Published in final edited form as:

Neuroscience. 2007 August 24; 148(2): 501–509.

Leukemia inhibitory factor participates in the expansion of neural stem/progenitors after perinatal hypoxia/ischemia

Matthew V Covey and Steven W Levison

Department of Neurology and Neurosciences, UMDNJ - New Jersey Medical School, Newark, NJ 07103

Abstract

Subsequent to perinatal hypoxia/ischemia there is an increase in the number of neural stem/progenitor cells (NSP) within the subventricular zone. Gene expression analyses have implicated Notch signaling in the expansion of these tripotential cells but there are limited data as to which signals are stimulating Notch activation. There is evidence that the LIFR/gp130 receptor heterodimer induces Notch1 to maintain NSP populations during normal development. LIF and CNTF bind to these receptor components and they coordinate injury responses in the CNS. Therefore, the aim of these studies was to investigate whether CNTF and/or LIF participate in NSP expansion in the SVZ after H/I as well as to characterize the downstream events that regulate NSP numbers. We report that LIF mRNA is induced 48 hours post insult by 13 fold but that it returns almost to baseline by 72 hours. Commensurate with increased LIF expression there is a corresponding increase in phosphorylated STAT-3 within the SVZ. Modeling the changes that occur in vivo, we show that LIF induces STAT-3 phosphorylation in neurospheres to enhance Delta-like-1 and Notch1 expression as well as to increase Notch1 activation. LIF also expands neurosphere number and size in vitro. Whereas CNTF can mimic the effects of LIF in vitro, CNTF expression in the SVZ was unchanged during recovery from H/I. Cumulatively, these data implicate LIF and not CNTF in the expansion of NSPs in the SVZ after perinatal brain injury. As both LIF expression and the endogenous regenerative response after brain injury are time-delimited, these findings provide insights into strategies to expand the endogenous pool of NSPs to repopulate the damaged brain.

Keywords

Neurogenesis; subventricular zone; stem cells; regenerative medicine; stroke; rats; brain damage

Introduction

There are a number of fetal and neonatal abnormalities of brain development that presently have no known treatment. Hypoxic/ischemic (H/I) insults, in both preterm and term infants, remain a major cause of structural and functional brain abnormalities. Previously it was thought impossible to achieve any kind of meaningful recovery after a perinatal brain injury, however, there is increasing evidence that the brain contains neural stem cells, that are resilient to H/I insults (Romanko et al., 2004). Moreover, there is emerging evidence that there is neuronal and glial cell replacement from these resident precursors (Parent et al., 2002, Yang and Levison,

Address for correspondence: Steven W. Levison, Ph.D., Laboratory for Regenerative Neurobiology, Department of Neurology and Neuroscience and NJMS-UH Cancer Center, UMDNJ-New Jersey Medical School, 205 South Orange Avenue, H-1226, Newark, NJ 07103, PH (973) 972-5162; FAX: 973-972-2668, Email: steve.levison@umdnj.edu

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

2006). However, the extent of cell replacement that naturally occurs is very modest. Arvidsson et al (2002) established that only 0.2% of neurons in the striatum were replaced from endogenous precursors after adult ischemia, thus measures are required to enhance repopulation and reconstruction of damaged areas of the brain from its resident stem cells.

The subventricular zone (SVZ) is a prominent reservoir of neural stem/progenitor cells (NSPs) that persist in the forebrain throughout life. After H/I there is an initial depletion of progenitors, followed by repopulation, that results in an increase in the size of the SVZ during recovery (Levison et al., 2001, Plane et al., 2004, Romanko et al., 2004, Ong et al., 2005, Yang and Levison, 2006). Felling et al (2006) observed increased proliferation in the SVZ two days after H/I resulting in an increase in precursors that persists for a week or more (Plane et al., 2004, Yang and Levison, 2006). In vitro studies reveal that within the first three days of recovery there is a doubling of the number of tripotential cells within the SVZ after H/I (Felling et al., 2006). Similarly, in vivo studies show enhanced neurogenesis during recovery (Plane et al., 2004, Yang and Levison, 2006, Yang et al., 2007). The increased production of self renewing, tripotential NSPs in the SVZ after a H/I insult portends the likelihood that multiple cell types can be replenished and replacement of all types of cells that are lost after a H/I is vital if meaningful recovery is to occur.

While we and others have documented the expansion of SVZ NSPs after H/I there is at most a doubling in the number of NSP numbers and the extent of cell replacement is limited. Therefore, to achieve more meaningful recovery it will be necessary to extend the expansion of the NSPs to ensure that there will be sufficient cells to successfully repair the brain. Understanding the pathways that regulate NSP expansion will be essential. It has been reported that the expansion of NSPs after H/I occurs in conjunction with an increase in the expression of mRNA's for Notch1 and Hes5 (Felling et al., 2006) and that inhibiting the Notch1 signaling pathway decreases the in vivo response of the NSP population during the recovery period after a H/I insult (Felling, PhD Dissertation). While Notch may be involved in the signaling pathway that mediates the expansion and proliferation of NSPs, it is important to know which signals are upstream of Notch.

Studies have shown that the activating the LIFR/gp130 receptor complex by CNTF maintains neural stem cells in vivo during normal development (Shimazaki et al., 2001). Activating the LIFR/gp130 receptor complex by CNTF, in turn significantly increases Notch1 (Chojnacki et al., 2003). To confirm the importance of this pathway a γ -secretase inhibitor was used to block the activation of Notch1 and this decreased the self renewal of neural stem cells. A similar result was observed when Notch1 mRNA was knocked down (Chojnacki et al., 2003). These previous studies indicate that CNTF could play a role in NSP cell responses to H/I. Another likely candidate is Leukemia Inhibitory Factor (LIF), a cytokine also from the Interleukin-6 (IL-6) family of cytokines.

