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Transient Maternal IL-6 Mediates Long-Lasting Changes in Neural Stem Cell Pools by Deregulating an Endogenous Self-Renewal Pathway

Denis Gallagher,[1,2,3] Andreea A. Norman,[1,8] Cameron L. Woodard,[1] Guang Yang,[1] Andrée Gauthier-Fisher,[1,2,9] Masashi Fujitani,[1,2,10] John P. Vessey,[1,2] Gonzalo I. Cancino,[1,2] Nadia Sachewsky,[4] Knut Woltjen,[3,11] Michael P. Fatt,[1,2,4] Cindi M. Morshead,[4,7] David R. Kaplan,[2,4,5] and Freda D. Miller[1,3,4,5,6,*]
[1]Program in Developmental and Stem Cell Biology
[2]Program in Cell Biology
The Hospital for Sick Children, Toronto, ON, M5G 1L7, Canada
[3]McEwen Center for Regenerative Medicine, University Health Network, Toronto, ON, M5G 1L7, Canada
[4]Institute for Medical Sciences
[5]Department of Molecular Genetics
[6]Department of Physiology
[7]Department of Surgery
University of Toronto, Toronto, ON, M5G 1X5, Canada
[8]Present address: Center for Commercialization of Regenerative Medicine, Toronto, ON, M5G 1L5, Canada
[9]Present address: CReATe Fertility Centre, Toronto, ON, M5G 1N8, Canada
[10]Present address: Department of Molecular Neuroscience, Graduate School of Medicine, Osaka University, Osaka 565-0871, Japan
[11]Present address: Center for iPS Cell Research and Application, Kyoto University, 53 Kawahara-cho, Shogoin, Sakyo-ku, Kyoto 606-8507, Japan
*Correspondence: fredam@sickkids.ca
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SUMMARY

The mechanisms that regulate the establishment of adult stem cell pools during normal and perturbed mammalian development are still largely unknown. Here, we asked whether a maternal cytokine surge, which occurs during human maternal infections and has been implicated in cognitive disorders, might have long-lasting consequences for neural stem cell pools in adult progeny. We show that transient, maternally administered interleukin-6 (IL-6) resulted in an expanded adult forebrain neural precursor pool and perturbed olfactory neurogenesis in offspring months after fetal exposure. This increase is likely the long-term consequence of acute hyperactivation of an endogenous autocrine/paracrine IL-6-dependent self-renewal pathway that normally regulates the number of forebrain neural precursors. These studies therefore identify an IL-6-dependent neural stem cell self-renewal pathway in vivo, and support a model in which transiently increased maternal cytokines can act through this pathway in offspring to deregulate neural precursor biology from embryogenesis throughout life.

INTRODUCTION

Many adult mammalian tissues contain resident stem cells, and increasing evidence indicates that age- or pathology-related perturbations in these cells can cause impaired tissue maintenance, repair and functionality (Simons and Clevers, 2011). However, we do not yet understand the developmental mechanisms that regulate the ultimate size or composition of adult stem cell pools. This issue is particularly relevant for the mammalian brain, since variations in adult neural precursor cells (NPCs) directly affect adult neurogenesis and cognitive function (Ming and Song, 2011). In this regard, accumulating evidence indicates that some human fetal perturbations have long-lasting effects on cognition (Patterson, 2007, 2011), raising the possibility that they might mediate their long-term effects in part by perturbing the establishment of appropriate adult NPC pools.

To address this possibility, we focused on a maternal cytokine surge, which occurs, for example, during maternal infection and is associated with autism spectrum disorder (ASD) and schizophrenia (Patterson, 2007, 2011). In particular, we examined the cytokine interleukin-6 (IL-6), which is thought to be responsible for at least some of the aberrant behavioral outcomes of maternal infection in mice. For example, the development of aberrant behaviors in offspring of infected mothers can be inhibited by blocking maternal IL-6, and a single injection of IL-6 in pregnant rodents at midgestation mimics maternal infection with regard to cognitive outcomes (Smith et al., 2007). Since IL-6 in the maternal circulation can enter the fetal circulation (Dahlgren et al., 2006) and cross the blood–brain barrier (Banks et al., 1994), it is possible that IL-6 can perturb neural function in the long term by deregulating embryonic NPCs, thereby altering neonatal and adult NPC numbers and/or function.

Here, we asked whether maternal IL-6 causes long-term changes in adult NPC pools and, if so, how it might do that. We show that maternally administered IL-6 causes a rapid expansion of embryonic forebrain precursors, ultimately

resulting in an abnormally expanded adult forebrain NPC pool and aberrant neurogenesis months after fetal exposure. Our studies indicate that maternal IL-6 mediates these effects by hyperactivating an endogenous IL-6-dependent self-renewal pathway that determines the number of embryonic forebrain NPCs. Thus, our results support the concept that the size of the adult forebrain NPC pool is determined by the number of embryonic NPCs, and indicate that perturbations that deregulate embryonic precursor self-renewal have long-lasting consequences for stem cell pools, neurogenesis, and potentially cognitive outcomes in adults.

RESULTS

Acute Exposure to Maternally Administered IL-6 In Utero Causes Long-Lasting Perturbations in Neural Precursor Pools in the Adult Forebrain

To ask whether exposure to transient maternal IL-6 has long-term consequences for adult NPCs, we administered a single pulse of IL-6 by intraperitoneal injection into pregnant mice on gestational day 13.5 (G13.5; Smith et al., 2007). We then characterized adult NPCs in their forebrain niche, the subventricular zone (SVZ), in 2-month-old offspring using three different approaches. First, we administered bromodeoxyuridine (BrdU), and 1 day later quantified the proportion of proliferating BrdU-positive cells. Adult mice exposed to maternal IL-6 as embryos had approximately two times more BrdU-positive SVZ cells compared with controls (Figures 1A and 1B). These cells were cycling precursors, since the large majority were positive for the pan-precursor marker Sox2 (Figure 1C) and the proliferation marker Ki67 (Figure 1D). Second, we immunostained SVZ sections for Sox2 and Ki67 or for glial fibrillary acidic protein (GFAP), Olig2, or Mash1, all of which are markers for various NPC subpopulations (Figures 1D and 1F). Quantification showed that total Sox2-positive cells were significantly increased in mice exposed to transient maternal IL-6 in utero (Figures 1E and 1F), but the relative proportions of the different subpopulations were unaltered (Figures 1F and 1G). Third, we quantified the number of SVZ neurospheres as a surrogate measure for NPCs. Maternal IL-6 exposure caused an almost 1.5-fold increase in SVZ neurosphere-initiating cells (Figure 1H).

Since maternally administered IL-6 caused a long-lasting increase in forebrain NPCs, we asked whether it also affects adult NPCs in the hippocampal dentate gyrus. Analysis of BrdU-positive proliferating precursors 1 day after BrdU administration showed that similar numbers of proliferating NPCs were present in the dentate gyrus subgranular zone in adult mice that were or were not exposed to maternal IL-6 (Figures S1A and S1B available online). Similar results were obtained by quantifying Sox2-positive NPCs (Figure S1C). Consistent with this, the two groups had similar numbers of doublecortin-positive newborn neurons (Figure S1D), as well as more mature adult-born neurons, which we determined by administering BrdU and quantifying BrdU-positive cells that coexpressed the neuronal marker neuronal nuclei (NeuN) 1 month later (Figure S1E). Thus, exposure to maternal IL-6 at embryonic day 13.5 (E13.5) selectively increases adult forebrain, but not hippocampal, NPC pools.

Acute Exposure to Maternally Administered IL-6 In Utero Causes Long-Lasting Perturbations in Adult Olfactory Neurogenesis

To ask whether the observed increase in forebrain SVZ precursors alters adult neurogenesis, we injected pregnant mice with IL-6 at G13.5 and then injected their adult offspring with multiple rounds of BrdU over the course of 1 day. One month later, we characterized neurons born during this time frame by immunostaining coronal olfactory bulb sections for BrdU and NeuN (Figure 1I). There were approximately 1.5-fold more newborn neurons in mice exposed to maternal IL-6 as embryos (Figure 1J). To characterize these adult-born neurons, we immunostained sections for calretinin and parvalbumin, which are markers for different interneuron populations. The relative proportion of BrdU-positive, calretinin-positive neurons was significantly increased in mice exposed to maternal IL-6 (Figures 1K–1M). In contrast, very few BrdU-positive cells were parvalbumin positive, and this was similar in both groups ($p > 0.05$). To ascertain whether the genesis of oligodendrocytes was altered, we immunostained sections for the oligodendrocyte transcription factor Olig2. Only small numbers of BrdU-positive, Olig2-positive cells were present in either the corpus callosum or the cortical gray matter, and the two groups had similar numbers of double-labeled corpus callosum cells ($p > 0.05$). Thus, a maternal IL-6 surge causes long-term alterations in the numbers and phenotypes of adult-born olfactory neurons.

