play important roles in the regulation of diverse neural cell types in brain.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- Detailed CNS expression pattern of CSF-1, IL-34 and the CSF-1R
- Expression of the CSF-1R on neural progenitor cells (NPC)
- Altered proliferation, survival and differentiation of NPC in Csf1r−/− mice
- Direct regulation of neurogenesis by CSF-1 or IL-34 *in vitro*
- Increased lethality & brain abnormalities in *Nestin-Cre/+; Csf1r*$^{flox/flox}$ mice

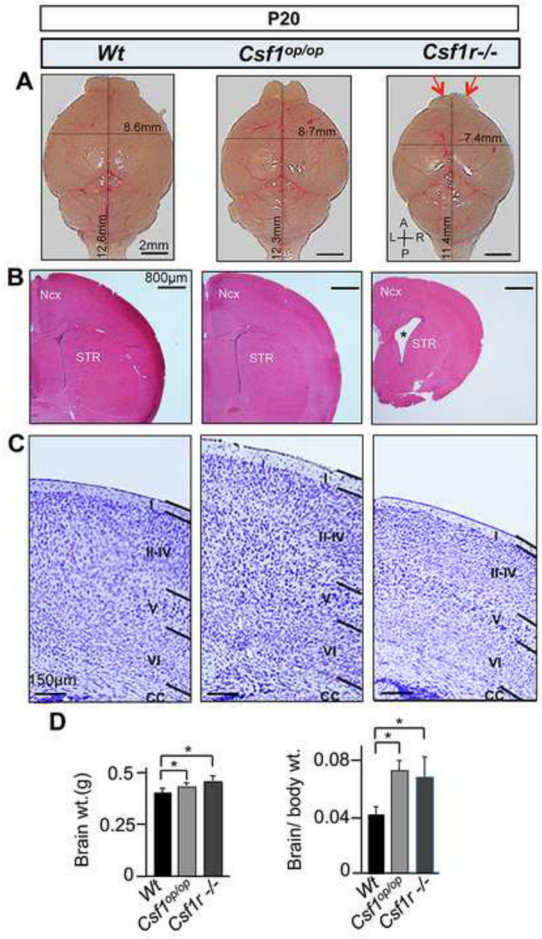

Fig. 1.

Gross anatomical and histological alterations in P20 *Csf1*$^{op/op}$ and *Csf1r−/−* brains. (A) A graded reduction in brain size along the A/P axis, from *Csf1*$^{op/op}$ to *Csf1r−/−* mice, with specific atrophy of the OB (red arrows) and a reduction in brain size along the mediolateral (L/R) axis in the *Csf1r−/−* mice (upper panels). (B) Coronal sections stained with hematoxylin and eosin (H&E), showing normal patterning but decrease in forebrain size of the *Csf1r−/−* mice. Asterisk indicates the increased size of the lateral ventricles in *Csf1r−/−* mice. (C) Nissl staining showing a normal laminar patterns, but an increase in thickness of neocortex (layers I–IV) in *Csf1*$^{op/op}$ mice and a reduction in thickness of the *Csf1r−/−* neocortex (all layers). (D) Whole brain weights (left panel) and brain to body weight ratios (right panel). *, $p<0.01$. $n \geq 5$ per condition and mutant model. Ncx, neocortex; STR, striatum; A/P anterio-posterior; L/R, left-right; CC, corpus callosum; OB, olfactory bulb.

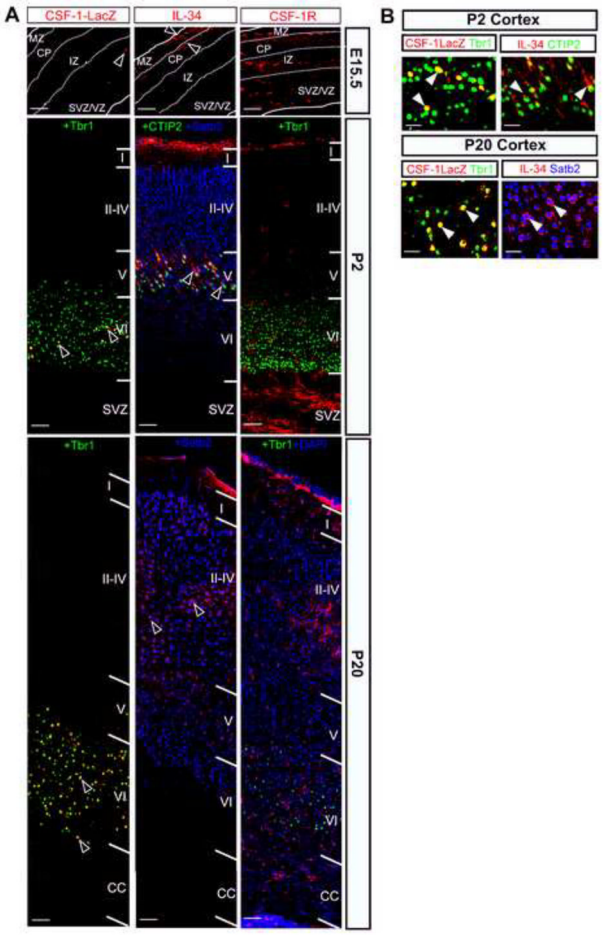

Fig. 2.