It has previously been shown that LIF enhances the self renewal of both embryonic (Smith et al., 1988, Williams et al., 1988, Viswanathan et al., 2003, Horiuchi et al., 2004) and neural stem cells (Turnley and Bartlett, 2000, Pitman et al., 2004) and that the LIF receptor (LIFR)/gp130 signal transducing heterodimer is important for neural stem cell maintenance. Hatta et al (2002) also showed that LIF, signaling through gp130, increases entry into the cell cycle. Whereas these studies demonstrate a role for gp130 and Notch signaling in the homeostatic maintenance of NSPs it is not known whether either or both of these cytokines participate in the expansion of NSPs in the SVZ after brain injury. There are also data that demonstrate that administering CT-1, a member of the same cytokine family, protects the brain from H/I injury (Wen et al., 2005). Therefore, the aims of this study were to determine whether either CNTF, CT-1 and/or LIF are induced within the SVZ after H/I and to use in vivo and in vitro techniques to define the signaling pathways that regulate the reactive expansion of NSPs after injury.

Experimental procedures

Perinatal Hypoxia/Ischemia Animal Model

Timed pregnant Wistar rats were purchased from Charles River Laboratories (Charles River, Wilmington, DE), housed in individual cages and fed standard lab chow. After normal delivery, the litter size was adjusted to 12 pups per litter. Cerebral H/I was produced in 6-day-old rats (day of birth being P0), by a permanent unilateral common carotid artery (CCA) ligation followed by systemic hypoxia (Vannucci and Vannucci, 2005). Briefly, P6 pups (12.5g–15g) were lightly anesthetized with isofluorane (4% induction, 2% maintenance). Once fully anesthetized, a midline neck incision was made and the right CCA was identified. The CCA was separated from the vagus nerve and then ligated using 3-0 silk. The incision was then sutured. The whole procedure was completed within 6 min. The pups were returned to the dam for 1.5 hours and then were prewarmed in jars for 20 minutes in a 37°C water bath. The pups were exposed to a humidified, hypoxic atmosphere (8% O₂/92% N₂) at 37°C for 75 min. After this hypoxic interval the pups were returned to their dam for recovery periods of 1,2,3,4 days or 1 week, at which time they were either decapitated for RNA/protein isolation or anesthetized and sacrificed by intracardiac perfusion for immunohistochemistry. Sham operated animals were exposed to the same procedure as hypoxic animals except that the CCA was not ligated after it was separated from the vagus nerve. Control animals were separated from the dam for the same amount of time as experimental animals, but were otherwise untouched. Only those animals with moderate brain damage, defined as a smaller hemisphere without significant cortical infarction, were used in this study and they accounted for 90% of the total animals exposed to hypoxia/ischemia.

Neurosphere Preparation

Normal Wistar pups (P2-7) were sacrificed by decapitation and their brains removed using aseptic techniques. The SVZs were isolated and placed in dishes containing PGM solution (PBS with 1 mM MgCl₂ and 0.6% dextrose), where the SVZ was mechanically minced. The pieces of tissue were collected and incubated in Accutase solution at 37°C for 15 minutes. The tissue was dissociated in Pro-N media (Dulbecco's modified eagle's medium/F12 media containing 10ng/ml d-biotin, 25 µg/ml insulin, 6.29ng/ml progesterone, 16.1 µg/ml putrescine, 5ng/ml selenium, 50 µg/ml apo-transferrin, 50 µg/ml gentamycin) using micropipettes. The cell suspension was then passed through a 40 µm Nitex screen to ensure an even single cell suspension and cells were collected by centrifugation at 200g for 5 min. The media was removed and replaced with 10mL of fresh media and the process repeated 3 times to remove tissue debris. The number of viable cells was determined with a hemocytometer by exclusion of 0.1% Trypan Blue dye. The cells were then plated into six well tissue culture plastic plates (BD Bioscience) at a density of 5×10^3 cells/cm² in Pro-N media supplemented with 20 ng/mL EGF and 10 ng/mL FGF-2. Cell cultures were fed every two to three days. After 6–7 days the primary neurospheres were collected and passaged to form secondary neurospheres. Neurospheres were collected and centrifuged at 200g for 5 min. The media was removed, replaced with Accutase and incubated at 37°C for 10 minutes. The spheres were dissociated in Pro-N media using micropipettes. The cell suspension was then passed through a 40 µm Nitex screen and cells were collected by centrifugation at 200g for 5 min. The media was removed and replaced with 10mL of fresh media and the process repeated 3 times. Viable cells were then plated into six well tissue culture plastic plates (BD Bioscience) at a density of 5×10^3 cells/cm² in 50/50 mixture of Pro-N media supplemented with 20 ng/mL EGF and 10 ng/mL FGF-2 and neurosphere conditioned Pro-N media. Cell cultures were fed every two to three days. This protocol has been previously shown to yield NSPs by Felling et al (2006).

Neurosphere Treatment

Secondary neurospheres were cultured in either Pro-N media supplemented with 20 ng/mL EGF and 10 ng/ml FGF-2 or Pro-N media supplemented with 20 ng/mL EGF and 10 ng/ml FGF-2 and 5ng/mL of recombinant human Leukemia Inhibitory Factor (LIF) (Alomone Labs, Israel) for 24 hours or 6 days in vitro. After 24 hours the neurospheres were collected for RNA or protein extraction. After six days the number of secondary neurospheres formed in either condition was calculated.

Neurosphere Quantitation

A neurosphere was defined as a free-floating, cohesive cluster of at least 8 cells, although the vast majority of neurospheres (>98%) were substantially larger than this. Plates were gently shaken before counting each well to ensure an even distribution of spheres. 5 random 4X fields were counted per well and at least 3 wells were examined per group. The frequency of sphere-forming cells was calculated from the average number of spheres per field, the area of the field, and the area of the well.