A Maternally Administered IL-6 Surge Increases the Number of Adult Forebrain NPCs Deriving from Embryonic Cortical Precursors

Adult NPCs in the SVZ arise from two different embryonic origins: the ventral ganglionic eminence (GE) and the dorsal cortex (Merkle et al., 2004; Willaime-Morawek et al., 2006; Young et al., 2007; Kohwi et al., 2007; Ventura and Goldman, 2007). Since most adult-born, calretinin-positive interneurons are generated from cortically derived SVZ NPCs (Young et al., 2007; Kohwi et al., 2007), and these neurons are proportionately increased in adult mice exposed to maternally administered IL-6, we asked whether maternal IL-6 alters the cortical contribution to the SVZ precursor pool. For this purpose, we used the *piggyBac* (PB) transposon system. We electroporated the E13.5/14.5 cortex with a PB transposase plasmid together with an enhanced GFP (EGFP) reporter construct flanked by PB inverted repeats (ITRs) (Nagy et al., 2011). This in utero electroporation transfects cortical radial glial precursors (Gauthier-Fisher et al., 2009; Vessey et al., 2012), and the PB transposase allows lineage tracing by virtue of genomic integration of the EGFP reporter (Figure 2A). Analysis of electroporated cortices from 2 weeks to 3 months postnatally showed that many EGFP-positive cells were present in the upper cortical layers, the corpus callosum, the dorsal portion of the SVZ, and the rostral migratory stream (RMS). In contrast, cortices electroporated with the reporter plasmid alone contained only a few EGFP-positive neurons, as previously reported (Saito and Nakatsuji, 2001).

Three lines of evidence indicate that the cortically derived EGFP-positive cells in the SVZ were precursors. First, we dissected the SVZ and cultured cells as neurospheres; some spheres were completely EGFP positive and maintained EGFP expression for many passages (Figure 2B). Southern blots

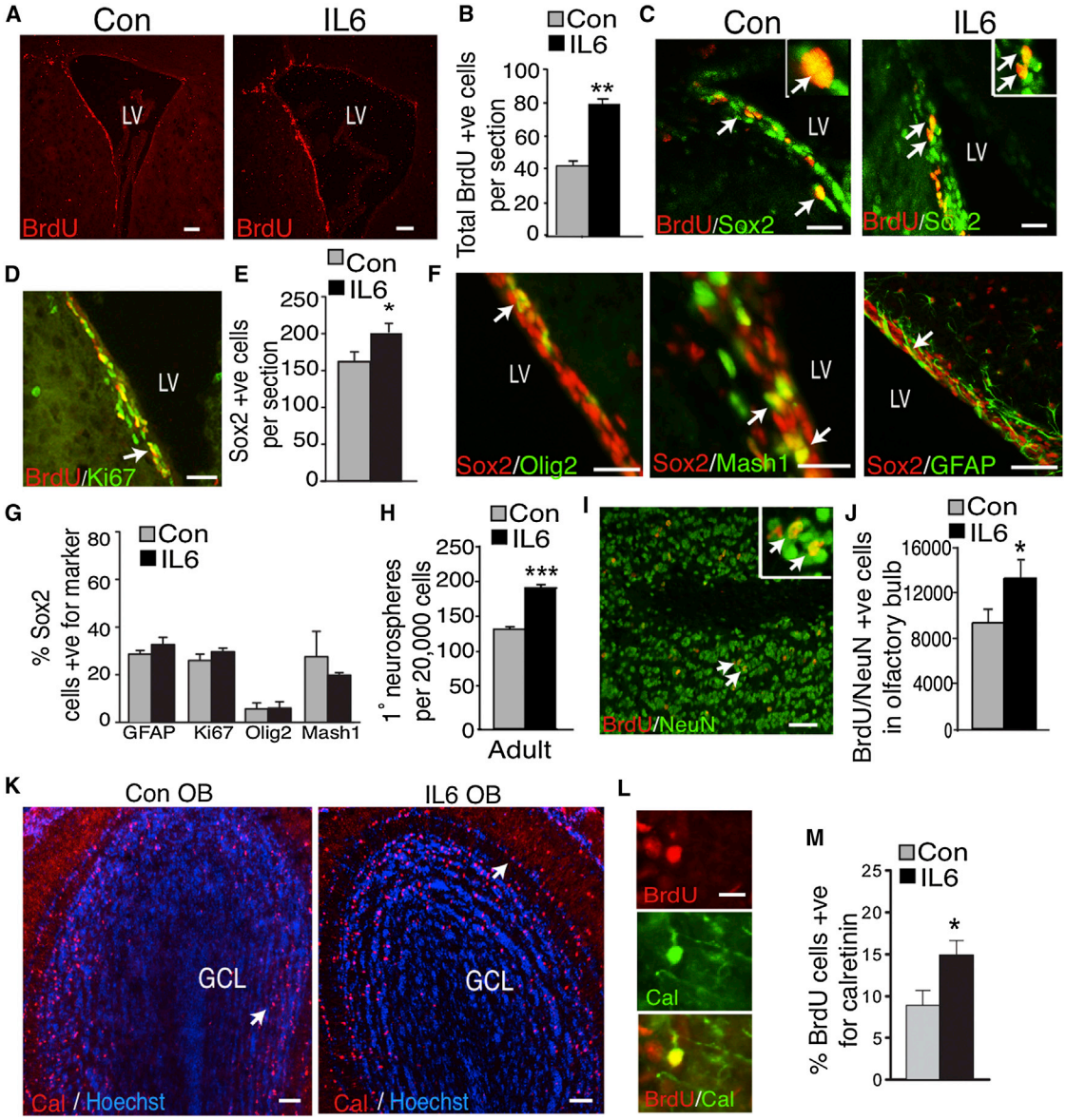

Figure 1. Acute Exposure to Maternally Administered IL-6 In Utero Causes Long-Lasting Changes in Adult SVZ NPC Pools and Olfactory Neurogenesis

Pregnant mice were injected with IL-6 or vehicle (Con) at G13.5 and their 2-month-old adult offspring were analyzed.

(A–D) Adult offspring were injected with BrdU and 1 day later, coronal sections through the SVZ and lateral ventricles (LVs) were immunostained for BrdU (A, C, and D, red) and Sox2 (C, green) or Ki67 (D, green).

(B) The numbers of BrdU-positive SVZ cells in matched sections were quantified (**$p < 0.01$; n = 4 animals each).

(C and D) Arrows denote double-labeled cells, and insets in (C) show these cells at higher magnification. A control section is shown in (D).

(E–G) Coronal sections through the SVZ of adult offspring were immunostained for Sox2 (F, red) and Olig2, Mash1, or GFAP (F, green), and the total number of Sox2-positive cells (E) or the proportion of Sox2-positive cells that expressed the subpopulation-specific markers (G) was quantified (*$p < 0.05$; n = 3 animals each). Control sections are shown in (F) and arrows denote double-labeled cells.

(H) Quantification of the number of neurospheres generated per 20,000 SVZ cells in adult offspring (***$p < 0.001$; n = 8 animals per group, each cultured independently).

(I–M) Adult offspring were injected with BrdU five times over 1 day, and 1 month later the olfactory bulbs were sectioned and immunostained for BrdU (red, I and L) and NeuN (green, I) and/or calretinin (Cal; red in K, green in L). In (K) the sections were counterstained with Hoechst 33258 (blue). Sections were quantified for total BrdU-positive, NeuN-positive cells (J) or for the proportion of BrdU-positive cells that were also calretinin positive (M). *$p < 0.05$; n = 5 mice each in (J) and 3 each in (M). Arrows denote double-labeled (I) or single-labeled (K) cells, and the inset in (I) shows these cells at higher magnification. (I) and (L) show sections from offspring of IL-6-treated mothers. In all panels, error bars denote SEM. Scale bars: (A) = 100 μm; (C), (D), (F), (J), and (L) = 50 μm; (M) = 20 μm. GCL, granule cell layer.

See also Figure S1.