Complementary expression of CSF-1 and IL-34 in the *TgZ* neocortex. (A) Temporal pattern of expression: E15.5: While the CSF-1 reporter is expressed in the SVZ/VZ, IL-34 is expressed in the MZ and in the CP. CSF-1R is expressed throughout the dorsal neocortex. P2: Complementary expression of the CSF-1 reporter (in layer VI) and IL-34 (in layer V and meninges) in distinct cortical laminar patterns. Strong CSF-1R staining is apparent in the SVZ and meninges, with lower expression levels in the cortical layers. P20: CSF-1 reporter and IL-34 expression patterns are similar to those observed at P2 but IL-34 expression expands to upper layers (in layers II–V). Note the decline of CSF-1R expression in the developing CC and its increased expression in the upper cortical layers that also strongly express IL-34. (B) Cellular expression profiles of IL-34 and CSF-1 in postnatal (P2 and P20) neocortex of the *TgZ* mouse. Blown-up images from (A) showing CSF-1 reporter-LacZ and IL-34 co-staining with markers: Tbr1 (layer VI-specific postmitotic neurons); CTIP2 (layer V-specific postmitotic neurons) and Satb2 (layers II–IV-specific postmitotic neurons). Arrowheads indicate overlap of staining profiles. CC, corpus callosum; VZ, ventricular zone; SVZ, sub-ventricular zone; CP, cortical plate; MZ, marginal zone; IZ, intermediate zone.

