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Inhibition of astrocyte FAK-JNK signaling promotes subventricular zone neurogenesis through CNTF

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Abstract

Astrocyte-derived ciliary neurotrophic factor (CNTF) promotes adult subventricular zone (SVZ) neurogenesis. We found that focal adhesion kinase (FAK) and JNK, but not ERK or P38, repress CNTF in vitro. Here, we defined the FAK-JNK pathway and its regulation of CNTF in mice, and the related leukemia inhibitory factor (LIF) and interleukin-6 (IL-6), which promote stem cell renewal at the expense of neurogenesis. Intrastriatal injection of FAK inhibitor, FAK14, in adult male C57BL/6 mice reduced pJNK and increased CNTF expression in the SVZ-containing periventricular region. Injection of a JNK inhibitor increased CNTF without affecting LIF and IL-6, and increased SVZ proliferation and neuroblast formation. The JNK inhibitor had no effect in CNTF−/− mice, suggesting that JNK inhibits SVZ neurogenesis by repressing CNTF. Inducible deletion of FAK in astrocytes increased SVZ CNTF and neurogenesis, but not LIF and IL-6. Intrastriatal injection of inhibitors suggested that P38 reduces LIF and IL-6 expression, whereas ERK induces CNTF and LIF. Intrastriatal FAK inhibition increased LIF, possibly through ERK, and IL-6 through another pathway that does not involve P38. Systemic injection of FAK14 also inhibited JNK while increasing CNTF, but did not affect P38 and ERK activation, or LIF and IL-6 expression. Importantly, systemic FAK14 increased SVZ neurogenesis in wildtype C57BL/6 and CNTF+/+ mice, but not in CNTF−/− littermates, indicating that it acts by upregulating CNTF. These data show a surprising differential regulation of related cytokines and identify the FAK-JNK-CNTF pathway as a specific target in astrocytes to promote neurogenesis and possibly neuroprotection in neurological disorders.

Graphical Abstract

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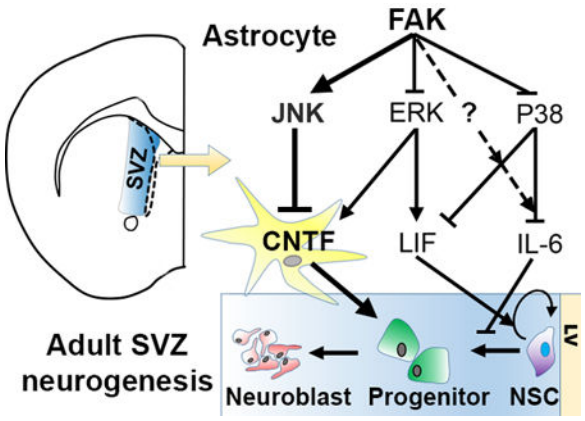

Keywords

SVZ; astrocyte; neurogenesis; FAK; JNK; P38; ERK

Introduction

The adult mammalian SVZ, including that of humans, continues to produce new neurons (Ernst et al. 2014; Hagg 2009; Ming and Song 2011; Ponti et al. 2013a). Some SVZ astrocytes are neural stem cells that generate rapidly proliferating progenitors which differentiate into neuroblasts (Doetsch et al. 1999; Gotz et al. 2015; Platel et al. 2009; Ponti et al. 2013a; Ponti et al. 2013b). The adult SVZ has densely packed astrocytes (Shen et al. 2008; Tavazoie et al. 2008; Yang et al. 2008) which produce CNTF to promote neurogenesis (Carroll et al. 1993; Emsley and Hagg 2003; Yang et al. 2008), possibly through upregulation of fibroblast growth factor 2 (FGF2) (Kang et al. 2013), which stimulates progenitor production (Kuhn et al. 1997). Stimulating endogenous neurogenesis might be a good therapeutic strategy for neuronal cell replacement. CNTF also has neuroprotective actions in various neurological disease models (Hagg and Varon 1993; Kang et al. 2012). CNTF is almost exclusively expressed in the nervous system, and is produced by astrocytes in the brain (Dallner et al. 2002; Stockli et al. 1989) and is enriched in the SVZ (Yang et al. 2008). In the naïve brain, CNTF is produced at very low levels and rapidly increases after injury (Ip et al. 1993; Kang et al. 2012) but not much is known about mechanisms that regulate cytokines such as CNTF. Understanding the mechanisms that regulate CNTF compared to other cytokines would help to develop specific pharmacological therapeutic strategies to address the low CNS bioavailability and serious side effects of systemic administration of CNTF protein (Thoenen and Sendtner 2002). We previously identified a novel inhibitory pathway consisting of integrins and downstream FAK and JNK using cultured C6 astroglioma cells (Keasey et al. 2013). Pharmacological inhibition of FAK or JNK induced CNTF in vitro. In mice, intrastriatal or systemic FAK inhibition increased CNTF and SVZ neurogenesis. Integrin receptors also can activate other MAP kinases through FAK, including ERK and p38 MAP kinase (Giancotti and Ruoslahti 1999; Hunter and Eckhart 2004; Staquicini et al. 2009). To identify pharmacological targets that might be more selective than FAK, we here determined the roles of JNK, P38 and ERK in regulating CNTF and neurogenesis in the adult mouse SVZ.