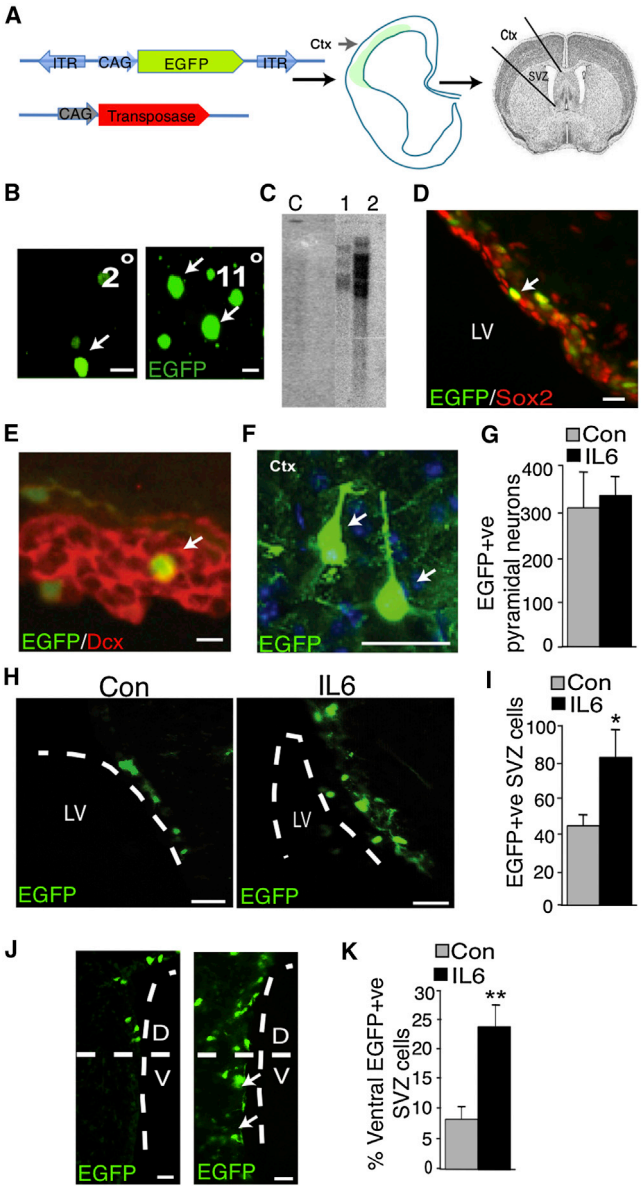

Figure 2. Transient Maternal IL-6 Increases the Number of Adult Forebrain NPCs Deriving from Embryonic Cortical Precursors

(A–E) In utero electroporation was used to introduce plasmids encoding the PB transposase and an EGFP reporter plasmid into E13.5/14.5 cortical precursors, and their cortices were analyzed 2–8 weeks postnatally as shown in (A).

(B and C) Neurospheres were generated from the SVZ at 2 weeks, and EGFP-positive spheres (arrows in B) were passaged one (2°) or ten (11°) times. Genomic DNA was isolated from positive neurospheres from two different mice (labeled 1 and 2; C denotes the negative-control DNA), and Southern blot analysis was performed for the EGFP reporter transgene (C).

(D and E) Coronal SVZ sections were immunostained for EGFP (green) and Sox2 (red, D) or doublecortin (red, E). Arrows indicate double-labeled cells. LV, lateral ventricle.

(F–K) Pregnant mice were injected with IL-6 or vehicle (Con) at G13.5, their embryonic offspring were electroporated with the PB transposase and EGFP reporter plasmids 1 day later, and their cortices were analyzed at 2 months of age.

(F–I) Coronal cortical sections through the SVZ were immunostained for EGFP (green, F and H), and total EGFP-positive pyramidal neurons (F and G) or EGFP-positive SVZ cells (H and I) in three matched sections were quantified (*$p < 0.05$; $n = 5$ each). The dotted lines in (H) demarcate the LV. The section in (F) was counterstained with Hoechst (blue).

(J and K) EGFP-positive cells around the LVs were quantified with regard to the percentage of dorsally versus ventrally located cells (K). **$p < 0.01$; $n = 3$ each, 3–4 sections per brain. Dotted lines in (J) denote the LVs and the ventral/dorsal midline used for quantification. Arrows indicate EGFP-positive cells below the midline.

Error bars denote SEM. Scale bars, 10 μm (B), 20 μm (F), and 50 μm (D, E, H, and J).

confirmed that the EGFP transgene was integrated into genomic DNA from these neurospheres (Figure 2C). Second, immunostaining demonstrated that many of the EGFP-positive SVZ cells were Sox2 positive (Figure 2D). Third, immunostaining also identified EGFP-positive, doublecortin-positive adult-born neuroblasts in the RMS (Figure 2E), indicating that EGFP-positive neurogenic NPCs had persisted from embryogenesis to adulthood.

Having established this approach, we treated mothers with IL-6 at G13.5 and electroporated the embryos 1 day later with the EGFP reporter and PB transposase. Analysis of the cortices of 2-month-old electroporated mice demonstrated that with or without IL-6, EGFP-positive cells were located from the rostral-most point where the corpus callosum crosses the midline through to the start of the hippocampus. In this region, we observed EGFP-positive cells with the morphology of pyramidal neurons in the cortical gray matter (Figure 2F), as well as many positive cells around the lateral ventricles (Figure 2H) and within the RMS (as in Figure 2E). Quantification showed that the two groups had similar numbers of EGFP-positive pyramidal neurons (Figure 2G), but maternal IL-6 exposure caused an almost 2-fold increase in EGFP-positive SVZ cells (Figures 2H and 2I). Moreover, the location of these EGFP-positive SVZ cells was changed. In control brains, they were largely localized to the dorsal SVZ, consistent with previous lineage-tracing studies (Willaime-Morawek et al., 2006; Young et al., 2007; Kohwi et al., 2007), whereas in IL-6 brains, the EGFP-positive cells extended ventrally into a region normally populated by adult NPCs from the ventral forebrain (Figures 2J and 2K). Thus, transient, maternally administered IL-6 increases the number of SVZ NPCs contributed by embryonic cortical precursors, consistent with the enhanced genesis of calretinin-positive olfactory interneurons.

Maternally Administered IL-6 Causes a Rapid Expansion of Embryonic Forebrain Precursor Cells In Vivo

One explanation for these findings is that transient IL-6 exposure in utero expands the embryonic cortical NPC pool, and this expansion translates into an enlarged adult NPC pool. To address this possibility, we injected mothers with IL-6 on G13.5 and with BrdU on G14.5, and then analyzed their embryonic offspring 1 day later. For comparison, we injected mothers with interferon-γ (IFNγ), which is also produced during maternal infections but does not have long-term behavioral consequences when injected on its own (Smith et al., 2007). Immunostaining demonstrated that in all groups of embryos, most of the BrdU-positive cortical cells were located in the precursor regions (the ventricular zone [VZ] and SVZ; Figure 3A) and expressed the radial precursor marker Pax6 (Figure 3B). Some BrdU-positive

cells were also present in the intermediate zone (IZ; Figure 3A), where they expressed the newborn neuron marker βIII-tubulin (data not shown). However, the numbers of BrdU-positive cells differed between groups, with approximately 1.5-fold more BrdU-positive cells in the maternal IL-6 group relative to the control and maternal IFNγ groups (Figure 3C). Moreover, double labeling for Ki67 demonstrated that maternal IL-6, but not IFNγ, caused a 1.5- to 2-fold increase in the proportion of BrdU-positive precursors that were still in the cell cycle 1 day later (Figures 3B and 3D). Consistent with this increase in proliferating precursors, maternal IL-6 caused a doubling of embryonic cortical cells that could generate a neurosphere at clonal density (Figure 3E).

To ask whether neurogenesis or astrogenesis was also perturbed, we performed similar experiments but this time analyzed cortices 3 days following BrdU injection. In controls, BrdU-positive cells were distributed in the VZ/SVZ, IZ and the cortical plate, which contains newborn neurons (Figures 3F and 3G). In embryos exposed to maternal IL-6, BrdU-positive cells in the VZ/SVZ were significantly increased, whereas those in the cortical plate were proportionately decreased (Figures 3F and 3G). Immunostaining (Figure 3H) for Sox2 and Satb2, a marker for neurons born over this time period (Tsui et al., 2013), demonstrated that this altered localization was due to a doubling in the proportion of BrdU-positive, Sox2-positive precursors and a coincident decrease in BrdU-positive, Satb2-positive neurons (Figures 3I and 3J). In contrast to neurogenesis, astrogenesis was unchanged, since there were no GFAP-positive cells in the cortex of either group.

Approximately 70% of forebrain NPCs derive from the ventral GE (Young et al., 2007). We therefore asked whether maternally administered IL-6 also affected GE precursors in these embryos. Immunostaining of sections obtained 1 day after BrdU injection demonstrated similar patterns of BrdU-positive cells in the embryonic ventral forebrain of embryos from IL-6- or vehicle-treated mothers (Figure 3K). However, as seen in the cortex, maternally administered IL-6 caused a significant increase in the number of BrdU-positive cells (Figure 3L). Consistent with this, maternally administered IL-6 increased the proportion of ventral GE precursors that generated a neurosphere at clonal density (Figure 3M). Thus, maternal IL-6 causes a robust and general increase in embryonic forebrain precursors.

Embryonic Forebrain Precursors Express IL-6 Receptor, and IL-6 Directly Promotes Their Proliferation

These data suggest that acute maternal IL-6 might directly promote expansion of embryonic forebrain precursors. In this regard, IL-6 signals via a complex of IL-6 and gp130 receptors, and we previously showed that gp130 is expressed by embryonic cortical precursors (Barnabé-Heider et al., 2005). To determine whether IL-6 receptor (IL6R) is also expressed, we performed three experiments. First, we analyzed E12.5 cultures of proliferating cortical radial precursors that generate transit-amplifying intermediate progenitors and newborn neurons in culture (Gauthier-Fisher et al., 2009; Vessey et al., 2012). RT-PCR (Figure 4A) and immunostaining (Figure 4B) demonstrated that IL6R was expressed in these Sox2-positive precursors. Second, we isolated Sox2-positive precursors from the embryonic cortex of Sox2:EGFP mice (Hutton and Pevny, 2011). These cells expressed IL6R mRNA, as demonstrated by RT-PCR (Figure 4A). Third, we performed RT-PCR and immunostaining for IL6R in the embryonic cortex. IL6R mRNA was expressed at E13 (Figure 4A), and IL6R immunoreactivity (Figure 4C) was detectable in almost all Sox2-positive VZ/SVZ precursors.