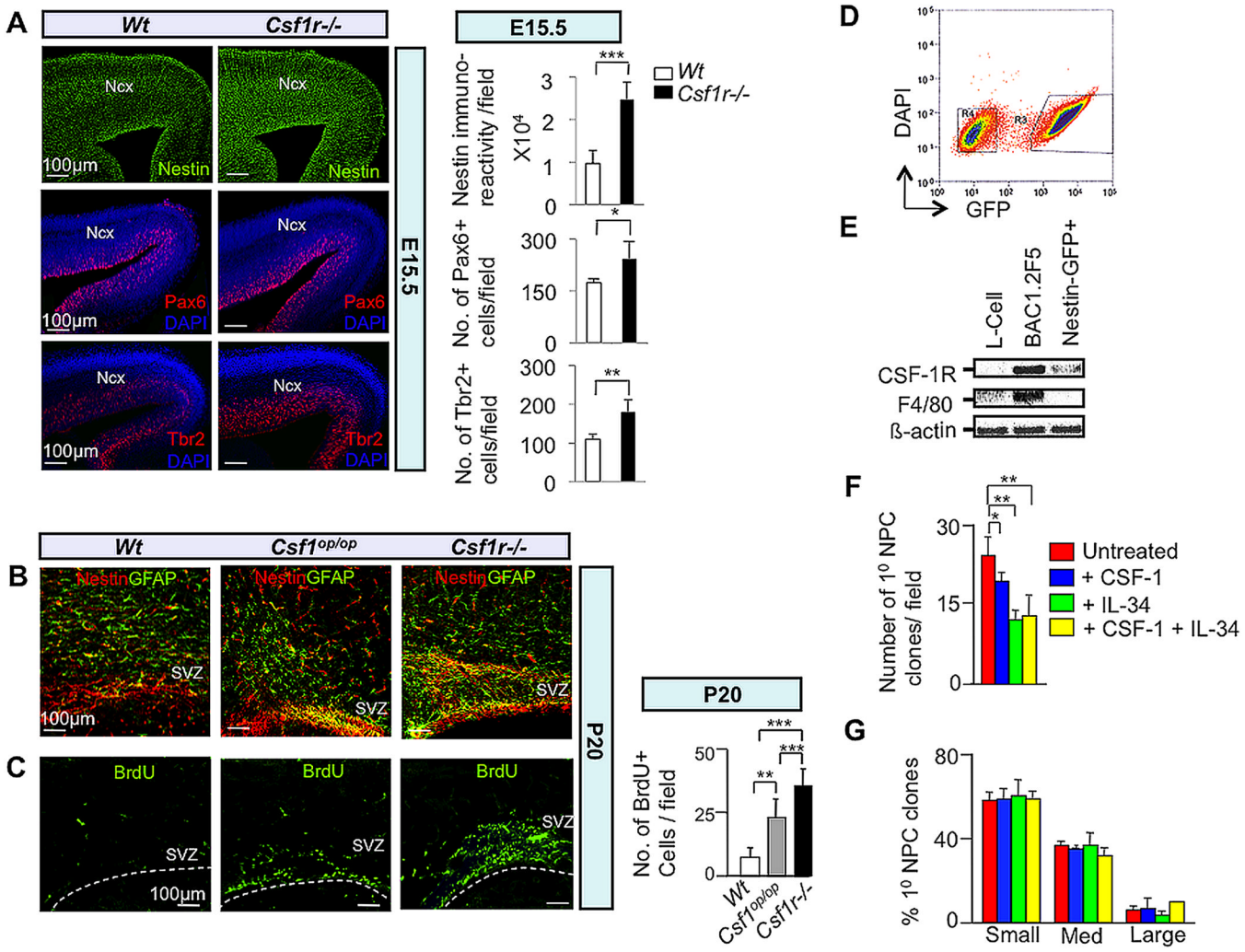

Fig. 3.

The CSF-1R directly mediates suppression of dorsal forebrain progenitor proliferation/self-renewal. (A–C) *In vivo* studies: Immunofluorescence microscopy of coronal sections of E15.5 (A) and P20 (B,C) *Wt* and mutant neocortex. (A) Nestin, Pax6 and Tbr2, (B) Nestin-GFAP double and (C) BrdU immunostaining. Quantification= cells/field. Mean ± SD of four representative fields per genotype. n=3: *, P<0.05; **, P<0.01 and ***, P< 0.001. Ncx, neocortex; SVZ, subventricular zone. Dotted lines in (C) delineate the ventricular lining. (D–G) *In vitro* studies: (D) FACS purification of cerebral cortical GFP+ progenitors from P2 *Nestin-GFP* transgenic pups. Cells were cultured in the absence of CSF-1 for 3 days and subjected to FACS. Cells gated in region R3 were used to set up neurosphere cultures. (E) Total RNA isolated from the GFP+ fraction was subjected to RT-PCR for assessment of *Csf1r* and *F4/80* (Hume et al., 1983) mRNAs. RNA from L-cells and BAC1.2F5 macrophages, respectively, represent negative and positive controls for *F4/80* and *Csf1r* mRNA expression. (F) FACS-purified GFP+ cells incubated in the presence of EGF and combinations of CSF-1 and IL-34 for 7 DIV. Reduced numbers of primary progenitor clones following incubation with CSF-1 and/or IL-34 for 7 DIV. (G) Failure of CSF-1 or IL-34 to affect the generation of small, medium or large-sized primary clones after 7 DIV. Clonal sizes: Small, 0.5–2.0 mm^2; medium, 2.0–6.0 mm^2; large, ≥ 6.0 mm^2. Mean ± SEM of 16

different representative fields from three independent experiments between untreated and CSF-1, IL-34 and CSF-1+IL-34 treated conditions. *P<0.05, **P<0.01 and ***P<0.001.