Protein extraction

Protein was isolated from SVZ's at 2 days of recovery or from control or LIF treated secondary neurospheres. For protein extraction from tissue the SVZ's were dissected out from either control (n = 6) or H/I animals (n = 6) and placed into tubes containing HES lysis buffer (Hepes, EDTA, Sucrose, Sodium orthovanadate) with one Complete Mini Protease Inhibitor tablet (Roche) and phosphatase inhibitor cocktail (Sigma, MO). For protein extraction from neurospheres the control or LIF treated neurospheres were collected and centrifuged at 1200 rpm for 5 min. The media was removed and supplemented with HES lysis buffer. All tubes were placed on ice and the tissues or cells homogenized using a tissue homogenizer. Samples were then placed on a rocker at 4°C for 2 hours before being mechanically sheared through a 26G needle ten times. The samples were then centrifuged at 9300g for 10 minutes and the supernatant removed. Protein concentration was determined using the BCA protein assay kit (Pierce, IL).

Western Blotting

For Western blotting, 30ug of protein sample was combined with NuPage SDS sample buffer and NuPage reducing agent, heated at 70°C for 10 min and loaded onto a NuPage 4–12% Bis-Tris pre-cast gel (Invitrogen, Carlsbad, CA). Standard lanes were loaded with 5uL MagicMark XP (Invitrogen) molecular weight standards. After gel electrophoresis, proteins were transferred to nitrocellulose and incubated with Ponceau-S to stain total proteins. The blots were rinsed and incubated overnight at 4°C with gentle shaking with polyclonal antibody to either pStat-3 (Cell Signaling, CA, 1:1000), Stat-3 (Santa Cruz, CA, 1:1000), or Notch1 C-terminus (Santa Cruz, CA, 1:1000). After 3 rinses in TBS-Tween, they were incubated with HRP-conjugated secondary antibody for 2 hours at room temperature with gentle shaking. The blots were incubated with Western Lightning chemiluminescence reagent according to manufacturer's instructions (PerkinElmer, Wellesley, MA). Images were captured in a UVP EpiChem³ darkroom and processed using Labworks 4.0 digital quantification software (UVP, Upland, CA).

Quantitative real-time PCR analysis

Total RNA was isolated from the SVZs after 1, 2, 3, 4 days or 1 week of recovery from H/I and from LIF treated or control secondary neurospheres. The SVZs were dissected out of control and H/I animals (n=5) and snap frozen at -80°C. The neurospheres were collected, spun down, the media removed and 0.5 ml Trizol reagent (Molecular Research Center, Inc.) added prior to being snap frozen at -80°C. Tissue samples were thawed directly into 0.5 ml

Trizol reagent (Molecular Research Center, Inc.) and all samples were homogenized using a teflon tissue homogenizer. The aqueous phase was transferred to a new tube followed by addition of 100 μ l of chloroform and mixing. After adding 250 μ l of 70% EtOH, the aqueous phase was applied to an RNeasy Mini-spin column (Qiagen). The RNA was extracted using the standard Qiagen protocol. The concentration of total RNA was determined by measuring optical density on a spectrophotometer (Becton-Dickson). RNA samples were stored at -80° C until needed. For quantitative real-time PCR (Q-PCR), cDNA was prepared from 1 μ g of total RNA, using a commercially available RT kits (Qiagen). Primers for Leukemia Inhibitory Factor (LIF), Notch, and Delta-like 1 were designed by Invitrogen and for CNTF by Applied Biosystems. Q-PCR was performed in a 25 μ l reaction volume. All reactions were performed in duplicate using an ABI Prism 7700 Sequence Detection System (Applied Biosystems). Data analysis was performed according to the protocol provided by Applied Biosystems using Sequence Detector Systems v 1.7 software. Fold-changes in gene expression relative to a housekeeping gene were obtained using the Relative Expression Software Tool (REST) for groupwise comparison and statistical analysis of relative expression results in real-time PCR (Pfaffl et al., 2002). The relative amount of calculated message was normalized to the level of a control gene (18S rRNA) to control for inter-sample variation in amplification efficiency.

Statistical analysis

The results obtained from the quantitative real-time PCR analysis were analyzed using the REST program (Pfaffl et al., 2002) as noted above. The REST program utilizes pair-wise fixed reallocation randomized testing to determine if there is a significant change in the expression of the target gene. This test is considered more flexible than non-parametric tests and does not suffer from the same reduction of power as parametric tests do (Pfaffl et al., 2002). Results from the other experiments were analyzed for statistical significance using a *t* test or by ANOVA, and all error bars represented SEMs. Post-hoc analysis was applied to evaluate inter-group differences. Comparisons were interpreted as significant when associated with $p < 0.05$.

Results

The expression of LIF but not CNTF mRNA increases after perinatal H/I

It has been shown that the LIFR/gp130 receptor heterodimer maintains the numbers of both embryonic and neural stem cells in vitro (Chojnacki et al., 2003), supporting the hypothesis that ligands for this receptor complex might be elevated during recovery from H/I prior to the increase in NSPs. To assess levels of IL-6 family cytokines after H/I we micro dissected SVZs from the ipsilateral (ILH) and contralateral hemispheres (CLH) of H/I animals as well as from untouched control (UTC) animals and isolated total mRNA at intervals of recovery spanning from 24 hours to 7 days. Using Taqman qRT-PCR analysis we observed a significant increase in the expression of LIF mRNA relative to 18S in the ILH compared to the CLH (Figure 1A). At 24 and 48 hours of recovery LIF was induced 13 and 12 fold respectively (Figure 1A, $n = 6$, *, $p < 0.05$). At 72 hours there was a decrease in the level of LIF induction to 3 fold compared to CLH and this level of induction was unchanged at 4 days of recovery (Figure 1A, $p < 0.05$) and maintained out to 7 days of recovery (data not shown). Comparison of mRNA from the ILH to UTC animals revealed a 16 fold induction at 48 hours (Figure 1B, $n = 6$, $p < 0.05$) Comparing the mRNA from the CLH to the UTC animals did not reveal a significant induction (data not shown). By contrast there was no significant change in the expression of CNTF or Cardiotrophin-1 (CT-1) mRNA relative to 18S in the ILH compared to the CLH (Figure 1A) from 24 hours to 4 days of recovery after H/I. The expression of Oncostatin-M (OSM) mRNA was also unchanged in either the ILH or CLH SVZs after H/I (data not shown).