Since cortical precursors express both IL6R and gp130, we asked whether maternally administered IL-6 could activate the downstream JAK-STAT pathway in their progeny. Western blot analysis of embryonic cortices 1 day following maternal IL-6 injection demonstrated a significant increase in activated, phosphorylated STAT3 (Figure 4D). Similarly, when cultured E12.5 cortical precursors were exposed to IL-6 for 1.5 hr, phosphorylated STAT3 levels were significantly increased (Figure 4E). Since IL-6 activates downstream signaling in cortical precursors, we asked whether it could directly promote proliferation. E13.5 precursors were cultured, 100 ng/ml IL-6 was added on day 1, and BrdU was added on day 2. The cultures were analyzed on day 3 by immunostaining for BrdU, Ki67, the metaphase marker phospho-histone H3, or the radial precursor marker Pax6 (Figure 4F). IL-6 increased the proportion of proliferating radial precursors as monitored by all four markers (Figures 4G–4J).

We also asked whether IL-6 directly enhances GE precursor proliferation. Initially, we confirmed that IL6R was expressed in ventral Sox2-positive precursors by immunostaining embryonic GE sections (Figure 4K). To culture the GE precursors, we dissected E13.5 GEs and cultured the dissociated cells in defined medium with fibroblast growth factor 2 (FGF2), as we did for cortical precursors. Four hours after plating, approximately 80% of the cells were proliferating, Ki67-positive precursors, and approximately 15% were βIII-tubulin-positive newborn neurons. We then added 100 ng/ml IL-6 to these GE cultures on day 1, and added BrdU on day 2. Analysis after one additional day showed that IL-6 significantly increased the proportion of BrdU-positive, proliferating GE cells (Figures 4L and 4M).

IL-6 Enhances Self-Renewal of Developing Forebrain Precursors

Since IL-6 can directly promote precursor proliferation, we performed a clonal analysis to determine whether it also enhances self-renewal. Specifically, we cultured E13.5 cortical precursors with or without IL-6 after cotransfecting them at very low efficiency with PB transposase and the PB EGFP reporter. Analysis conducted 4 days later showed that in controls, the majority of EGFP-positive clones contained one or two cells, and only a small proportion had six or more cells (Figure 5A). IL-6 treatment significantly increased the number of larger clones: almost 10% had more than ten cells, and about 5% contained 21 or more cells, which was rarely seen without addition of IL-6 (Figures 5B and 5C).

We also analyzed the composition of these clones by immunostaining them for EGFP as well as GFAP to detect astrocytes and βIII-tubulin to detect neurons (Figure 5D). In controls, most of the clones (60%–70%) contained only neurons, and these were almost all one- or two-cell clones (Figure 5E). A small proportion (about 10%) contained neurons and other cells, and approximately 20% contained unlabeled cells (either precursors alone or precursors plus glial cells; Figure 5E). IL-6 addition slightly decreased the neuron-only clones, but almost doubled the mixed clones containing neurons and other cells (Figure 5E). These mixed clones included the large, greater-than-ten-cell

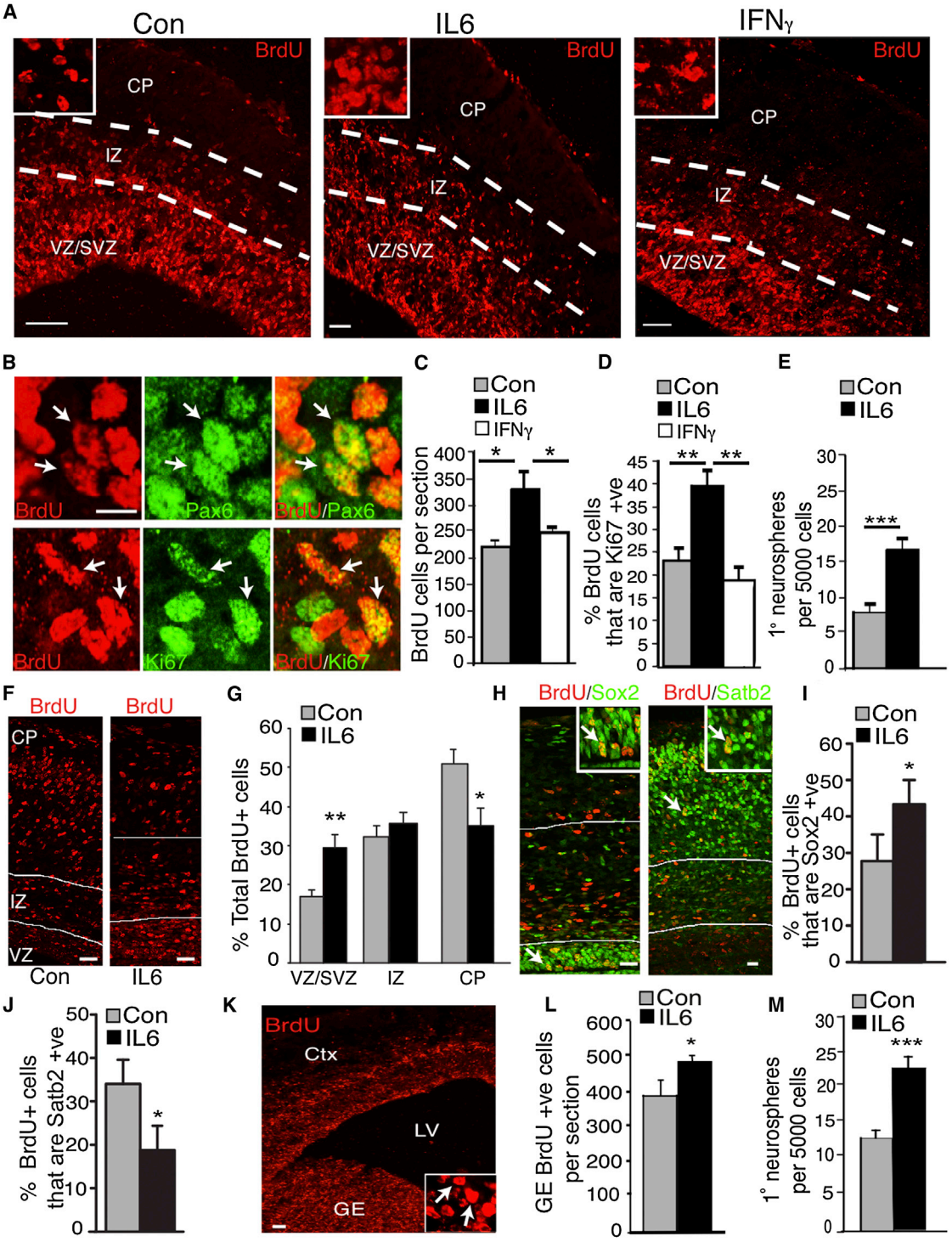

Figure 3. Maternal IL-6 Causes a Rapid Expansion of Embryonic Forebrain Precursor Cells

(A–M) Pregnant mice were injected with IL-6, IFNγ, or vehicle alone (Con) at G13.5, and with BrdU 1 day later, and the forebrains of their embryonic progeny were analyzed 1 day (A–E and K–M) or 3 days (F–J) later.

(A–D) Matched coronal sections through the embryonic cortex 1 day after maternal injection were immunostained for BrdU (red, A and B) and Pax6 or Ki67 (both green, B), and the total number of BrdU-positive cells (C) or the proportion of BrdU-positive cells that were also Ki67 positive (D) was quantified. *$p < 0.05$, **$p < 0.01$; n = 5 or 6 in (C) and 3 each in (D). In (A), insets show positive cells at higher magnification and the different zones are demarcated by hatched white lines. In (B), sections are from control mice and arrows denote double-labeled cells. CP, cortical plate; IZ, intermediate zone; VZ, zone; SVZ, subventricular zone.

(E) Total number of neurospheres generated from 5,000 primary cortical cells of embryonic offspring 2 days after injection of IL-6 or vehicle into their mothers. ***$p < 0.001$; n = 20 each.

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clones, almost all of which contained neurons, glia, and precursors. The situation was different with regard to GFAP-positive glial clones. Without IL-6, only approximately 10% of the clones contained GFAP-positive cells, and about half of these were astrocyte-only clones of one or two cells in size (Figure 5F). However, IL-6 more than doubled the proportion of mixed clones containing astrocytes and other cells (Figure 5F). Thus, IL-6 enhanced cortical precursor self-renewal and increased the proportion of multipotent clones in these cultures.