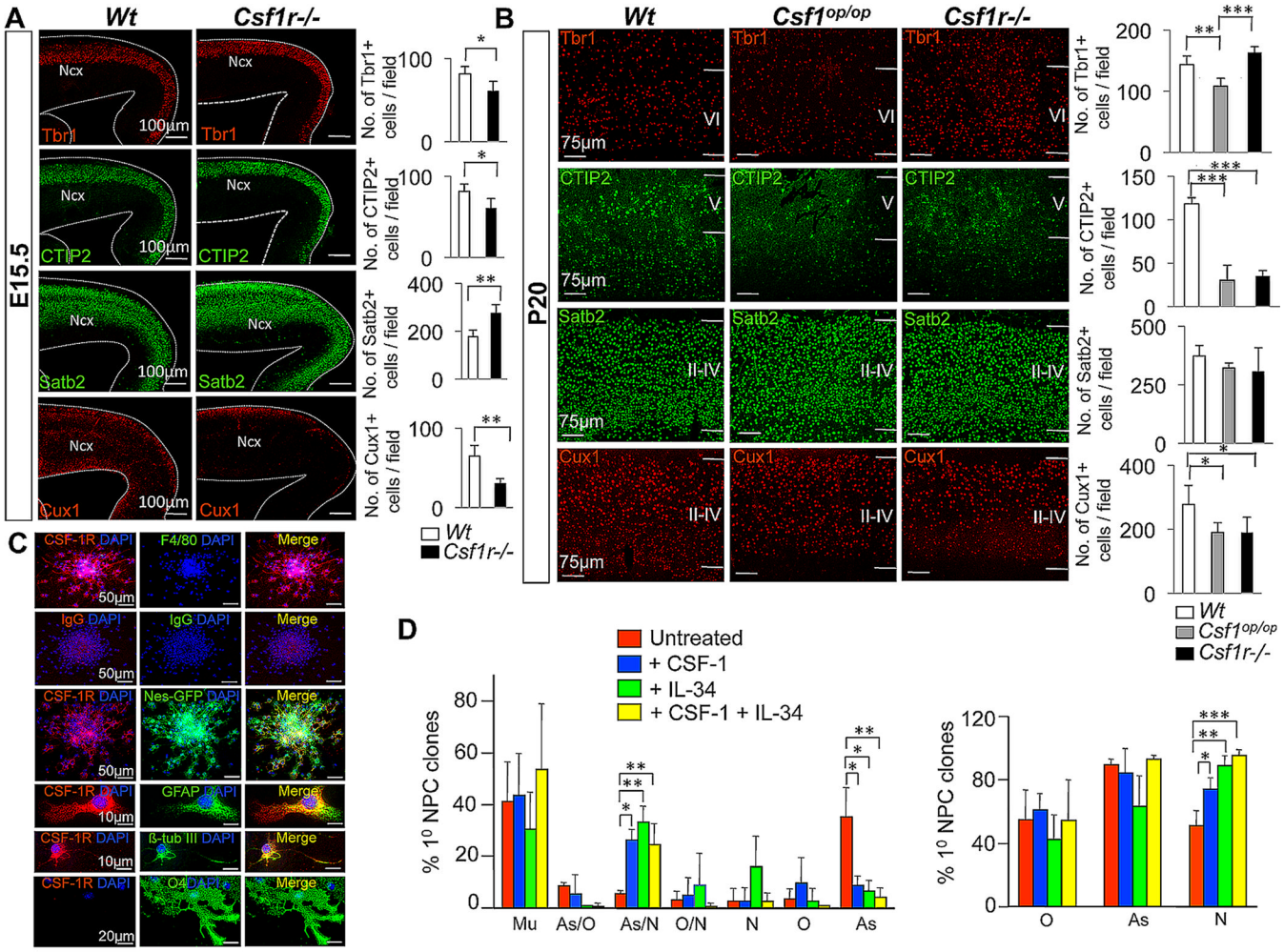

Fig. 4.

The CSF-1R directly enhances neuronal differentiation of dorsal forebrain progenitors. (A,B) *In vivo* studies: Immunofluorescence microscopy of coronal sections of E15.5 (A) and P20 (B) *Wt* and mutant neocortex. Tbr1, CTIP2, Satb2 and Cux1 immunostaining. Quantification, cells/field. Mean ± SD of four representative fields per genotype. n=3: Ncx, neocortex. *P<0.05, **P<0.01 and ***P<0.001. (C,D) *In vitro* studies: Adherent GFP+ primary cortical progenitor clones (generated under proliferation conditions, with EGF only, for 7 DIV, as described in Fig. 3D) incubated under differentiation conditions for 4 DIV. (C) Expression of the CSF-1R and the absence of F4/80 immunoreactivity (upper panels). Control IgGs for the CSF-1R and F4/80 antibodies indicate specificity (second row of panels). Three lower rows: Overlap of CSF-1R staining with GFP, GFAP and β tub-III. (D) Adherent GFP+ clones generated in the presence of EGF and combinations of CSF-1 and IL-34 for 7 DIV, then subsequently incubated under differentiation conditions for 4 DIV. Left panel: Percentage of primary progenitor clones that were multipotent (Mu) (β tub-III+/GFAP+/O4+), bipotent (GFAP+/O4+ (As/O), β tub-III+/GFAP+ (As/N) and β tub-III+/O4+ (O/N)) and unipotent (O4+ (O), β tub-III+ (N), or GFAP+ (As)). CSF-1 or IL-34 facilitated the generation of astrocyte/neuron (As/N) bipotent clones at the expense of unipotent astrocyte clones (As). Right panel: Percentage of primary progenitor clones containing β tub-III+ neurons (N), GFAP+ astrocytes (As) and O4+ oligodendrocytes (O). Mean ± SEM

of 16 different representative fields from three independent experiments between untreated and CSF-1, IL-34 and CSF-1+IL-34 treated conditions. *P<0.05, **P<0.01 and ***P<0.001.