CNTF belongs to a cytokine family that activates the gp130 receptor, including LIF and IL-6 (Zigmond 2011). LIF promotes stem cell self-renewal (Gregg and Weiss 2005; Pitman et al. 2004; Shimazaki et al. 2001) at the expense of neuroblast formation (neurogenesis) in the SVZ (Bauer and Patterson 2006). IL-6 inhibits progenitor proliferation and neuronal differentiation (Bowen et al. 2011; Covey et al. 2011). LIF and IL-6 seem to initially inhibit the CNTF-mediated increase in neurogenesis that occurs after an ischemic stroke in mice (Kang et al. 2013). The mechanisms that regulate LIF and IL-6 in the SVZ are unknown. Identifying such intracellular signaling pathways may help to therapeutically modulate their expression in the CNS in disorders characterized by excessive inflammation. Our previous study showed that FAK inhibition reduced LIF and IL-6 expression in cultured C6 cells (Keasey et al. 2018). Here, we determined whether JNK, P38 and ERK downstream from FAK might regulate LIF and IL-6.

Materials and methods

Animals

A total of 243 mice were used. Adult male C57BL/6 (8–10 weeks old, JAX Stock 000664) were purchased from Jackson Laboratory. Heterozygous CNTF knockout mice (Emsley and Hagg 2003; Kang et al. 2013; Valenzuela et al. 2003; Yang et al. 2008) were bred to produce F1 sex-matched littermates for experiments. CNTF breeders were originally obtained from Regeneron Pharmaceuticals and were on a C57BL/6 × 129Sv background. We have backcrossed them 7 times into the JAX C57BL/6 line. In these mice, a lacZ gene was inserted in the deleted CNTF locus so that CNTF expression can be indirectly assessed by β-galactosidase protein expression (Kang et al. 2012; Valenzuela et al. 2003; Yang et al. 2008). We use heterozygous CNTF+/− mice for such reporter studies to retain CNTF expression. They respond to stroke, which promotes neurogenesis through CNTF, in the same way as the wildtype CNTF+/+ mice (Kang et al. 2013). Mice with floxed alleles of FAK (B6;129X1-*Ptk2*^{tm1Lfr}/Mmucd, RRID:MMRRC_009967-UCD, N3 generation C57BL/6NHsd background) were purchased from MMRRC at the University of California at Davis. Glial fibrillary acid protein (GFAP)-cre mice expressing tamoxifen-inducible Cre recombinase specifically in GFAP-positive astrocytes (B6.Cg-Tg(GFAP-cre/ERT2)505Fmv/J, JAX Stock 012849, C57BL/6 background and backcrossed for at least 8 generations) were from the Jackson Laboratory. After cross-breeding, GFAP-cre heterozygous FAK-flox offspring were backcrossed with FAK-flox mice to obtained homozygous FAK-flox mice with GFAP-cre (FAK$^{fl/fl}$-GFAP^{cre}, conditional knockout mice) and control FAK-flox littermates (FAK$^{fl/fl}$). To induce Cre recombinase-mediated excision of LoxP sites, FAK$^{fl/fl}$-GFAP^{cre} mice (8–10 weeks old) were injected with tamoxifen twice a day with the first injection between 9 and 10 am and the second injection between 3 and 4 pm (i.p., 100 mg/kg) each day for 5 days. Experiments were performed 14 days after the last injection. FAK$^{fl/fl}$ controls were treated the same way. Tamoxifen (T5648, Sigma-Aldrich) was dissolved in sunflower oil (20 mg/ml) at 37 °C overnight and stored at 4 °C in the dark for up to 7 days (Kim et al. 2014). Genotyping of tail snips was performed according to the protocols provided by the suppliers. Both male and female mice at 8–12 weeks old were used in the experiments and no sex difference was detected in our experiments. All mice were housed in accordance with the Association for the Assessment and Accreditation of Laboratory Animal Care (AALAC)

with food and water available ad libitum, and maintained on a 12 h light:12 h dark on/off cycle. All procedures were approved by the East Tennessee State University Committee on Animal Care which is in compliance with the NIH Guide on Care and Use of Animals.

Drug treatments: i.p. and intrastriatal stereotaxic injections, 5-bromo-2'-deoxyuridine (BrdU) labeling

Adult C57BL/6, or CNTF+/+ and their CNTF−/− littermates were injected once a day with phosphate-buffered saline (PBS) or FAK inhibitor 14, FAK14 (i.p., 3 mg/kg, Tocris Bioscience, #3414), for 3 days. Two hours after the last injection, they were anesthetized with Avertin (i.p., 400 mg 2, 2, 2-tribromoethanol in 20 ml of 2% 2-methyl-2-butanol in saline per gram body weight, Sigma-Aldrich, T48402). The brain was dissected and cut in the coronal plane into 2 mm slices using single sided razor blades and a mouse coronal brain slicer (World Precision Instruments, Inc). A 0.5 mm wide strip of the periventricular region, which includes the SVZ and part of the medial striatum, from the genu of the corpus callosum to the anterior commissure decussation was dissected out, flash frozen in liquid nitrogen, and stored at −80 °C for mRNA and protein analysis. In order to label proliferating cells in the SVZ in the FAK14 experiments, mice received daily BrdU (i.p., 50 mg/kg, Sigma-Aldrich, B5002) or 5-ethynyl-2'-deoxyuridine (EdU, i.p., 50 mg/kg, Molecular Probes, C10337) injections 4 h following the FAK14 injections and perfused with ice cold PBS and 4% PFA 2 h after last BrdU or EdU injection. For the BrdU pulse-chase study to identify neural stem cell proliferation, mice received daily PBS or FAK14 followed by BrdU 4 h later, for 3 days. After another 20 days, mice were processed for histology. For FAK conditional knockout mice, 14 days after the last tamoxifen injection, fresh SVZ tissue was collected or BrdU was given for 3 days and these mice processed for histology.