There is an increase in phosphorylated Stat-3 in the SVZ after perinatal H/I

IL-6 family cytokines activate Janus kinases, which may phosphorylate Stat-3 enabling it to dimerize and translocate to the nucleus. To determine whether this signaling pathway is activated during recovery from perinatal H/I we examined Stat-3 protein phosphorylation in samples from ILH, CLH, Sham Operated (SO) and UTC animals. Figure 2A shows Western blots from ILH, CLH, SO and UTC SVZs stained for pStat-3 and Stat-3. Analysis of the OD revealed a 2.3 fold increased ratio of pStat-3 to Stat-3 in the ILH SVZ compared to CLH, SO and UTC SVZs (Figure 2B, $n = 6$, $p < 0.05$). There was also a slight increase in the ratio of pStat-3 to Stat-3 in the CLH SVZ compared to SO and UTC SVZs (Figure 2B, $n = 6$, $p < 0.05$). The increased ratio of pStat-3 to Stat-3 in the ILH suggests that this pathway is activated after H/I. Similarly Figure 2C shows Western blots from control, LIF treated and CNTF treated secondary neurospheres stained for pStat-3 and Stat-3. We found that both cytokines increased the ratio of pStat-3 to Stat-3, similar to what is observed after H/I. Figure 2D shows that there is a 3.5 fold increase in the ratio of pStat-3 to Stat3 when LIF ($n = 6$) ($p < 0.05$) is added to secondary neurospheres and a 3.2 fold increase in the ratio of pStat3 to Stat3 when CNTF ($n = 6$) ($p < 0.05$) is added to secondary neurospheres. The higher baseline ratio of pStat3 to Stat3 in neurospheres compared to tissue samples is probably due to the more homogeneous nature of the cells in vitro compared to in vivo. These data indicate that LIF is capable of activating the Stat-3 pathway and that the increase in NSPs we observe after H/I could be related to increased LIF expression.

A greater number of neurospheres are formed after the addition of LIF

The clonal neurosphere assay is an important method for quantifying numbers of NSPs in the brain, to evaluate self-renewal, and it can provide insights into modes of proliferation. We micro dissected SVZ's from normal hemispheres and formed primary neurospheres in ProN media containing EGF and FGF-2. The neurospheres that formed were then dissociated and plated at clonal density in the presence of EGF and FGF-2 to form secondary neurospheres. To determine whether LIF is involved in the maintenance of neural stem cells one group had its standard media supplemented with 5ng/mL of LIF. Quantitative analyses revealed that the addition of 5ng/mL of LIF increased the number of secondary neurospheres by 1.5 fold (Figure 3A, $p < 0.05$). Neurospheres cultured in the presence of LIF were also significantly larger than control (Figure 3B-D, $p < 0.05$). Including LIF in the medium also enhanced tertiary and quaternary neurosphere formation.

The validity of the neurosphere assay has been challenged resulting in a re-evaluation of the relationship between NSCs and neurospheres by one of the originators of this method (Reynolds and Rietze, 2005). A criticism of the neurosphere assay is that it is limited as it cannot precisely quantify the numbers of true NSCs because it expands both stem cells and progenitors, which is the rationale for the use of the term NSP. However, it does provide useful relative estimates and thus remains the most widely used and accepted approach for estimating NSC numbers. When assessing the size of the neurospheres we only measured obviously single neurospheres and not fused or clumped neurospheres which would give a false impression of increasing size in the presence of LIF.

The addition of LIF or CNTF induces factors involved in the maintenance of NSPs

Perinatal H/I induces the expression of Notch1 and Delta-like1, signaling molecules that regulate the maintenance of NSPs (Felling, PhD Dissertation). It has been established that CNTF induces increased expression of Notch in ES cells (Chojnacki et al., 2003). Therefore, we asked whether the addition of 5ng/mL of LIF or CNTF to secondary neurospheres for 24 hours would induce Delta-like1 and Notch1 mRNA. Using qRT-PCR we found that LIF supplementation increased Delta-like1 2.2 fold (Figure 4, $n = 6$, $p < 0.05$) and increased Notch1 by 2.4 fold (Figure 4, $n = 6$, $p < 0.05$). LIF did not increase the expression of Jagged 1 nor

Jagged 2 (data not shown). The addition of CNTF also stimulated a 3.4 fold increase in Delta-like 1 (Figure 4, $n = 6$, $p < 0.05$) and a 4 fold increase in Notch1 (Figure 4, $n = 6$, $p < 0.05$).

LIF and CNTF increases Activation of Notch1

Notch1 signaling is regulated by cleaving full length Notch into an intracellular domain (ICD), which then combines with other proteins to form a transcriptional activator. As shown in Figure 4 LIF induces the expression of Notch1, therefore it was of interest to establish whether LIF treatment also increased NICD. LIF and CNTF were added at 5ng/mL to neurosphere cultures and levels of NICD evaluated by Western blot. There were significant increases in Notch1 when LIF ($n = 6$) ($p < 0.05$) or CNTF ($n = 6$) ($p < 0.05$) were added to secondary neurospheres (Figure 5A). This was also seen as a significant increase in the OD measurement for Notch1 in LIF ($n = 6$) ($p < 0.05$) and CNTF ($n = 6$) ($p < 0.05$) treated secondary neurospheres compared to controls (Figure 5B). Figure 5C shows that LIF and CNTF increase the ratio of NICD to Notch1 five fold ($n = 6$) ($p < 0.05$).