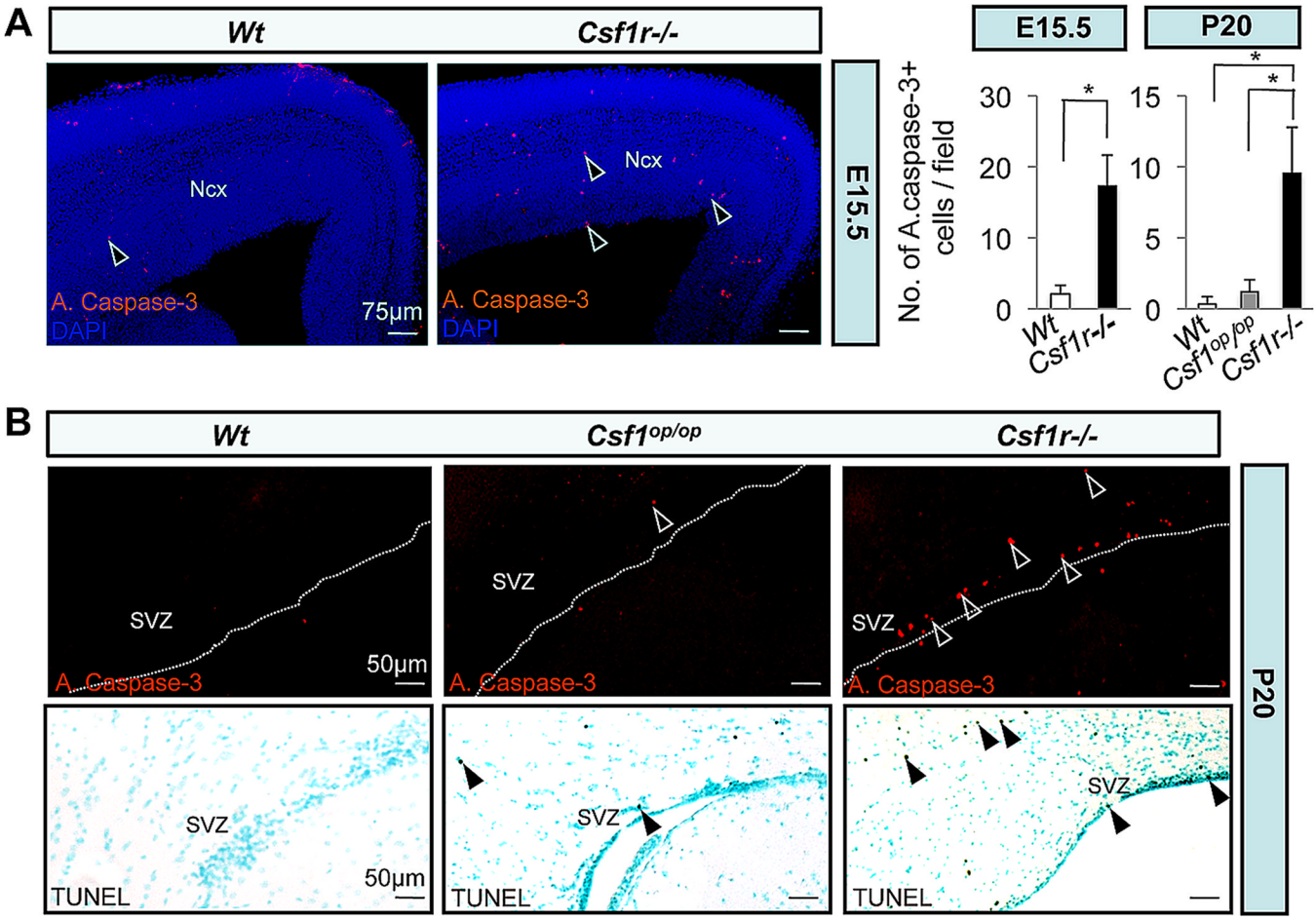

Fig. 5.

Cellular apoptosis in the developing *Csf1r−/−* neocortex. (A, B) Photomicrographs of active caspase-3+ (red dots) (A) and (B, upper panels) as well as TUNEL+ (brown dots) (B, lower panels) apoptotic cells in the SVZ/VZ region of E15.5 (A) and P20 (B) *Wt* and mutant mice. Arrowheads in (A, B) indicate apoptotic cells. Counterstained with DAPI (A) and hematoxylin (B). Dotted lines in (B) delineate the ventricular lining. Quantitation of the number of active caspase-3+ apoptotic cells/field. Means ± SD of ten different representative low-power (20X) fields per region per genotype from three different mice per genotype; *, P<0.001.

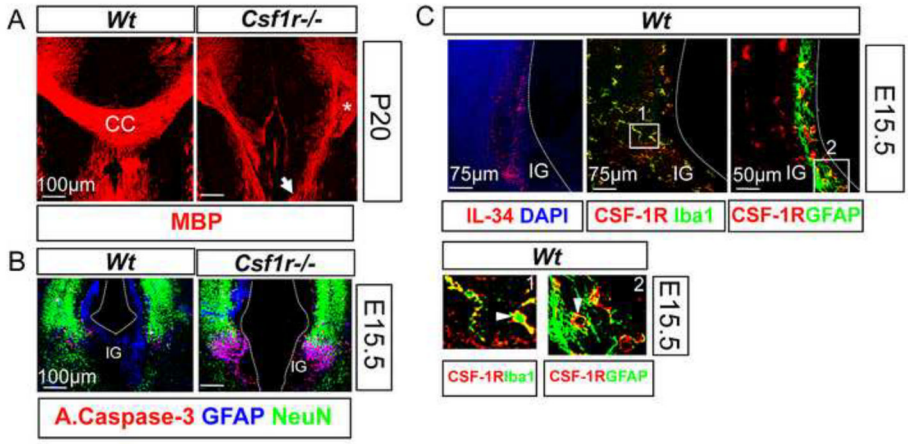

Fig. 6.