Intrastriatal injections were performed as previously described (Kang et al. 2012). Briefly, anesthetized mice were mounted into a Kopf stereotaxic apparatus with two ear bars and a tooth bar set at 0 mm. A burr hole was drilled at coordinates +1 mm rostrocaudal and 1.5 mm lateral from Bregma. A 10 μl Hamilton syringe was loaded with one of the inhibitors and held by a Model 5000 Microinjection unit (David Kopf Instruments) and lowered to 3.5 mm dorsoventral from the dura into the middle of striatum. After 2 min, 1 μl of test drug was injected over a 3 min period followed by a 2 min pause to prevent backflow. Mice were injected on both sides. One side was used for mRNA analysis and the other side was used for protein analysis. The injected drugs included PBS, vehicle specific to various drugs (0.05–5% DMSO), FAK14 (1 μg/μl, Tocris Bioscience, #3414, (Cabrita et al. 2011; Keasey et al. 2013)), SP600125 (JNK inhibitor, 10 μg/μl, Sigma-Aldrich, S5567, (Guan et al. 2006)), SB203580 (P38 MAP kinase inhibitor, 10 μg/μl, Sigma-Aldrich, S8037, (Ma et al. 2011)) or U0126 (ERK inhibitor, 10 μg/μl, Tocris Bioscience, #1144, (Maddahi and Edvinsson 2010)). The activity and specificity of these drugs has been documented in the cited references. The periventricular region including SVZ and medial striatum was collected at 4 or 24 h, flash frozen in liquid nitrogen and stored at −80 °C until mRNA and protein extraction analyses. To label proliferating cells following intrastriatal injection of JNK inhibitor or P38 inhibitor, mice were injected with BrdU (i.p., 144 mg/kg) at 21, 24 and 27 h and perfused with ice-cold PBS and 4% PFA at 48 h (Jia and Hegg 2012; Jia and Hegg 2015).

Immunohistochemistry

Coronal brain sections (30 μm) were washed with PBS, permeabilized with 0.3% triton x-100, incubated with 0.3% hydrogen peroxide for 30 min at room temperature to quench endogenous peroxidases, and blocked with 10% normal donkey serum. Tissue sections were then incubated with 2 M HCl for 30 min at 65 °C to denature DNA before applying BrdU primary antibody (rat anti-BrdU, 1:1000, Abcam ab6326, Clone BU1/75 (ICR1), Research Resource Identifier (RRID) http://antibodyregistry.org: RRID: AB_305426, (Hagimoto et al. 2017)) overnight at 4 °C as described previously (Jia and Hegg 2015). Biotinylated donkey anti-rat IgG (1:200, Vector Laboratory, PK6104), avidin-biotin complex conjugated with peroxidase (1:50, Vector Laboratory, PK6104) and a 3,3'-diaminobenzidine (DAB) reaction (Vector Laboratory, SK4100) were used to visualize BrdU immunostaining. EdU-positive cells were visualized by Click-IT EDU Alex Fluor 488 image Kit (ThermoFisher Scientific, C10337). For doublecortin (DCX) and β-gal CNTF reporter staining, brain sections were washed with PBS, permeabilized with 0.3% triton x-100, blocked with 10% bovine serum albumin and then incubated with goat anti-DCX (1:500, Santa Cruz Biotechnology, SC-8066, RRID: AB_2088494, (Farrar et al. 2005)), or mouse anti- β-gal (1:500, Promega, Z378A, RRID: AB_2313752 (Kang et al. 2012; Yang et al. 2008)) antibody overnight at 4 °C. Immunostaining was detected by incubation for 1 h in Alex Fluor 594-conjugated donkey anti-goat or mouse (1:200, Molecular Probe, Cat# A11080 or A11005) secondary antibody solution. The nuclei were counterstained with DAPI (Molecular Probes, Cat#H1399). Immunoreactivity was visualized on a Leica TCS SP8 confocal laser scanning microscope. Antibody specificity was further tested by using isotype specific purified IgG instead of primary antibody, and by omitting the primary or secondary antibody. No immunoreactivity was observed in any of the controls.

Cell counts

Every sixth 30 μm thick coronal section stained for BrdU through the SVZ along the rostrocaudal axis was used to perform unbiased and stereological analysis of BrdU-positive nuclei in the SVZ (total 6 sections per brain). The analysis was performed blinded to the treatments as described previously (Baker et al. 2004; Kang et al. 2013) using an optical fractionator stereological method (Stereologer, System Planning and Analysis, Alexandria, VA) and a motorized Leica DMIRE2 microscope. The reference space for the SVZ was defined as a 50 μm wide strip of the entire lateral wall of lateral ventricle, and, in the rostrocaudal axis, from the genu of the corpus callosum up to the decussation of the anterior commissure (Baker et al. 2004; Baker et al. 2005). For the pulse-chase study, the total numbers of BrdU-positive nuclei in the SVZ were counted on both sides due to the low numbers of cells. The number of DCX-positive neuroblasts was counted independently by two persons blinded to the treatments within the most populated dorsolateral part of SVZ with an area size of 640 μm × 480 μm. DCX-positive cells were identified by DAPI-labeled nuclei in four sections at 180 μm distances along the rostrocaudal axis of the SVZ. The optical density of β-gal in the dorsolateral part of the SVZ was measured (4 sections/brain, spaced 180 μm apart) with an area size of 640 μm × 480 μm using LAS X software (Leica Microsystems). The optical density of β-gal in the cortex dorsal to the corpus callosum was measured using the same set of sections with an area size of 640 μm × 480 μm.