Discussion

The aim of these studies was to investigate whether CNTF and/or LIF participate in the expansion of NSPs in the SVZ after H/I as well as to characterize the downstream events that regulate NSP numbers. Hypoxia/ischemia is known to increase the size of the SVZ as well its complement of tripotential NSPs (Plane et al., 2004, Ong et al., 2005, Felling et al., 2006, Yang and Levison, 2006). During recovery there also is an increase in the production of new neurons (Plane et al., 2004, Yang and Levison, 2006, Yang et al., 2007). While our previous work suggested that Notch signaling might induce NSP cell expansion after H/I, there are limited data as to which factors induce Notch after brain injury. LIF and CNTF were candidate signals as it had been shown that the LIFR/gp130 receptor heterodimer could induce Notch1 to maintain NSP populations (Chojnacki et al., 2003). The related cytokine CT-1 was another candidate as it has been shown to be neuroprotective in vitro model of H/I (Wen et al., 2005). Here we demonstrate that there is a robust increase in the expression of LIF mRNA but no change in the expression of CNTF, CT-1 nor OSM mRNA and that there is an accompanying increase in the ratio of pStat3 to Stat3 in the ILH SVZ after H/I compared to controls. These unexpected data implicate LIF and not CNTF in the expansion of NSPs. Based on these findings we asked whether LIF is indeed capable of increasing the size and number of neurospheres when added to standard culture media. We observed that there were more neurospheres when NSPs were grown in the presence of LIF and that these neurospheres were larger than controls. The addition of LIF or CNTF also increased the ratio of pStat3 to Stat3 protein, the expression of mRNA for Delta-like-1 and Notch1 and notch signaling as evidenced by an increase in the ratio of NICD to Notch1 protein.

Previously we found that there is an increase in the number of NSPs in the SVZ after H/I (Felling et al., 2006) and preliminary evidence pointed to the involvement of LIF. The IL-6 family of cytokines, of which LIF is a member, is involved in the maintenance and self-renewal of several types of stem cells. Smith et al (1988) were among the first to show that LIF is involved in the maintenance of embryonic stem cells by inhibiting differentiation. Subsequent work has shown that LIF is involved in the maintenance of neural stem cells. Gregg and Weiss (2005) studied the precursors in the ventricular zone of LIF receptor $-/-$ mice. They demonstrated that signaling via this receptor is important for maintaining embryonic precursors during normal development. Pitman et al (2004) showed that adding exogenous LIF to murine neural stem cells limits lineage commitment by promoting symmetrical division. Shimazaki et al (2001) found that LIF receptor $+/-$ adult mice had reduced numbers of neural stem cells, again concluding that the LIF receptor is essential to prevent the differentiation of neural stem cells. But it has not been determined which ligand is most relevant during normal development,

nor have studies elucidated which ligands are important for the expansion of NSPs after injury (which we probably need not emphasize is likely a more complex process than maintenance). Many studies on injuries to the adult brain have shown that CNTF is dramatically increased (Ip et al., 1993, Asada et al., 1995, Herx et al., 2000, Albrecht et al., 2003). Based on these data we hypothesized that CNTF would increase after neonatal H/I whereupon it would signal through its cognate receptor to effect expansion of the NSPs in the SVZ. However, based upon our analysis of cytokine mRNAs, this hypothesis no longer seems tenable. Rather, our data strongly implicate LIF, which also increases after brain injury and are consistent with a recent study that concluded that LIF enhances neural stem cell self-renewal (Banner et al., 1997, Bauer and Patterson, 2006).

LIF activates the JAK-STAT pathway, which is stimulated after H/I. It has been shown that LIF is required to maintain murine ES cells and that LIF exerts its effects by activating Stat3 (Raz et al., 1999). Targeted mutagenesis in ES cell lines showed that inhibiting Stat3 rapidly induces differentiation (Raz et al., 1999). Moon et al (2002) also showed that LIF inhibits the differentiation and maturation of immature olfactory receptor neurons via the activation of Stat3. In their study, a dominant negative form of Stat3 induced the premature differentiation of olfactory receptor neurons, indicating that Stat3 function is essential (Moon et al., 2002). Suzuki et al (2005) injected LIF into the boundary area of an ischemic infarct which rapidly induced Stat3 phosphorylation and decreased the severity of insult. Similarly we report increased levels of pStat3 when LIF mRNA is maximal and we show that LIF induces pStat3 phosphorylation in cultured neurospheres.

Notch promotes NSP self-renewal by preventing cell differentiation. Transgenic mice that are deficient in Notch1 or components of the Notch signaling pathway, such as presenilin-1 (γ -secretase) or RBP-J κ , show a loss of NSPs as measured by a decrease in the generation of neurospheres (Hitoshi et al., 2002). Hitoshi et al (2002) also showed that they could reverse the decrease in neurospheres by adding active Notch1. Yoon et al (2004) reported similar findings. They decreased Notch activity and decreased numbers of NSPs. Chojnacki et al (2003) used both pharmacological and genetic methods to inhibit the action of Notch1 in neurospheres in vitro and found that both approaches decreased secondary neurosphere formation. Gain of function experiments with Notch reveal that progenitors accumulate when Notch signaling is enhanced (Mizutani and Saito, 2005). Cumulatively, these studies establish Notch as vital for neural stem cell maintenance (Gaiano et al., 2000).