Abnormalities of midline crossing of the corpus callosum in the *Csf1r−/−* mice. (A) P20 forebrain sections immunostained for MBP, showing the failure of the callosal axons to cross the midline in the *Csf1r−/−* brains (white arrow). Asterisk indicates the formation of the Probst bundles (P). (B) Apoptosis of GFAP+ cells in the IG and along the midline of E15.5 *Csf1r−/−* dorsal forebrains. Overlap of active caspase-3 (red) with GFAP (blue) staining, but not with NeuN (green) staining. (C) Expression of IL-34 and the CSF-1R at the midline and the IG of E15.5 dorsal forebrains. Upper panels: Immunostaining reveals the expression of IL-34 (red, left) and the CSF-1R (red, middle and right). Note the presence of CSF-1R+(red) Iba1+(green) microglia (middle). A subset of GFAP+ (green) population also expresses the CSF-1R (red, right). Lower panels: Insets 1 and 2 from upper panels. Arrows indicate overlap of CSF-1R staining with Iba1 and GFAP staining. Dotted line indicates the contour of each hemisphere. CC, corpus callosum; IG, indusium griseum.

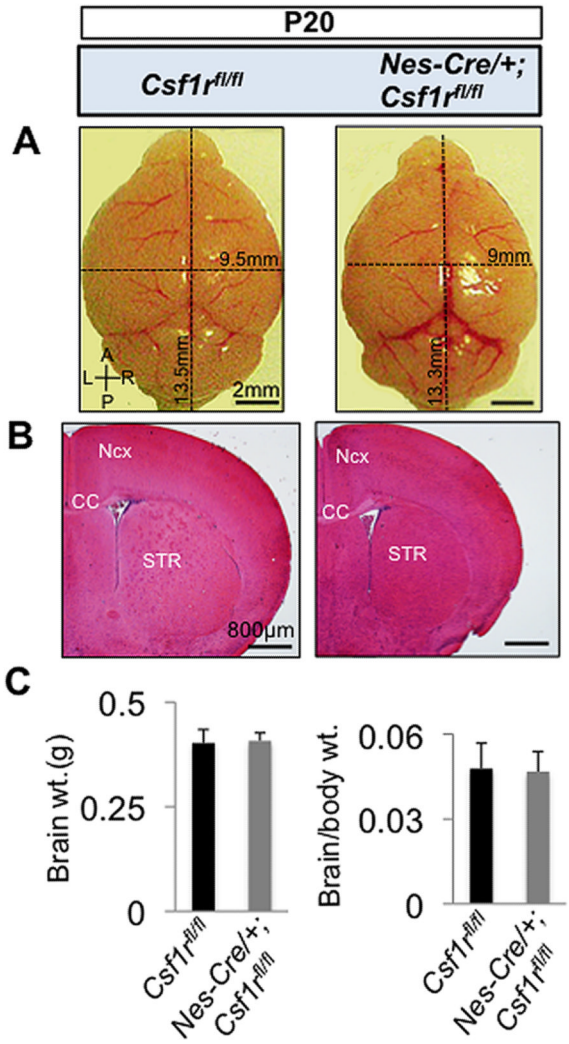

Fig. 7.

Gross anatomical and histological abnormalities in P20 *Nes-Cre/+; Csf1r*$^{fl/fl}$ brains. (A) Reduction in brain size along the A/P and the L/R axes, but normal development of the OB in *Nes-Cre/+; Csf1r*$^{fl/fl}$ mice. (B) Coronal sections stained with hematoxylin and eosin (H&E), showing normal patterning, but a decrease in the forebrain size in *Nes-Cre/+; Csf1r*$^{fl/fl}$ mice. Note a normal ventricular size and midline crossing of the CC in *Nes-Cre/+; Csf1r*$^{fl/fl}$ mice. (C) Whole brain weight (left panel) and brain to body weight ratios (right panel). n = 5 mice per group. Ncx, neocortex; STR, striatum; A/P anterio-posterior; L/R, left-right; CC, corpus callosum.