To ask whether maternally administered IL-6 might also increase NPC self-renewal in vivo, we injected pregnant mice with IL-6 on G13.5 and grew SVZ neurospheres from their offspring at postnatal day 7. This analysis showed that the number of SVZ neurosphere-initiating cells was significantly increased in mice exposed to maternal IL-6 in utero (Figure 5G). Moreover, when these neurosphere cells were passaged at equal cell densities, almost 50% more secondary neurospheres were generated by the maternal IL-6 group (Figure 5H). Thus, the SVZ NPC population is intrinsically different in mice exposed to maternal IL-6 in utero, possibly because it contains a higher proportion of self-renewing NPCs that persist from embryogenesis into postnatal life.

Autocrine/Paracrine IL-6 Regulates Embryonic Cortical Precursor Proliferation in Culture

The above data raise the possibility that IL-6 plays an endogenous role during cortical development. In support of this idea, RT-PCRs showed that IL-6 mRNA was expressed in both the E12 cortex and cultured cortical precursors (Figure 6A). To ask whether cortical precursors could actually synthesize and secrete IL-6, we cultured them for 1 day and collected their conditioned medium. Analysis of cytokine antibody dot blots demonstrated that, relative to control medium, IL-6 was readily detectable in the medium conditioned by cortical precursors, whereas other cytokines, including IL-4, IL-5, IL-9, and IL-10, were present at moderate to undetectable levels (Figure 6B).

Since cortical precursors secrete IL-6, we asked whether this autocrine/paracrine factor is important for basal precursor proliferation. For this purpose, we cultured cortical precursors for 2 days with a well-characterized function-blocking IL-6 antibody (Geddes et al., 2011) or with a nonspecific, control immunoglobulin G (IgG). Anti-IL-6 had no effect on cell survival, as monitored by counting cells with Hoechst-33258-positive condensed, apoptotic nuclei (control IgG: 11.6% ± 4%; anti-IL-6: 10.6% ± 4%; n = 3 independent experiments). However, the proportion of proliferating, Ki67-positive precursors was significantly reduced by anti-IL-6 relative to controls (Figures 6C and 6D). We obtained similar results when we assessed proliferation by adding BrdU to the cultures on day 1 (Figures 6E and 6F).

To confirm that autocrine/paracrine IL-6 is important for cortical precursor proliferation, we performed similar experiments with precursors from *IL-6*$^{-/-}$ mice (Kopf et al., 1994), which, intriguingly, have fewer proliferating precursors in the adult SVZ (Bowen et al., 2011). Analysis of E13.5 *IL-6*$^{-/-}$ versus *IL-6*$^{+/+}$ cortical precursors cultured for 2 days showed that the two genotypes had similar percentages of cell death (*IL-6*$^{+/+}$: 16.9% ± 3%; *IL-6*$^{-/-}$: 18.3% ± 4%; n = 3 independent experiments). However, the *IL-6*$^{-/-}$ precursors proliferated significantly less than their wild-type counterparts as monitored by Ki67 immunostaining (Figures 7G and 7H), consistent with the function-blocking antibody data.

IL6Rs Function in a Cell-Autonomous Fashion to Maintain Embryonic Cortical Radial Precursor Numbers

To ask whether there is an endogenous autocrine/paracrine IL-6 proliferation loop in vivo, we knocked down IL6R in embryonic cortical radial precursors. To do this, we generated siRNAs for IL6R and confirmed that they were efficacious by cotransfecting them with an IL6R expression plasmid into human embryonic kidney 293 (HEK293) cells (Figure 7A). We then used in utero electroporation to cotransfect E13/14 cortical radial precursors with a nuclear EGFP plasmid along with either control or IL6R siRNAs, as we have done for other siRNAs (Barnabé-Heider et al., 2005; Wang et al., 2010). Analysis 3 days later showed that IL6R knockdown resulted in proportionately fewer EGFP-positive cells in the VZ/SVZ, and proportionately more in the cortical plate (Figures 7B and 7C). Moreover, immunostaining for Sox2 and Satb2 (Figures 7D and 7F) demonstrated that IL6R knockdown resulted in more EGFP-positive neurons and fewer EGFP-positive precursors (Figures 7E and 7G), which explains the altered cell localization. Thus, IL6Rs on radial precursors function to maintain precursor numbers and prevent premature neuronal differentiation.

Endogenous IL-6 Enhances the Self-Renewal and Maintenance of Cortical Radial Precursors In Vivo

Since IL6R is important for maintaining embryonic NPCs, we asked whether endogenous IL-6 is also important. For this purpose, we injected pregnant *IL-6*$^{-/-}$ mice with BrdU on G13.5. Analysis of the cortices of their embryonic offspring 3 days later showed that there were significantly fewer cells in the VZ/SVZ of *IL-6*$^{-/-}$ embryos and significantly more in the cortical plate (Figures 7H and 7I). Immunostaining for Sox2 and Satb2 (Figures 7J and 7L) demonstrated a decrease in the proportion of BrdU-positive precursors (Figure 7K) and a concomitant increase in the proportion of BrdU-positive neurons (Figure 7M) in *IL-6*$^{-/-}$ versus *IL-6*$^{+/+}$ embryos.

To ask whether this decrease in *IL-6*$^{-/-}$ cortical precursors reflected decreased numbers of self-renewing NPCs, we cultured E14.5 *IL-6*$^{-/-}$ versus *IL-6*$^{+/+}$ cortical cells under clonal neurosphere conditions. This analysis showed that the number of neurosphere-initiating cells in the embryonic *IL-6*$^{-/-}$ cortex

(F–J) Matched coronal cortical sections 3 days after maternal injection were immunostained for BrdU (red, F and H) and Sox2 or SatB2 (both green, H), and the location (G) and percentage of BrdU-positive cells that were positive for Sox2 (I) or SatB2 (J) were quantified (*p < 0.05, **p < 0.01; n = 3 matched sections from 4 embryos each). In (H) arrows denote double-labeled cells, and insets show those cells at higher magnification. White lines denote the various cortical regions. (K and L) Matched coronal forebrain sections 1 day after maternal injection were immunostained for BrdU (red, K) and BrdU-positive cells in the GE were quantified (L). *p < 0.05; n = 6 embryos each. The inset in (K) shows positive cells (arrows) at higher magnification. Ctx, cortex.
(M) Total number of neurospheres generated from 5,000 primary GE cells of embryonic offspring 2 days after maternal injection. ***p < 0.001; n = 20 each. Error bars indicate SEM. Scale bars, 50 μm (A, F, K, and H) and 10 μm (B).

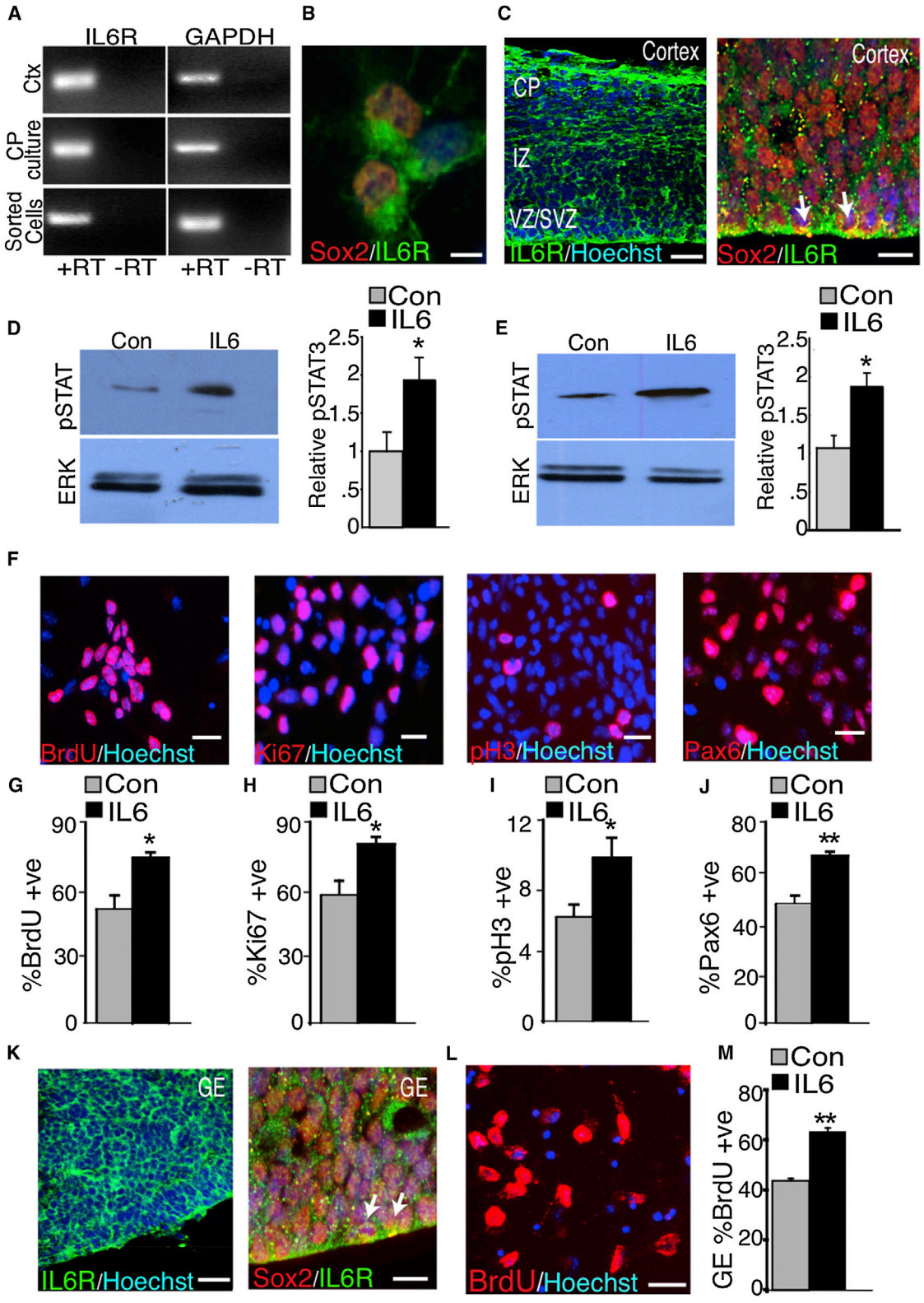

Figure 4. Embryonic Forebrain Precursors Express IL6R and Proliferate in Response to IL-6