Western blotting

The SVZ was homogenized by sonication in 100 μl RIPA buffer supplemented with protease and phosphatase inhibitors. The concentration of protein in the homogenate was measured using a bicinchoninic acid (BCA) protein assay kit (Thermo Scientific, #23228). Homogenates were resolved by electrophoresis and transferred to a PVDF membrane. After incubation with blocking buffer containing 0.1% Tween-20 and 5% non-fat milk, the membranes were probed overnight at 4 °C with the following phospho-specific antibodies. The following antibodies were all from Cell Signaling (Danvers, MA): rabbit anti-pFAK (1:1000, #3283S, RRID AB_2173659), rabbit anti-pJNK (1:1000, #9251, RRID AB_331659), rabbit anti-pP38 MAP kinase (1:1000, #4511, RRID AB_2139682) and rabbit anti-pERK (1:2000, #9106, RRID AB_331768). Mouse anti-CNTF (1:500, MAB338, clone 4–68, Millipore, Temecula, CA, RRID AB_2083064) was from Millipore (Temecula, CA) (Keasey et al. 2013). After washing with wash buffer containing 0.1% Tween-20, the membranes were incubated with HRP-labeled secondary antibody (1:1000, Cell signaling). Immunoreactive proteins were detected with ECL and imaged using the Odyssey® Fc Imaging System (LI-COR Biotechnology). The membranes were then stripped with Restore™ plus western blot stripping buffer (Thermo Scientific, #21059), re-blocked with blocking buffer and re-probed with the following primary antibodies against total protein, all from Cell Signaling ((Danvers, MA): rabbit anti-FAK (1:1000, #3285, RRID AB_10829239), rabbit anti-JNK (1:1000, #9252, RRID AB_2250373), rabbit anti-P38 MAP kinase (1:1000, #9212, RRID AB_330713), rabbit anti-ERK antibody (1:2000, #9102, RRID AB_330744) or rabbit anti-α tubulin (1:2000, #2125, RRID AB_2619646) overnight at 4 °C followed by HRP-labeled secondary antibody (1:1000). Protein expression was quantified via Image Studio Lite Ver 5.0 (LI-COR Biotechnology). The optical density of phospho-protein was normalized to total protein and then the data was presented as percentage or fold of the average of PBS or vehicle mice. The optical density of CNTF was normalized to α-tubulin.

RT-qPCR

RT-qPCR was performed as described previously (Kang et al. 2013). Briefly, total RNA from SVZ was isolated using RNeasy Mini Kit (Qiagen, Valencia, CA) according to manufacturer's protocol. Total RNA (1 μg) was reverse transcribed in a 25 ul reaction using random primers and MMLV-reverse transcriptase (Promega). qPCR was performed using Taqman Gene Expression Master Mix Kit (Applied Biosystems) with specific primers (Applied Biosystems), mouse GAPDH (4352932E), LIF (Mm00434762_g1), IL-6 (Mm00446190_m1), CNTF (Mm00446373_m1) and Ki67 (Mm01278608_m1) and QuantStudio 6 Flex Real-Time PCR System (Applied Biosystems). All reactions were performed in triplicate. The numbers of cycles for all genes were subtracted from GAPDH. Data were analyzed according to ΔΔCt and expressed as fold changes compared to control PBS or vehicle mice using $2^{-\Delta\Delta Ct}$ method.

Statistical analyses.

Data are presented as mean ± SEM. Statistical analyses were performed using GraphPad Prism (version 7). A value of $p \leq 0.05$ was considered to be statistically significant. A one-

way ANOVA with Bonferroni *post hoc* multiple comparisons was applied when there were three or more groups with testing for one factor. A two-way ANOVA with *post hoc* Tukey multiple comparisons was used when the groups were four or more and there were two factors to be tested, such as genotypes and treatments. If only two groups were compared, a Student's t-test was used.

Results

Intracerebral FAK inhibition reduces JNK activation which increases neurogenesis in the adult mouse SVZ through CNTF

We previously showed that FAK and JNK repress CNTF expression in vitro astroglioma C6 cells (Keasey et al. 2013). Here, male C57BL/6 mice were injected with the FAK inhibitor FAK14 into the striatum (Fig. 1A). After 24 h, phosphorylation of FAK (pFAK) in the periventricular region containing the SVZ was reduced by 42% (Fig. 1B,D) and phosphorylation of JNK (pJNK) was decreased by 25% (Fig. 1C,E) compared to PBS injections. In the same mice, CNTF mRNA was increased by 53% (Fig. 1F), which is consistent with our previous study (Keasey et al. 2013). CNTF levels were not different between naïve mice and those injected with PBS at 24 h (Supplemental Fig 1), suggesting that the intracerebral injection itself did not contribute to the increase in CNTF levels after FAK14. These data suggest that the FAK-JNK pathway represses CNTF expression in the adult mouse SVZ and surrounding tissue.