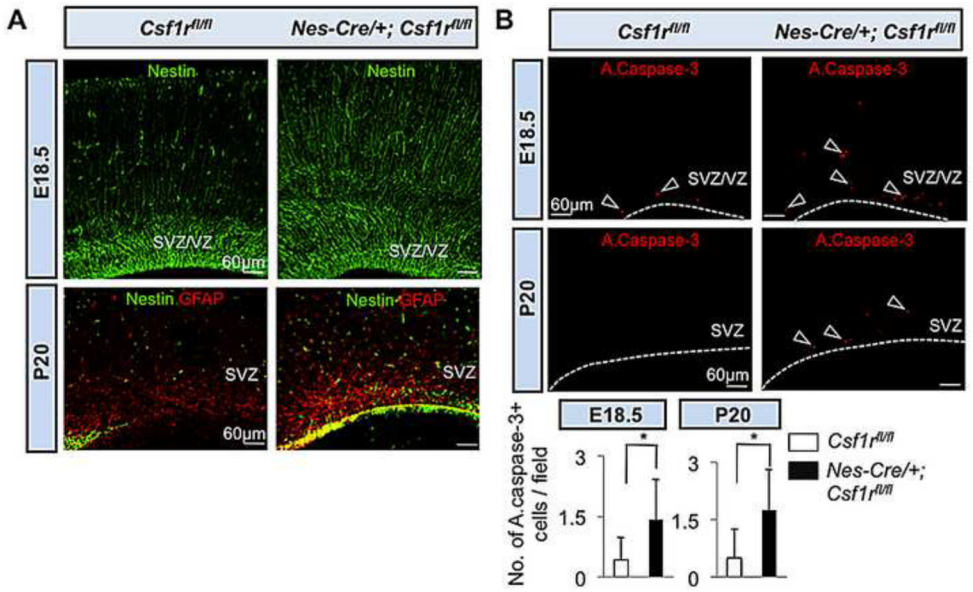

Fig. 8.

Expansion of forebrain progenitor pools and enhanced cellular apoptosis in *Nes-Cre/+; Csf1r*$^{fl/fl}$ mice. (A) Immunofluorescence microscopy of coronal sections of E18.5 (upper panels) and P20 (lower panels) *Wt* and mutant neocortex. Upper panels, Nestin. Lower panels, Nestin-GFAP double immunostaining. (B) Photomicrographs of active caspase-3+ (red) apoptotic cells in the SVZ/VZ region of E18.5 (upper panels) and SVZ region of P20 (middle panels) *Wt* and mutant mice. Arrowheads indicate apoptotic cells. Dotted lines in (B) delineate the ventricular border. Lower panels: Quantitation of the number of active caspase-3+ apoptotic cells/field. Means ± SD of eight representative low-power (20X) fields per region per genotype from two different mice per genotype; *, P<0.01.