(A) RT-PCR analysis for IL6R and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNAs in RNA from the E13 cortex (Ctx), from cortical precursors cultured for 1 day (CP culture), and from sorted E13 Sox2:EGFP-positive cortical precursors (sorted cells). RT denotes samples with (+) or without (−) reverse transcriptase.

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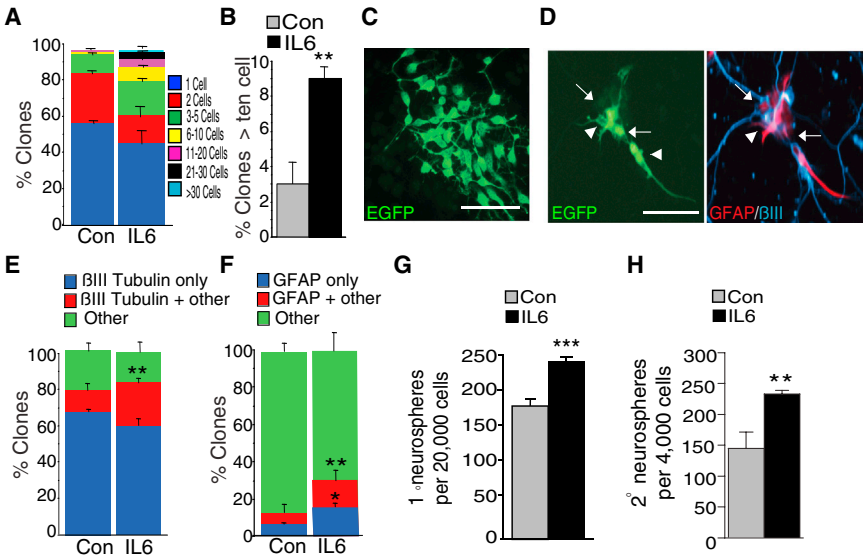

Figure 5. IL-6 Promotes Self-Renewal of Embryonic Forebrain Precursors

(A–F) Cultured E13.5 cortical precursors were transfected with the EGFP reporter and PB transposase, and treated with IL-6 or vehicle (Con) commencing on day 1. Isolated clones were characterized 2 days later by immunostaining for EGFP and cell-type-specific markers.

(A and B) Quantification showing the total distribution of clone sizes (A) and the percentage of clones greater than ten cells in size (B). $^{**}p < 0.01$; n = 3 independent experiments.

(C) A representative large, EGFP-positive clone in an IL-6-treated culture.

(D) An EGFP-positive (green) multipotent clone containing GFAP-positive astrocytes (red, arrowheads) and βIII-tubulin-positive neurons (blue, arrows).

(E) Quantification of the percentage of clones containing only βIII-tubulin-positive neurons, βIII-tubulin-positive neurons plus at least one other cell type, or no βIII-tubulin-positive cells (Other). $^{**}p < 0.01$; n = 3 independent experiments.

(F) Quantification of the percentage of clones containing only GFAP-positive astrocytes, GFAP-positive astrocytes plus at least one other cell type, or no GFAP-positive cells (Other). $^{*}p < 0.05$, $^{**}p < 0.01$; n = 3 independent experiments.

(G and H) Pregnant mice were injected with IL-6 or vehicle (Con) at G13.5, the SVZ of their offspring was dissected at P7, and cells were cultured under clonal neurosphere conditions. Primary spheres were quantified (G) and passaged at equal cell density to generate secondary spheres, which were then quantified (H). $^{**}p < 0.01$, $^{***}p < 0.005$; n = 8 mice each, analyzed individually.

Error bars indicate SEM. Scale bars, 25 μm.

was significantly decreased (Figure 7N). In addition, when *IL-6*$^{-/-}$ neurospheres were passaged, they generated only about half as many secondary neurospheres as the controls did (Figure 7O), suggesting that the *IL-6*$^{-/-}$ neurosphere population was relatively depleted of self-renewing stem cells. Thus, endogenous IL-6 binds to IL6Rs on cortical precursors to promote their self-renewal and maintain their numbers during embryogenesis.

DISCUSSION

The results presented here support a number of major conclusions. First, we show that a transient pulse of IL-6 in the maternal circulation causes a long-lasting increase in the SVZ precursor pool, accompanied by perturbed olfactory neurogenesis in adult offspring. Second, by lineage tracing, we show that in mice exposed to maternally administered IL-6 as embryos, the adult NPCs contributed by the dorsal cortex to the SVZ are increased in number and their localization is altered. Third, we provide evidence that this larger adult SVZ precursor pool is the consequence of a rapid IL6R-mediated increase in proliferation and self-renewal of neural precursors in the embryonic cortex and GE. Fourth, we demonstrate that embryonic cortical precursors normally synthesize and secrete IL-6, and that during development this autocrine/paracrine IL-6 activates the IL6R on embryonic forebrain precursors to regulate their self-renewal and numbers. Thus, our data identify a developmentally important IL-6-dependent NPC self-renewal pathway, and suggest that a transient maternal cytokine flare could act through this pathway in offspring to deregulate NPC biology in embryogenesis and throughout life.

The mechanisms that regulate the number and composition of adult stem cell pools are still largely uncharacterized. In the adult brain, about 70% of adult-born olfactory neurons are generated by SVZ NPCs that derive from the embryonic GE in the ventral telencephalon, and about 30% are generated by SVZ NPCs that originate from the embryonic dorsal cortex (Merkle et al.,

(B) Immunostaining for IL6R (green) and Sox2 (red) in cortical precursors cultured for 1 day.

(C) E12.5 cortical sections immunostained for IL6R (green, both panels) and Sox2 (red, right panel). The right panel is at higher magnification, and arrows denote double-labeled precursors adjacent to the ventricle.

(D and E) Western blot analysis for phosphorylated, activated STAT3 (pSTAT) in lysates of embryonic cortices 1 day after maternal IL-6 injection (D) or lysates of cortical precursors cultured for 1 day and exposed to IL-6 for 1.5 hr (E). Blots were reprobed for ERK protein as a loading control. The graphs show quantification of the relative ratio of pSTAT to ERK, normalized to the control in the same experiment ($^{*}p < 0.05$; n = 3 independent experiments).

(F–J) E13.5 cortical precursors were exposed to IL-6 or vehicle (Con) 1 day after culturing, BrdU was added on day 2, cultures were immunostained (red, F) on day 3, and the percentage of cells positive for BrdU (G), Ki67 (H), phospho-histone H3 (pH3; I) or Pax6 (J) was quantified ($^{*}p < 0.05$, $^{**}p < 0.01$; n = 3 independent experiments). In (F) cells were counterstained with Hoechst (blue).

(K) Coronal E12.5 GE sections were immunostained for IL6R (green, both panels) and Sox2 (red, right panel). The left panel is counterstained with Hoechst (blue) and the right panel is at higher magnification, with arrows denoting double-labeled precursors adjacent to the ventricle.

(L and M) E13.5 GE cultures were exposed to IL-6 or vehicle (Con) on day 1, BrdU was added on day 2, and on day 3, cultures were immunostained (red, L) and the percentage of BrdU-positive cells was quantified (M). $^{**}p < 0.01$; n = 3 independent experiments. In (L) cells were counterstained with Hoechst (blue).

Error bars indicate SEM. Scale bars, 10 μm (B), 20 μm (F and L), and 50 μm (C and K).