Intrastriatal injection of the JNK inhibitor, SP600125, significantly reduced pJNK in the periventricular region of C57BL/6 mice at 4 h (10 μg, Fig. 2A,B). At 24 h, the level of pJNK had returned to control levels (Fig. 2C,D). In concert, SP600125 increased CNTF mRNA and protein expression, but not LIF and IL-6 mRNA expression, in the periventricular region at 4 h (Fig. 2E–G). This suggests that JNK has specificity in regulating CNTF compared to these related cytokines. Next, we tested whether the increase in CNTF caused by JNK inhibition would promote SVZ neurogenesis. Intrastriatal inhibition of JNK by SP600125 in C57BL/6 mice increased Ki67 mRNA expression at 4 h in the periventricular region compared to vehicle, indicating increased proliferation (Fig. 3A; the same mice as in Fig. 2E,F). To determine whether CNTF mediates the increased cell proliferation of JNK inhibition, CNTF+/+ and CNTF−/− littermate mice received three i.p. injections of BrdU at 21, 24 and 27h following intrastriatal injection of SP600125. At 48 h, SP600125 had caused a 65% increase in BrdU-positive nuclei in the SVZ of CNTF+/+ mice compared to vehicle, as counted and calculated by unbiased stereology for the total number within the entire SVZ (28,472 ± 2,217 vs. 46,966 ± 4,143, Fig. 3B–D). There were no BrdU-positive nuclei in the neighboring medial striatum. Further, SP600125 increased the number of DCX-positive neuroblasts in the SVZ of CNTF+/+ mice two-fold compared to vehicle (85 ± 10 vs. 167 ±17 per section, Fig. 3E,F). SP600125 failed to change the number of BrdU-positive nuclei (27,608 ± 3,859 vs. 30,440 ± 6,275, Fig. 3C,D) and DCX-positive neuroblasts (73 ± 9 vs. 64 ± 15, Fig. 3E,F) in the SVZ of CNTF−/− mice. The numbers of proliferating BrdU-positive nuclei and DCX-positive neuroblasts in the SVZ of CNTF+/+ and CNTF−/− mice treated with vehicle were not significantly different (28,472 ± 2,217 vs. 27,608 ± 3,859 total, and 85 ± 10 vs. 73 ± 9 per section, respectively). This suggests that the lack of a response to the

JNK inhibitor treatment in the CNTF−/− mice was not due to different baseline levels of neurogenesis. Taken together, these data indicate that JNK plays an important and very specific role in regulating SVZ neurogenesis exclusively via repressing CNTF.

Astrocyte-specific genetic deletion of FAK increases CNTF and neurogenesis

To confirm the role of astrocytic FAK in inhibiting CNTF expression in the SVZ and periventricular region, we generated mice with tamoxifen-inducible conditional deletion of FAK in GFAP-positive astrocytes (Mori et al. 2006). The GFAP-cre-mediated deletion of FAK in the periventricular region was shown by the presence of the expected 327 bp recombined locus (Fig. 4A) in a PCR of DNA isolated from the periventricular region in $FAK^{fl/fl}$-$GFAP^{Cre}$ but not $FAK^{fl/fl}$ mice, using genotyping primers (Beggs et al. 2003). However, a substantial amount of FAK-flox allele remained present in the $FAK^{fl/fl}$-$GFAP^{Cre}$ mice, which is consistent with the relatively low abundance of GFAP-positive astrocytes in the collected tissue, including medial striatum and SVZ, compared to the more numerous other cell types (Capilla-Gonzalez et al. 2014; Doetsch et al. 1997). This cre-lox system also has a moderate efficiency in the striatum and periventricular region (Chow et al. 2008; Hirrlinger et al. 2006). Two weeks after a 5 day tamoxifen treatment, $FAK^{fl/fl}$-$GFAP^{Cre}$ mice had increased expression of CNTF (Fig. 4B) in the periventricular region (Fig. 4B) and more BrdU-positive nuclei (Fig. 4C) in the SVZ compared to control $FAK^{fl/fl}$ mice. This indicates that deletion of FAK in astrocytes increases CNTF and enhances SVZ proliferation. The levels of LIF and IL-6 were not affected by the conditional FAK knockout. These data suggest that FAK14 inhibitor-induced LIF and IL-6 may be from other cell types, and that the astrocytic FAK-JNK pathway is a specific target to increase neurogenesis.

Differential regulation of LIF and IL-6 by intracerebral inhibitors of FAK, P38 and ERK

We continued to test whether intracerebral FAK inhibition regulates cytokine expression through other MAP kinase pathways, including P38 and ERK, which are also activated by integrins and downstream FAK (Giancotti and Ruoslahti 1999; Hunter and Eckhart 2004; Staquicini et al. 2009). Intrastriatal injection of FAK14 activated P38 MAP kinase and ERK as seen by increased phosphorylation of P38 (pP38, Fig. 5A,B) and ERK (pERK, Fig. 5C,D) in periventricular region extracts. FAK14 also increased CNTF (1.5 fold), LIF (6.5 fold) and IL-6 (35.8 fold) mRNA expression in the same mice (Fig. 5E). Compared to naïve tissues, intracerebral injection of PBS did not affect CNTF, and only modestly increased IL-6 (1.8 fold) and LIF (2 fold) in the periventricular region (Supplemental Fig. 1). This suggests that the results from the injection experiments closely represent endogenous mechanisms in the naïve SVZ and not those caused by the minor striatal injury of the injection needle.