Felling et al (2006) showed that Notch1 increases 48 hours after H/I, which is the same time point when there is a doubling in the number of NSPs. While previous studies have shown that CNTF can activate Notch, our data indicate that CNTF mRNA is not altered 48 hours after H/I. However, LIF mRNA increases 13–15 fold and our in vitro data demonstrate that LIF induces Delta-like1, Notch1 and Notch signaling as revealed by an increase in NICD in secondary neurospheres. Using in situ hybridization, Felling (PhD Dissertation) found that Delta-like-1 and Notch1 are upregulated in the SVZ during recovery from perinatal H/I contemporaneously as we observe increased LIF. Therefore, our data strongly suggest that LIF, and not CNTF, acts during recovery from perinatal H/I to activate Delta-like1 and Notch1 in the SVZ to expand the number of NSP's.

On the basis of the data that we have collected we theorize that pStat3, induced by LIF, coordinates the induction of Notch1 in neural stem cells. Josten et al (2004) showed that JAK/STAT signaling is required for Notch activation, however, the molecular link remained to be determined. Kamakura et al (2004) reported that the combination of a dominant-negative Stat3 with over-expression of an active form of Notch1 resulted in a loss of NSPs. More recently Yoshimatsu et al (2006) have shown that there is Stat3 dependant expression of Delta-like1, which is an important ligand of Notch1, via a Stat responsive binding element. These findings

suggest that increasing levels of Notch alone are insufficient to maintain neural stem cells in their undifferentiated state. Thus, activated Stat-3 within both the NSPs as well as within cells adjacent to the NSPs must occur to sustain Notch signaling concurrent with Stat-3 gene transactivation. That LIF is sufficient to promote Stat-3 signaling as well as the expression of both Notch1 and Delta-like-1 strongly implicates LIF in the regenerative process that ensues in the SVZ after injury.

In summary, these results add several pieces to the puzzle for the mechanisms that regulate the expansion of the SVZ NSPs that occurs after perinatal H/I. We show that during the recovery from perinatal H/I there is induction of LIF, but not CNTF, as well as increased phosphorylated STAT-3 within the SVZ. We also show that LIF induces STAT-3 phosphorylation in neurospheres leading to Delta-like-1 and Notch1 expression as well as increased active Notch. From these data we propose a model in which LIF binding causes phosphorylation of Stat3 in the neural stem cells as well as in adjacent cells within their niche. As a consequence of binding to stat binding elements in the Notch and Delta-like 1 promoters, Notch signaling occurs which in conjunction with other Stat-3 regulated genes, shifts the neural stem cells from asymmetrical divisions to symmetrical divisions to double the NSP population. As our studies indicate that both the expansion of the SVZ NSPs and the expression of LIF are transient after perinatal H/I, our data suggest that exogenous LIF infusion might further expand the NSP population and, in conjunction with other therapies, might enlarge the endogenous pool of NSPs to successfully repopulate the damaged brain.

References

- Albrecht PJ, Murtie JC, Ness JK, Redwine JM, Enterline JR, Armstrong RC, Levison SW. Astrocytes produce CNTF during the remyelination phase of viral-induced spinal cord demyelination to stimulate FGF-2 production. *Neurobiol Dis* 2003;13:89–101. [PubMed: 12828933]
- Arvidsson A, Collin T, Kirik D, Kokaia Z, Lindvall O. Neuronal replacement from endogenous precursors in the adult brain after stroke. *Nat Med* 2002;8:963–970. [PubMed: 12161747]
- Asada H, Ip NY, Pan L, Razack N, Parfitt MM, Plunkett RJ. Time course of ciliary neurotrophic factor mRNA expression is coincident with the presence of protoplasmic astrocytes in traumatized rat striatum. *J Neurosci Res* 1995;40:22–30. [PubMed: 7714923]
- Banner LR, Moayeri NN, Patterson PH. Leukemia inhibitory factor is expressed in astrocytes following cortical brain injury. *Exp Neurol* 1997;147:1–9. [PubMed: 9294397]
- Bauer S, Patterson PH. Leukemia inhibitory factor promotes neural stem cell self-renewal in the adult brain. *J Neurosci* 2006;26:12089–12099. [PubMed: 17108182]
- Chojnacki A, Shimazaki T, Gregg C, Weinmaster G, Weiss S. Glycoprotein 130 signaling regulates Notch1 expression and activation in the self-renewal of mammalian forebrain neural stem cells. *J Neurosci* 2003;23:1730–1741. [PubMed: 12629177]
- Felling RJ, Snyder MJ, Romanko MJ, Rothstein RP, Ziegler AN, Yang Z, Givogri MI, Bongarzone ER, Levison SW. Neural stem/progenitor cells participate in the regenerative response to perinatal hypoxia/ischemia. *J Neurosci* 2006;26:4359–4369. [PubMed: 16624956]
- Gaiano N, Nye JS, Fishell G. Radial glial identity is promoted by Notch1 signaling in the murine forebrain. *Neuron* 2000;26:395–404. [PubMed: 10839358]
- Gregg C, Weiss S. CNTF/LIF/gp130 receptor complex signaling maintains a VZ precursor differentiation gradient in the developing ventral forebrain. *Development* 2005;132:565–578. [PubMed: 15634701]
- Hatta T, Moriyama K, Nakashima K, Taga T, Otani H. The Role of gp130 in cerebral cortical development: in vivo functional analysis in a mouse exo utero system. *J Neurosci* 2002;22:5516–5524. [PubMed: 12097503]
- Herx LM, Rivest S, Yong VW. Central nervous system-initiated inflammation and neurotrophism in trauma: IL-1 beta is required for the production of ciliary neurotrophic factor. *J Immunol* 2000;165:2232–2239. [PubMed: 10925311]