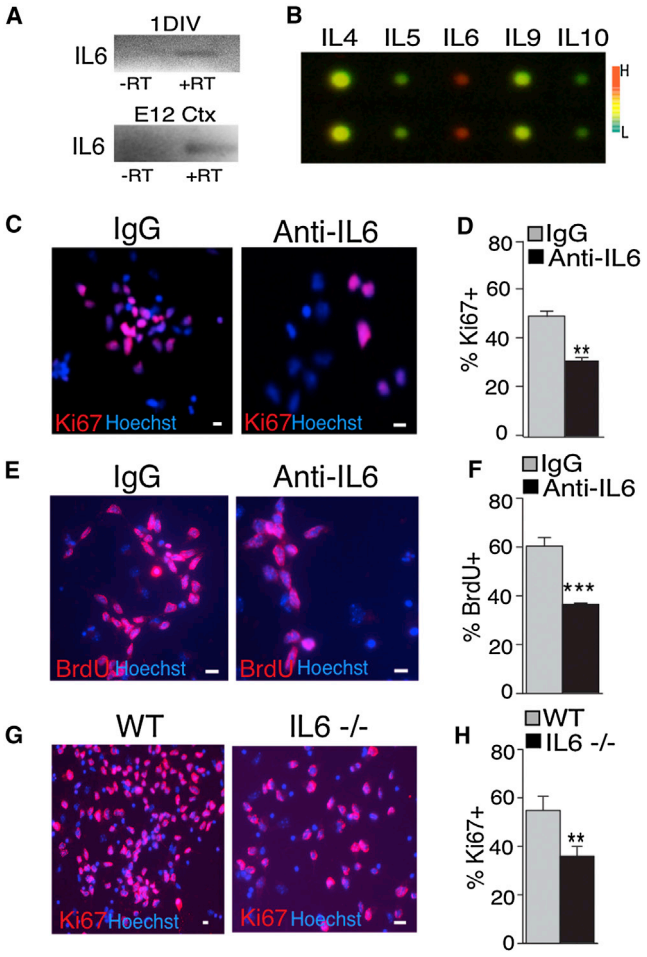

Figure 6. Endogenously Produced IL-6 Regulates Embryonic Cortical Precursor Proliferation in Culture

(A) RT-PCRs for IL-6 mRNA in RNA from the E12 cortex (Ctx) and E12.5 cortical precursors cultured for 1 day (1DIV). RT denotes samples with (+) or without (−) reverse transcriptase.

(B) Cytokine antibody array dot blot showing detectable IL-6 (red indicates the highest levels on the color scale to the right) in the medium conditioned by E12.5 cortical precursors for 1 day. Other cytokines were present at low or undetectable levels (green or yellow on the color scale).

(C–F) E13 cortical precursors were cultured for 2 days with either a control IgG or a function-blocking IL-6 antibody, and BrdU was added on day 1. On day 2, cultures were immunostained for Ki67 (red, C) or BrdU (red, E) and the relative proportion of positive cells was quantified (D and F). $^{**}p < 0.01$, $^{***}p < 0.001$; n = 3 independent experiments.

(G and H) E13.5 cortical precursors from *IL-6*$^{+/+}$ (wild-type [WT]) or *IL6*$^{-/-}$ mice were cultured for 2 days and immunostained for Ki67 (red, G), and the proportion of Ki67-positive cells was determined (H). $^{**}p < 0.01$; n = 3 independent experiments.

In (C), (E), and (G) cells were counterstained with Hoechst (blue). Error bars indicate SEM. Scale bars, 25 μm.

2004; Willaime-Morawek et al., 2006; Young et al., 2007; Kohwi et al., 2007; Ventura and Goldman, 2007). Intriguingly, these two populations give rise to distinct olfactory bulb progeny: most calretinin-positive interneurons and some adult-born excitatory neurons are contributed by cortical NPCs, whereas calbindin-positive interneurons are thought to derive almost exclusively from ventrally derived NPCs (Kohwi et al., 2007; Young et al., 2007; Brill et al., 2009). The mechanisms responsible for determining this mosaic NPC composition are important, since accumulating evidence indicates that SVZ NPCs are critical for maintaining olfaction-dependent behaviors (Breton-Provencher and Saghatelyan, 2012) and play key roles in the response to injury and neurodegeneration (Kolb et al., 2007; Brill et al., 2009). The data presented here strongly suggest that the composition of the adult SVZ NPC pool is determined by the size of the embryonic GE and cortical precursor pools, and that stimuli that aberrantly affect embryonic precursors ultimately cause perturbations in the postnatal and adult precursor pools. Here, the stimulus we studied is a transient maternal IL-6 surge, which causes enlargement of the adult SVZ pool and enhanced olfactory neurogenesis. This stimulus also apparently results in an enhanced cortical contribution to the SVZ, since cortically derived NPCs are increased in number and aberrantly localized, and the relative number of adult-born calretinin-positive olfactory neurons, which are largely generated by cortically derived NPCs, is increased.

What are the factors that normally regulate self-renewal of embryonic forebrain precursors? Although FGF2 and epidermal growth factor (EGF) are well established in this regard (Tropepe et al., 1999), we provide evidence that IL-6 also plays an essential role. In particular, we identify an autocrine/paracrine IL-6/IL6R loop that promotes the proliferation, self-renewal, and maintenance of embryonic cortical precursors. Intriguingly, a previous study showed that adult *IL-6*$^{-/-}$ mice had almost 3-fold fewer proliferating adult SVZ NPCs (Bowen et al., 2011), suggesting that the embryonic depletion of *IL-6*$^{-/-}$ cortical NPCs we document here translates directly into a long-term depletion of the adult SVZ. This is an unexpected role for IL-6 in the CNS, where it is thought to be predominantly an immune modulator. However, IL-6 has been implicated in muscle stem cell proliferation (reviewed in Muñoz-Cánoves et al., 2013), raising the possibility that it might be a general stem cell self-renewal factor like its related family member LIF, which also stimulates proliferation and self-renewal of adult and embryonic forebrain precursors (Shimazaki et al., 2001; Bauer and Patterson, 2006).

Our data provide strong support for a model in which a transient maternally administered pulse of IL-6 hyperactivates this endogenous IL-6 self-renewal pathway to deregulate the forebrain NPC pool from embryogenesis through to adulthood. These findings are important because they provide one mechanism to explain how the fetal environment might impact the development of later-onset disorders such as schizophrenia (Patterson, 2007). Of particular relevance to this study, a maternal cytokine surge is thought to accompany physiological perturbations such as maternal infections, which are associated with intellectual disability, ASD, and schizophrenia (Patterson, 2011). Although there are many ways in which a maternal immune response could (and likely does) influence neural development, this particular model provides a relatively straightforward mechanism for encoding long-term changes by regulating the size and/or nature of postnatal and adult NPC pools. Intriguingly, recent work has also shown that genes mutated in human cognitive disorders, including those encoding SHP-2, CBP, and DISC-1, regulate neural development at least in part by

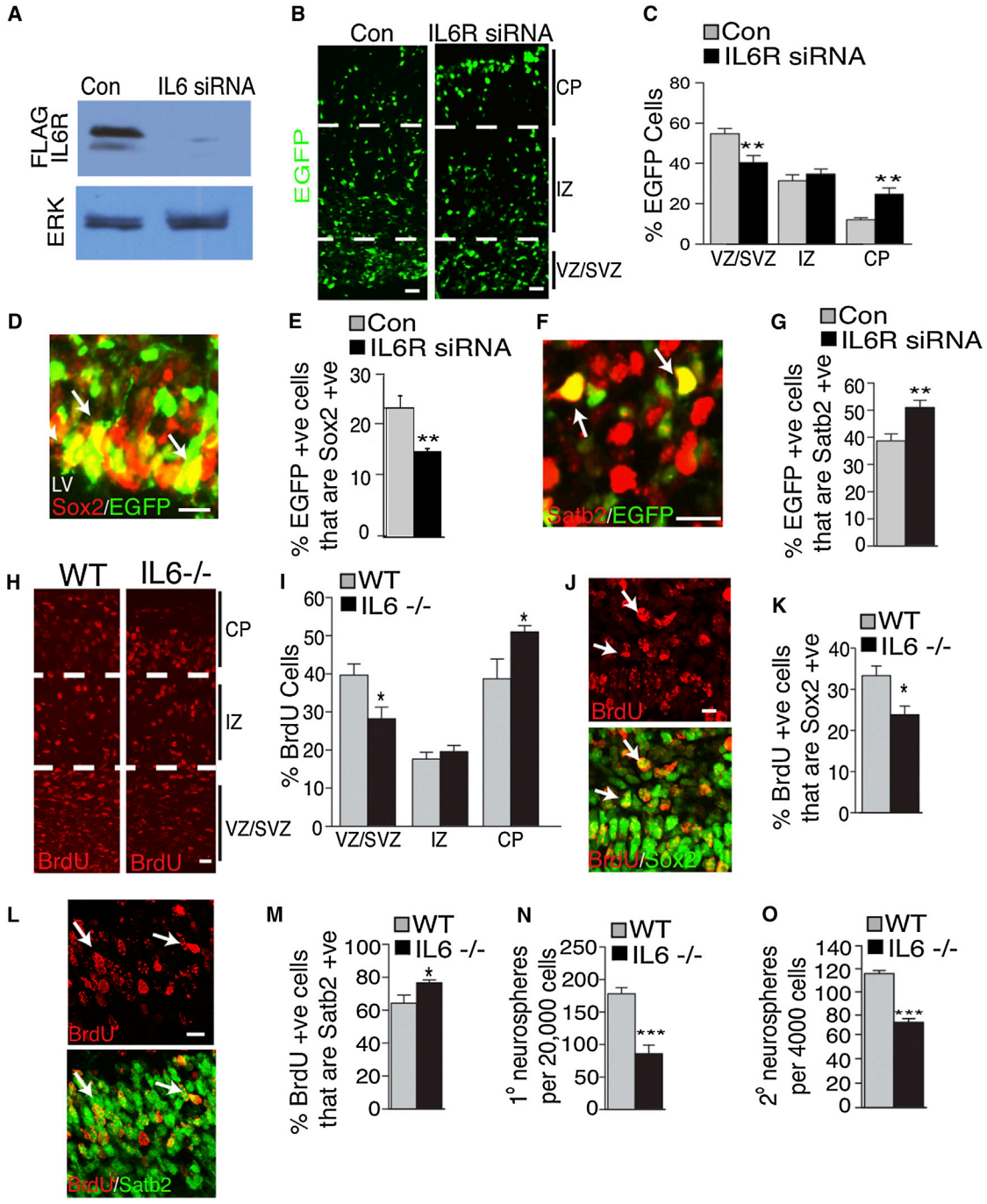

Figure 7. Endogenous IL-6 and IL6Rs Regulate Embryonic Cortical Precursor Maintenance and Numbers In Vivo