Next, intrastriatal injection of the P38 inhibitor, SB203580, into C57BL/6 mice reduced the level of pP38 in the periventricular region at 24 h but not 4 h (Fig. 6A,B). In the same 24 h mice, LIF and IL-6, but not CNTF, mRNA expression was increased (Fig. 6C). The apparently discrepant finding that FAK14 increased LIF and IL-6 despite activating P38 (Fig. 5A,B) suggests that there is a stimulatory FAK pathway which intersects downstream of P38. Intrastriatal inhibition of P38 did not alter Ki67 mRNA expression in the periventricular region at 24 h (Fig. 6D) or the number of BrdU-positive nuclei in the SVZ of another set of C57BL/6 mice at 48 h (Fig. 6E,F). Together, these data suggest that

intracerebral inhibition of P38 does not increase neurogenesis, perhaps because it increases LIF and IL-6, while not affecting CNTF expression. The ERK inhibitor, U0126, injected into the striatum of C57BL/6 mice decreased the level of pERK in the periventricular region at 4 h, which returned to control levels at 24 h (Fig. 7A,B). ERK inhibition reduced expression of CNTF and LIF mRNA in the periventricular region, without altering IL-6 mRNA (Fig. 7C), and slightly reduced Ki67 expression (Fig. 7D). Taken together the data so far show a remarkable differential regulation by FAK, JNK, P38 and ERK of these three closely related gp130-activating cytokines.

Systemic FAK inhibitor treatment selectively inhibits JNK and increases CNTF

We had previously shown that systemic injection of FAK14 stimulated CNTF expression and SVZ neurogenesis (Keasey et al. 2013). Therefore, we determined whether systemic FAK14 treatment would affect the FAK-MAPK signaling pathways and cytokine expression in the same way as the intrastriatal injections. C57BL/6 mice received 3 daily i.p. injections of PBS or FAK14 and cytokine levels in the periventricular region were measured 2 h after the last injection (50 h after the first). Systemic FAK14 decreased pFAK (Fig. 8A,C) and increased CNTF protein (Fig. 8B,D) and mRNA expression (Fig. 8E). Localization of the β-gal reporter in CNTF heterozygous mice, in which a lacZ gene was inserted in the deleted CNTF locus, revealed that CNTF expression was enriched predominantly in the SVZ of PBS injected mice (Kang et al. 2012; Valenzuela et al. 2003; Yang et al. 2008). FAK14 treatment increased CNTF expression in astrocytes of several other brain regions, including the neighboring striatum and corpus callosum white matter (Fig. 8F,F'), and overlaying cortex (Fig. 8G,G'). In the SVZ (Fig. 8H) and cortex (Fig. 8I), FAK14 caused a 4 or 6 fold increase in β-gal expression compared to PBS. This suggests that systemic FAK14 promotes CNTF expression throughout the CNS with potential implications as a therapeutic beyond SVZ neurogenesis. Systemic FAK14 treatment reduced pJNK in the periventricular region (Fig. 8J,K) but did not alter pP38 (Fig. 8L,M) or pERK (Fig. 8N,O). Expression of LIF (Fig. 8P) and IL-6 (Fig. 8Q) mRNA was not affected. This suggests that systemic FAK14 selectively promotes CNTF expression and acts specifically through inhibition of JNK (see also Fig. 3C–F).

Systemic FAK inhibitor treatment increases SVZ neurogenesis through CNTF

In a separate experiment, C57Bl/6 mice received i.p. injections of PBS or FAK14 followed by daily BrdU injections for 3 days to label proliferating cells and analyzed 2 h after the last injection. Systemic FAK14 increased the number of BrdU-positive nuclei (Fig. 9A,B) in the SVZ compared to systemic PBS. Next, we determined whether FAK14-induced neurogenesis is mediated by CNTF, by treating CNTF+/+ and CNTF−/− littermate mice the same way. In a separate experiment, such mice were injected with another labeled nucleotide, EdU, instead of BrdU. The systemic FAK14 treatment increased the total numbers of both BrdU-positive (Fig. 9C,D, 164,510 ± 11,972 vs. 240,563 ± 10,656) and EdU-positive (151,558 ± 26,889 vs. 250,913 ± 8,641, Fig. 9E) nuclei in the SVZ of CNTF +/+ mice, compared to PBS. The greater numbers compared to the data in Fig. 3 results from the longer period over which BrdU was injected (3 vs. 1 d) and the shorter time since the last injection 2 vs. 21 h). In CNTF−/− mice, FAK14 did not alter the numbers of BrdU-positive nuclei (121,832 ± 26,585 vs. 153,991 ± 13,675) or EdU-positive nuclei (159,287 ± 26,978

vs. 180,063 ± 17,449) in the SVZ (Fig. 9C–E). In the same experiments, FAK14 significantly increased DCX-positive neuroblasts in the SVZ of CNTF+/+ (120 ± 6 vs. 182 ± 15, per section) but not CNTF−/− mice (114 ± 22 vs. 102 ± 17) (Fig. 9F,G). The numbers of BrdU-, EdU- and DCX-positive cells in the SVZ of CNTF+/+ and CNTF−/− mice injected with PBS were not significantly different, suggesting that the lack of a response to FAK14 in the CNTF−/− mice was not due to different levels in baseline neurogenesis. Together, these data indicate that systemic FAK inhibition induces SVZ neurogenesis entirely through upregulation of CNTF. Systemic FAK inhibition increased neurogenesis by 45% compared to the 30% increase in the astrocyte FAK null mice. Although the difference was not statistically significant, it is possible that the efficiency of the FAK inhibitor is greater than the cre-lox recombination.