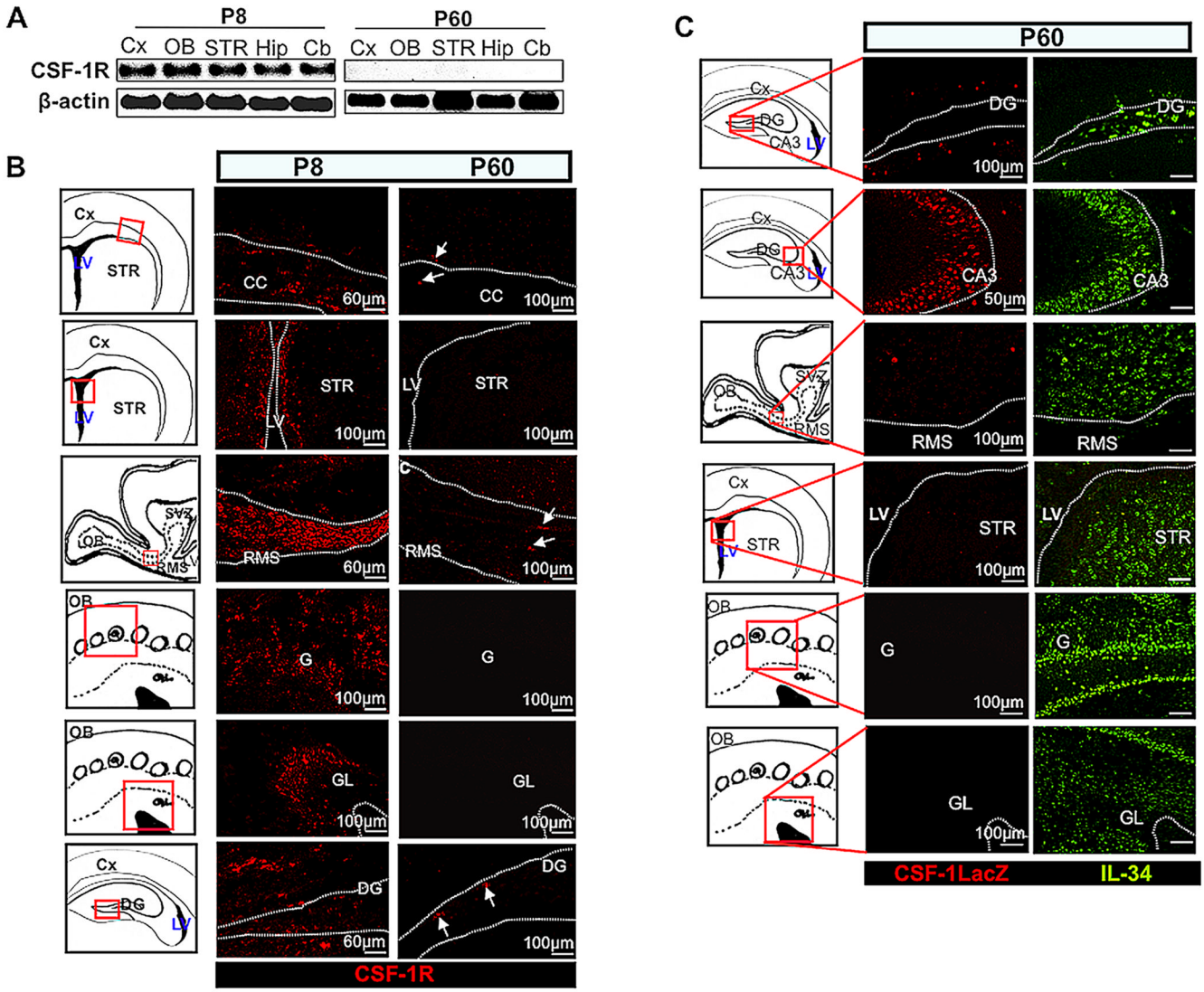

Fig. 9.

Expression of IL-34, CSF-1 and the CSF-1R in the adult brain. (A,B) Decline in the level of CSF-1R expression in adult brain. (A) Semi-quantitative RT-PCR showing relative abundance of Csf1r mRNA from various P8 and P60 brain regions. (B) CSF-1R (red) immunostaining of various P8 and P60 *Wt* brain regions. (C) Exclusive expression of IL-34 as well as regional co-expression with CSF-1 in adult brains. Immunostaining of P60 *TgZ* brain sections showing expression of IL-34 (green) and CSF-1 reporter (red). Dotted lines delineate the contour of the structures. Arrows in (B) indicate a few CSF-1R+ cells in P60 brains. OB, olfactory bulb; G, glomerulus; GL, granule cell layer; CC, corpus callosum; Cx, cerebral cortex; LV, lateral ventricles; SVZ, subventricular zone; DG, dentate gyrus; RMS, rostral migratory stream; STR, striatum; CA3, CA3 region of hippocampus; hip, hippocampus; Cb, cerebellum.

Table 1

Distribution of neural cell types among active caspase-3+ cells in the anterior SVZ of P20 *Csf1r−/−* mice

Neural cell-specific marker	% double + cells
Nestin	69.1 (29/42) *
GFAP	40.0 (22/55)
β-tub III	45.8 (22/48)
S100β	19.5 (16/82)
PDGFRα	33.3 (27/81)
O4	27.8 (27/97)
NeuN	0.0 (0/50)
APC	0.0 (0/50)

* Number of double positive cells over the total number of active caspase-3+ cells. Average of five different low-power (10X) fields per region per neural lineage marker.

Table 2

Chi square analysis of progeny genotypes from the cross between *Nes-Cre/+; Csf1r$^{fl/+}$* and *Csf1r$^{fl/fl}$* mice

Cross	**Progeny**							
Nes-Cre/+; Csf1r$^{fl/+}$ X Csf1r$^{fl/fl}$	***Nes-Cre/+***		***+/+***		**Number of progeny**	**Number of litters**	***χ^2**	**P**
	Csf1r $^{fl/+}$	***Csf1r $^{fl/fl}$***	***Csf1r $^{fl/+}$***	***Csf1r $^{fl/fl}$***				
Age	**O/E**	**O/E**	**O/E**	**O/E**				
P20	18/22	12/22	24/22	20/22	74	12	5.26	0.02
E18.5	16/11	13/11	8/11	12/11	49	6	1.38	0.10

*, Only *Nes-Cre/+; Csf1r $^{fl/+}$* and *Nes-Cre/+; Csf1r$^{fl/fl}$* mice were considered for χ^2 analysis (df = 1).

O, Observed; E, Expected.

Table 3

Brain phenotypes of *Csf1*$^{op/op}$, *Csf1r−/−* and *Nes-Cre/+; Csf1r*$^{fl/fl}$ mice compared to those of wild type mice.

	Mouse Mutant		
Phenotype	***Csf1*$^{op/op}$**	***Csf1r−/−***	***Nes-Cre/+; Csf1r*$^{fl/fl}$**
Brain size	Reduced	Reduced	Reduced
Brain mass	Increased	Increased	Unchanged
OB atrophy	Absent	Present	Absent
Ventricular size	Unchanged	Increased	Unchanged
CC midline crossing defect	Present (22%*)	Present (80%*)	Absent
Cortical thickness	Increased	Reduced	Variable
Cortical NPC proliferation	Increased	Increased	Increased
Excitatory neuronal differentiation	Reduced	Reduced	Variable
Sub-cortical OL differentiation	Reduced	Reduced	Unchanged
Cortical cellular apoptosis	Unchanged	Increased	Increased
Cortical microglia	Reduced	Absent	Unchanged

OB, olfactory bulb; CC, corpus callosum; NPC, neural progenitor cell; OL, oligodendrocyte;

*, penetrance