(A) Western blot of lysates from HEK293 cells cotransfected with a FLAG-tagged IL6R plasmid and scrambled or IL6R siRNAs, probed with a FLAG antibody. The blot was reprobed for ERK as a loading control.

(B–G) E13/14 cortices were electroporated with an EGFP expression plasmid and IL6R or scrambled siRNAs, and cortices were analyzed 3 days later.

(B and C) Coronal cortical sections were immunostained for EGFP (green, B) and the proportion of EGFP-positive cells in the different cortical regions was quantified (C). **$p < 0.01$; n = 3 each, 3–4 sections per embryo. In (B) the dotted lines demarcate the different cortical regions.

(D–G) Cortical sections were immunostained for EGFP (green, D and F) and Sox2 (red, D) or Satb2 (red, F), and the proportion of EGFP-positive cells that were also positive for Sox2 (E) or Satb2 (G) was quantified. **$p < 0.01$; n = 3 each, 3–4 sections per embryo. Arrows in (D and F) indicate double-labeled cells.

(H–M) Pregnant *IL-6*$^{+/+}$ (WT) or *IL-6*$^{-/-}$ mice were injected with BrdU at G13.5 and the cortices of their embryonic offspring were analyzed 3 days later.

(H and I) Coronal cortical sections were immunostained for BrdU (red, H) and the proportion of BrdU-positive cells in different cortical regions was quantified (I). *$p < 0.05$; n = 5 embryos each. In (H) the dotted lines demarcate the different cortical regions.

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regulating NPCs (Gauthier et al., 2007; Wang et al., 2010; Brandon et al., 2009). Thus, NPCs may be one cell type in which genetic predisposition and fetal environmental perturbations converge to cause long-term alterations in adult neural function.

In summary, our data define a new endogenous stem cell self-renewal pathway, and show how a transient maternal perturbation, in this case increased levels of circulating IL-6, can hyperactivate this endogenous mechanism to have a profound long-term impact on the neonatal and adult SVZ NPC pools that are ultimately established in their offspring. These findings not only shed unexpected light on the relationship between embryonic and adult stem cell pools but also provide an example of how a transient maternal perturbation can deregulate stem cell function throughout life.

EXPERIMENTAL PROCEDURES

Animals

All animal use was approved by The Hospital for Sick Children in accordance with CCAC policies. CD1 mice (Charles River Laboratories) were used unless otherwise indicated. *IL6*$^{-/-}$ mice in a C57BL/6J background (B6;129S2-*Il6tm1Kopf*/J) (Kopf et al., 1994) and their wild-type counterparts were obtained from Jackson Laboratories. Pregnant female mice at G13.5 were injected i.p. with 5 μg of carrier protein-free recombinant mouse IL-6 or IFNγ in 200 μl of PBS (Smith et al., 2007) 24 hr prior to in utero electroporation or BrdU injection. For adult NPC studies, adult mice were either injected once with 100 mg/kg BrdU and analyzed the next day, or injected with 5 × 100 mg/kg BrdU at 3 hr intervals and analyzed 4 weeks later (Cancino et al., 2013).

Precursor Cell Cultures and Clonal Analysis

E12.5–E13.5 cortical precursors (as indicated in Results) and E13.5 ganglionic eminence precursors from CD1, *IL-6*$^{+/+}$, or *IL-6*$^{-/-}$ mice were cultured as described previously (Gauthier-Fisher et al., 2009; Vessey et al., 2012; Supplemental Experimental Procedures). Cells were cultured at a density of 150,000 cells/ml for BrdU analysis and 125,000–250,000 cells/ml for clonal analysis. For BrdU experiments, IL-6 was added on day 1, BrdU (10 μM) was added on day 2, and cells were analyzed on day 3. For the function-blocking IL-6 experiments, 10 μg/ml of a control IgG or IL-6 function-blocking antibody (R&D Systems; Geddes et al., 2011) was added upon plating and precursors were analyzed on day 2. For clonal analysis, precursors were transfected with PCAG-PB-EGFP and PCAG-PBase or empty vector plasmids at an efficiency of at most 1%–3%, and IL-6 (100 ng/ml) was added 1 day after plating. Further details are provided in the Supplemental Experimental Procedures.

Neurosphere Cultures

Postnatal and adult SVZ neurospheres were isolated, cultured, and passaged as described previously (Fujitani et al., 2010; Supplemental Experimental Procedures). Spheres ≥50 μm in diameter were quantified 6 days later. For embryonic cortical and GE neurospheres, the entire cortex and GE were dissected and spheres >80 μm in diameter were counted.

In Utero Electroporation

In utero electroporation of E13/14 CD1 mice was performed as previously described (Gauthier-Fisher et al., 2009; Vessey et al., 2012) with plasmids as described in the Supplemental Experimental Procedures. Brains were dissected, fixed overnight, and cryosectioned coronally at 16–20 μm.

RT-PCR

Total RNA was isolated with Trizol and cDNA prepared with random hexamers (Fermentas; GIBCO) according to the manufacturer's protocols. Primer sequences are listed in the Supplemental Experimental Procedures. PCR products were confirmed by sequencing.

Western Blot Analysis and Cytokine Antibody Arrays

For acute stimulation, cells plated at high density in 60 cm plates for 1 day were washed for 1 hr and treated with IL-6 (100 ng/ml) or ciliary neurotrophic factor (CNTF; 50 ng/ml) for 1.5 hr. For in vivo experiments, cortices were isolated 1 day after maternal IL-6 injection. Samples were lysed and western blots performed as previously described (Barnabé-Heider et al., 2005; antibodies and details are given in the Supplemental Experimental Procedures). For cytokine antibody arrays, E12.5 cortical precursors were cultured for 24 hr, and conditioned versus unconditioned media were compared on array membranes (Raybiotech) according to the manufacturer's protocols.

Validation of siRNA-Mediated Knockdown

HEK293 cells were cultured and transfected as previously described (Vessey et al., 2012) with a murine FLAG-tagged IL6R plasmid (Origene) and either a control scrambled siRNA or a proprietary "Smartpool" IL6R siRNA cocktail (both from Thermo Scientific) for 24 hr.

Immunocytochemistry, Microscopy, and Antibodies

Immunocytochemistry of cultured cells and tissue sections were performed essentially as described previously (Gauthier-Fisher et al., 2009; Vessey et al., 2012; Cancino et al., 2013). Digital image acquisition was performed with Axiovision software (Zeiss) on a Zeiss Axioplan 2 microscope with an Orca-R2 CCD video camera (Hamamatsu) or with Volocity (Perkin Elmer) software on an Olympus IX81F-3 confocal microscope with a Hamamatsu EM-CCD video camera.

Analysis and Statistics

Details regarding the quantification of in vivo experiments are provided in the Supplemental Experimental Procedures. Statistics were performed using Student's t test and, where appropriate, ANOVA with Fisher's post hoc test. Error bars indicate SEM.

SUPPLEMENTAL INFORMATION

Supplemental Information for this article includes Supplemental Experimental Procedures and one figure and can be found with this article online at http://dx.doi.org/10.1016/j.stem.2013.10.002.

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(J–M) Cortical sections were immunostained for BrdU (red, J and L) and Sox2 (green, J) or Satb2 (green, L), and the proportion of BrdU-positive cells that were also positive for Sox2 (K) or Satb2 (M) was quantified. *$p < 0.05$; n = 5 embryos each. Arrows in (J) and (L) denote double-labeled cells.
(N and O) Cortices from E14.5 *IL-6*$^{+/+}$ (WT) or *IL-6*$^{-/-}$ mice were dissected and cultured under clonal neurosphere conditions. Primary spheres were quantified (N) and passaged at equal cell density, and secondary spheres were quantified (O). ***$p < 0.005$; n = 8 embryos each, analyzed individually.
Error bars indicate SEM. Scale bar, 50 μm (B and D), 25 μm (F and H), 10 μm (J and L).

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