Lastly, we tested whether FAK14-induced CNTF might regulate neurogenesis by affecting slowly proliferating neural stem cells in the SVZ, by using a BrdU pulse-chase method. CNTF+/+ and CNTF−/− mice were injected daily for 3 days with PBS or FAK14 followed 4 h later by BrdU. This was followed by a 20 day wash-out period to allow BrdU-incorporated neuroblasts to migrate away from the SVZ. The BrdU-retaining cells in the SVZ represent slowly proliferating cells, such as stem cells, and post-mitotic cells (Doetsch et al. 1999; Hsu 2015). FAK14 treatment did not alter the number of BrdU-retaining cells in the SVZ of either CNTF+/+ or CNTF−/− mice (Fig. 9H). However, CNTF−/− mice had only approximately half as many BrdU-retaining cells. This suggests that acute FAK14 treatment increases neurogenesis by promoting C-cell or neuroblast proliferation through CNTF. It also suggests that chronic basal levels of CNTF helps to maintain slowly proliferating cells, such as neural stem cells, and post-mitotic cells in the SVZ.

Discussion

The main findings of this study are 1) the unique role of FAK-JNK signaling, compared to other MAPKs, in repressing CNTF expression and neurogenesis in the SVZ of adult mice, 2) the surprising differential signaling pathways that regulate the highly related cytokines LIF and IL-6, and 3) the specific role of FAK in the astrocyte in the production of these cytokines which play key roles in regulating SVZ neurogenesis.

FAK-JNK pathway inhibition uniquely increases CNTF expression in the SVZ

Intratriatal FAK inhibition with FAK14 increased CNTF expression in the SVZ, as previously seen with PF573228 (Keasey et al. 2013), another specific FAK inhibitor (Keasey et al. 2018). Intrastriatal FAK14 decreased JNK but increased P38 and ERK activation, suggesting some specificity of pathway regulation downstream of FAK. Moreover, intrastriatal JNK, but not P38 or ERK inhibition increased CNTF expression, consistent with C6 astroglioma cells (Keasey et al. 2013). The finding that systemic FAK14 increases CNTF expression but only affected pJNK and not P38 or ERK, also supports the specific role of JNK (Fig. 10). FAK-JNK-mediated repression of CNTF is unique compared to other cytokines, since intrastriatal JNK inhibition or systemic FAK14 did not affect LIF and IL-6 expression. The different effects of intrastriatal and systemic FAK inhibition on P38, ERK, and the related expression of LIF and IL-6 is potentially due to differential access of FAK14

to different cell types or cell structures. In the SVZ, CNTF is expressed by astrocytes that form densely packed end-feet around the microvasculature (Shen et al. 2008; Tavazoie et al. 2008; Yang et al. 2008). Thus, whereas intrastriatal FAK14 has access to astrocytes, microglia and neurons which all can produce LIF and IL-6 (Banner et al. 1997; Erta et al. 2012; Lau and Yu 2001; Schlaepfer et al. 2007; Van Wagoner and Benveniste 1999; Van Wagoner et al. 1999), systemic injection may primarily deliver FAK14 to astrocytes through their end-feet.

The unique role of astrocytic FAK in regulating SVZ CNTF is supported by the inducible conditional astrocyte-FAK knockout mice. LIF and IL-6 were not affected in these mice, suggesting that intrastriatal FAK14 increased their expression in cell types which remain to be identified. We have not detected LIF and IL-6 immunostaining in naïve mice, possibly due to very low protein levels (4.0 pg/ml, 0.8 pg/ml, unpublished results), consistent with no in situ hybridization signal for mRNA (Allen Brain Atlas). IL-6 and LIF staining is present in reactive astrocytes and microglia after brain injury (Block et al. 2000; Orzylowska et al. 1999; Suzuki et al. 2000; Suzuki et al. 2009), and we have seen the same after stroke in mice.

The FAK-JNK pathway represses CNTF transcription through pS727-STAT3 (Keasey et al. 2013). STAT3 may play a dual role because CNTF transcription is stimulated through the gp130-JAK-STAT3 pathway, involving pY705-STAT3 (Kang et al. 2012; Keasey et al. 2013; Shuto et al. 2001). Integrin-FAK-S727-STAT3 signaling also promotes mitochondrial function (Boengler et al. 2010; Boengler et al. 2013; Szczepanek et al. 2011; Visavadiya et al. 2016; Wegrzyn et al. 2009). FAK-mediated integrin signaling maintains normal blood brain barrier function (del Zoppo and Milner 2006). It remains to be determined whether FAK-JNK pathway inhibition affects mitochondrial or blood brain barrier function in vivo. Systemic FAK14 did not induce any obvious deficits in mice and is well tolerated in clinical trials for cancer. JNK might be a better target than FAK because it can be pro-apoptotic (Dhanasekaran and Reddy 2017) but systemic JNK inhibition remains to be tested.