- Hitoshi S, Alexson T, Tropepe V, Donoviel D, Elia AJ, Nye JS, Conlon RA, Mak TW, Bernstein A, van der Kooy D. Notch pathway molecules are essential for the maintenance, but not the generation, of mammalian neural stem cells. *Genes Dev* 2002;16:846–858. [PubMed: 11937492]
- Horiuchi H, Tategaki A, Yamashita Y, Hisamatsu H, Ogawa M, Noguchi T, Aosasa M, Kawashima T, Akita S, Nishimichi N, Mitsui N, Furusawa S, Matsuda H. Chicken leukemia inhibitory factor maintains chicken embryonic stem cells in the undifferentiated state. *J Biol Chem* 2004;279:24514–24520. [PubMed: 15044464]
- Ip NY, Wiegand SJ, Morse J, Rudge JS. Injury-induced regulation of ciliary neurotrophic factor mRNA in the adult rat brain. *Eur J Neurosci* 1993;5:25–33. [PubMed: 8261087]
- Josten F, Fuss B, Feix M, Meissner T, Hoch M. Cooperation of JAK/STAT and Notch signaling in the *Drosophila* foregut. *Dev Biol* 2004;267:181–189. [PubMed: 14975725]
- Kamakura S, Oishi K, Yoshimatsu T, Nakafuku M, Masuyama N, Gotoh Y. Hes binding to STAT3 mediates crosstalk between Notch and JAK-STAT signalling. *Nat Cell Biol* 2004;6:547–554. [PubMed: 15156153]
- Levison SW, Rothstein RP, Romanko MJ, Snyder MJ, Meyers RL, Vannucci SJ. Hypoxia/ischemia depletes the rat perinatal subventricular zone of oligodendrocyte progenitors and neural stem cells. *Developmental Neuroscience* 2001;23:234–247. [PubMed: 11598326]
- Mizutani K, Saito T. Progenitors resume generating neurons after temporary inhibition of neurogenesis by Notch activation in the mammalian cerebral cortex. *Development* 2005;132:1295–1304. [PubMed: 15750183]
- Moon C, Yoo JY, Matarazzo V, Sung YK, Kim EJ, Ronnett GV. Leukemia inhibitory factor inhibits neuronal terminal differentiation through STAT3 activation. *Proc Natl Acad Sci U S A* 2002;99:9015–9020. [PubMed: 12084939]
- Ong J, Plane JM, Parent JM, Silverstein FS. Hypoxic-ischemic injury stimulates subventricular zone proliferation and neurogenesis in the neonatal rat. *Pediatr Res* 2005;58:600–606. [PubMed: 16148080]
- Parent JM, Vexler ZS, Gong C, Derugin N, Ferriero DM. Rat forebrain neurogenesis and striatal neuron replacement after focal stroke. *Ann Neurol* 2002;52:802–813. [PubMed: 12447935]
- Pfaffl MW, Horgan GW, Dempfle L. Relative expression software tool (REST) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. *Nucleic Acids Res* 2002;30:e36. [PubMed: 11972351]
- Pitman M, Emery B, Binder M, Wang S, Butzkueven H, Kilpatrick TJ. LIF receptor signaling modulates neural stem cell renewal. *Mol Cell Neurosci* 2004;27:255–266. [PubMed: 15519241]
- Plane JM, Liu R, Wang TW, Silverstein FS, Parent JM. Neonatal hypoxic-ischemic injury increases forebrain subventricular zone neurogenesis in the mouse. *Neurobiol Dis* 2004;16:585–595. [PubMed: 15262271]
- Raz R, Lee CK, Cannizzaro LA, d'Eustachio P, Levy DE. Essential role of STAT3 for embryonic stem cell pluripotency. *Proc Natl Acad Sci U S A* 1999;96:2846–2851. [PubMed: 10077599]
- Reynolds BA, Rietze RL. Neural stem cells and neurospheres--re-evaluating the relationship. *Nat Methods* 2005;2:333–336. [PubMed: 15846359]
- Romanko MJ, Rothstein RP, Levison SW. Neural stem cells in the subventricular zone are resilient to hypoxia/ischemia whereas progenitors are vulnerable. *J Cereb Blood Flow Metab* 2004;24:814–825. [PubMed: 15241190]
- Shimazaki T, Shingo T, Weiss S. The ciliary neurotrophic factor/leukemia inhibitory factor/gp130 receptor complex operates in the maintenance of mammalian forebrain neural stem cells. *J Neurosci* 2001;21:7642–7653. [PubMed: 11567054]
- Smith AG, Heath JK, Donaldson DD, Wong GG, Moreau J, Stahl M, Rogers D. Inhibition of pluripotential embryonic stem cell differentiation by purified polypeptides. *Nature* 1988;336:688–690. [PubMed: 3143917]
- Suzuki S, Yamashita T, Tanaka K, Hattori H, Sawamoto K, Okano H, Suzuki N. Activation of cytokine signaling through leukemia inhibitory factor receptor (LIFR)/gp130 attenuates ischemic brain injury in rats. *J Cereb Blood Flow Metab* 2005;25:685–693. [PubMed: 15716858]
- Turnley AM, Bartlett PF. Cytokines that signal through the leukemia inhibitory factor receptor-beta complex in the nervous system. *J Neurochem* 2000;74:889–899. [PubMed: 10693919]

- Vannucci RC, Vannucci SJ. Perinatal hypoxic-ischemic brain damage: evolution of an animal model. *Dev Neurosci* 2005;27:81–86. [PubMed: 16046840]
- Viswanathan S, Benatar T, Mileikovsky M, Lauffenburger DA, Nagy A, Zandstra PW. Supplementation-dependent differences in the rates of embryonic stem cell self-renewal, differentiation, and apoptosis. *Biotechnol Bioeng* 2003;84:505–517. [PubMed: 14574685]
- Wen TC, Rogido MR, Moore JE, Genetta T, Peng H, Sola A. Cardiotrophin-1 protects cortical neuronal cells against free radical-induced injuries in vitro. *Neurosci Lett* 2005;387:38–42. [PubMed: 16084018]
- Williams RL, Hilton DJ, Pease S, Willson TA, Stewart CL, Gearing DP, Wagner EF, Metcalf D, Nicola NA, Gough NM. Myeloid leukaemia inhibitory factor maintains the developmental potential of embryonic stem cells. *Nature* 1988;336:684–687. [PubMed: 3143916]
- Yang Z, Covey MV, Bitel CL, Jonakait GM, Levison SW. Sustained Neocortical Neurogenesis after Neonatal Hypoxic/Ischemic Injury. *Annals of Neurology* 2007;61:199–208. [PubMed: 17286251]
- Yang Z, Levison SW. Hypoxia/ischemia expands the regenerative capacity of progenitors in the perinatal subventricular zone. *Neuroscience* 2006;139:555–564. [PubMed: 16500031]
- Yoon K, Nery S, Rutlin ML, Radtke F, Fishell G, Gaiano N. Fibroblast growth factor receptor signaling promotes radial glial identity and interacts with Notch1 signaling in telencephalic progenitors. *J Neurosci* 2004;24:9497–9506. [PubMed: 15509736]
- Yoshimatsu T, Kawaguchi D, Oishi K, Takeda K, Akira S, Masuyama N, Gotoh Y. Non-cell-autonomous action of STAT3 in maintenance of neural precursor cells in the mouse neocortex. *Development* 2006;133:2553–2563. [PubMed: 16728475]

Abbreviations

ANOVA	Analysis of Variance
CCA	Common Carotid Artery
CLH	Contralateral Hemisphere
CNTF	Ciliary Neurotrophic Factor
CT-1	Cardiotrophin-1
EGF	Epidermal Growth Factor
FGF	Fibroblast Growth Factor
H/I	Hypoxia/Ischemia
ILH	Ipsilateral Hemisphere
IL-6	Interleukin-6
LIF	Leukemia Inhibitory Factor

LIFR	Leukemia Inhibitory Factor Receptor
NICD	Notch Intracellular Domain
NSP	Neural Stem/Progenitor Cell
OSM	Oncostatin-M
PGM	Phosphate Buffered Saline with Glucose and Magnesium
Q-PCR	Quantitative Real-time
PCRREST	Relative Expression Software Tool
SO	Sham Operated
SVZ	Subventricular Zone
UTC	Untouched Control

Figure 1.

LIF mRNA is increased in the SVZ after H/I. Panel A shows the change in LIF mRNA expression over 4 days of recovery after H/I. LIF mRNA in the ipsilateral SVZ was compared to the contralateral SVZ by real time PCR. There is a significant peak in expression 24h after hypoxia-ischemia which is maintained through 48h of recovery ($n = 6$ at all time points) (*, $p < 0.05$). There is no significant increase in the expression of either CNTF or CT-1 mRNA. Panel B depicts LIF mRNA expression at 48 hours of recovery in the ILH SVZ compared to CLH SVZ and to Untreated Control (UTC) SVZ ($n = 6$) ($p < 0.05$). Statistical significance was determined using the REST program.

Figure 2.

Phosphorylated Stat-3 is increased in the SVZ after H/I. Panel A depicts Western blot analysis of pStat-3 and Stat-3 from proteins obtained from micro-dissected SVZs at 48h. Thirty micrograms of total protein from untouched controls (UTC), sham operated (SO), contralateral H/I (CLH) and ipsilateral H/I (ILH) are depicted. Panel B depicts Western blot quantitation at 48 hours of recovery revealing an increase in the ratio of pStat3 to Stat3 in the ILH SVZ when compared to UTC, SO and CLH SVZs ($n = 6$) (*, $p < 0.05$). The data also reveal an increase in the ratio of pStat3 to Stat3 in the CLH SVZ when compared to UTC and SO SVZs ($n = 6$) (+, $p < 0.05$). Panel C depicts Western blot analysis of pStat-3 and Stat-3 from neurospheres treated with 5ng/mL of LIF or CNTF for 24 hours. Two samples from control (CTL), 5ng/mL LIF treated and 5ng/mL CNTF treated are depicted. Panel D depicts Western blot quantitation of pStat-3 to Stat-3 after treatment with either LIF ($n = 6$) (*, $p < 0.05$) or CNTF ($n = 6$) (**, $p < 0.05$), as revealed by quantification of band intensity using a UVP ChemiImager.

Figure 3.

LIF enhances neural stem cell expansion/maintenance. For panels A and B, primary neurospheres were dissociated and replated into either control medium or medium supplemented with 5ng/mL of LIF. The number and size of spheres was quantified at 6 days in vitro. LIF increased the number of secondary neurospheres (*, $p < 0.05$) and the spheres were also larger (**, $p < 0.05$). Panels C and D show representative neurospheres cultured in standard media or in medium supplemented with 5ng/mL of LIF.

Figure 4.

LIF induces both Notch1 and Delta-like-1 expression. Neurospheres were treated with 5ng of LIF or CNTF for 24h and the levels of Notch and Delta-like-1 mRNA were quantified by real time PCR. There were significant increases in mRNA expression for both Delta-like-1 and Notch1 vs control after treatment with LIF and CNTF as quantified by the REST program. (n = 6). *, $p < 0.05$ for LIF; +, $p < 0.05$ for CNTF.

Figure 5.

Notch activation is increased by LIF and CNTF. Panel A depicts Western blot analysis of Notch1 and Notch1 intracellular domain (ICD) from neurospheres treated with 5ng/mL of LIF or CNTF for 24 hours. Thirty micrograms of total protein from 2 representative samples from control (CTL), 5ng/mL LIF treated and 5ng/mL CNTF treated were analyzed by Western blot. Panel B depicts densitometric analysis of total Notch expression in neurospheres treated with CNTF or LIF (n = 6) (*, $p < 0.05$) or CNTF (n = 6) (**, $p < 0.05$). Panel C depicts the ratio of Notch1 ICD to total Notch1 after treatment with either LIF (n = 6) (*, $p < 0.05$) or CNTF (n = 6) (**, $p < 0.05$) by Student's t-test.