Systemic FAK inhibition in β-gal CNTF reporter heterozygous mice, had increased CNTF expression in areas neighboring the SVZ, including striatum and the white matter of the corpus callosum, and overlying cortex. This may be relevant to rescuing striatal neurons in Huntington’s disease and oligodendrocytes in multiple sclerosis, respectively, given CNTF’s trophic effects in animal models (Huang and Dreyfus 2016; Ramaswamy and Kordower 2012).

FAK and JNK inhibition enhance SVZ neurogenesis through CNTF

Systemic FAK and intrastriatal JNK inhibition increased SVZ neurogenesis entirely through CNTF, as shown by increased numbers of BrdU labeled nuclei and neuroblasts in CNTF+/+, but not in CNTF−/−, littermates. The astrocyte-specific deletion of FAK increased CNTF expression and SVZ neurogenesis, again revealing the specificity of the astrocyte FAK-JNK-CNTF pathway.

Our data suggest that promoting neurogenesis requires increased CNTF without concomitant increases in LIF and/or IL-6, which are known to reduce neuroblast formation by promoting

stem cell self-renewal. (Bauer and Patterson 2006; Bowen et al. 2011; Covey et al. 2011; Gregg and Weiss 2005; Pitman et al. 2004; Shimazaki et al. 2001). Thus, the P38 inhibitor, which increased LIF and IL-6, but not CNTF, did not increase neurogenesis. In contrast, systemic FAK14, intrastriatal JNK inhibitor and astrocytic knockout of FAK increased CNTF and promoted neurogenesis, but did not affect LIF or IL-6. This is consistent with CNTF-mediated increased neurogenesis after stroke only when IL-6 expression drops to normal levels (Kang et al. 2013). The current pulse-chase experiment suggests that acute induction of CNTF by FAK14 did not affect neural stem cell proliferation. This indicates that FAK14-increased CNTF acts on progenitor cells, possibly through FGF2, as we have proposed (Kang et al. 2013).

The pulse-chase BrdU data from CNTF−/− mice also suggest that chronic basal levels of CNTF promotes neural stem cell proliferation in the SVZ. These cells can respond to CNTF because CNTFα receptors in the SVZ are exclusively expressed in GFAP-positive cells (Emsley and Hagg 2003), including the much sparser neural stem cells (Doetsch et al. 1999; Emsley and Hagg 2003; Ponti et al. 2013a; Ponti et al. 2013b). In fact, CNTF together with EGF stimulates SVZ neural stem cell self-renewal through up-regulation of notch 1 in vitro and in vivo (Chojnacki et al. 2003). It remains to be determined how long-term FAK and JNK inhibitor treatments that induce CNTF would affect neural stem cells.

P38 and ERK signaling differentially regulates CNTF, LIF and IL-6

Integrin signaling is known to activate FAK and downstream JNK, ERK and P38 MAP kinases (Giancotti and Ruoslahti 1999; Hunter and Eckhart 2004; Staquicini et al. 2009). Surprisingly, there were substantial differential effects of inhibiting these MAPKs on the expression of the highly related CNTF, LIF and IL-6. P38 inhibition increased LIF and IL-6, but not CNTF, while ERK inhibition reduced CNTF and LIF, but not IL-6 (Fig. 10). LIF expression in Schwann cells is increased by the cAMP-PKCβ-ERK pathway (Matsuoka et al. 1997; Nagamoto-Combs et al. 1999) and cAMP induces it in cultured astrocytes (Murphy et al. 1995). However, ERK increases both LIF and CNTF (Fig. 10) and cAMP decreases CNTF expression in the SVZ (Yang et al., 2008), suggesting redundant signaling pathways involved in fine-tuning cytokine expression in the SVZ. The finding that intrastriatal FAK inhibition increases LIF would be most consistent with its activation of ERK, rather than disinhibiting P38 (Fig. 10). The opposing effects of P38 and ERK on LIF expression in the mouse periventricular region is consistent with their opposing effects on cultured neural stem cell proliferation (Kim and Wong 2009). Also, P38 inhibition can rescue their proliferation in the SVZ of ATM−/− mice (Kim and Wong 2012). P38 and ERK also have different roles on oligodendrocyte progenitor differentiation (Cui et al. 2014), and both ERK and P38 are required for progenitor to pre-oligodendrocyte differentiation, but only P38 is involved in immature oligodendrocyte differentiation. Here, intracerebral P38 inhibition did not affect neurogenesis after 48 h, despite induction of LIF and IL-6, perhaps because the effects on the neural stem cells require longer times to subsequently affect progenitor proliferation. CNTF and IL-6 expression is downstream of STAT3 signaling (Keasey et al. 2013; Shuto et al. 2001; Sumimoto et al. 2006), suggesting that JNK, P38 and ERK are co-regulators, consistent with their known role in STAT3 phosphorylation. However, it remains to be determined how the differential signaling occurs.

The finding that intrastriatal FAK inhibition increases IL-6 expression is in apparent disagreement with a previous report that FAK mediates TNFα-induced IL-6 expression in cultured cancer cells and fibroblasts (Schlaepfer et al., 2007). Moreover, FAK inhibition also down-regulates IL-6 in C6 cells (Keasey et al. 2018). This raises the possibility that there are major differences between proliferating and non-proliferating cells. In fact, systemic FAK inhibition reduces stroke-induced IL-6 expression in adult mice (unpublished data), which would be consistent with the proliferating astrocytes and microglia. The finding that intrastriatal FAK inhibitor stimulates IL-6 expression and activates P38, whereas the P38 inhibitor also increased IL-6 expression, suggests that there is another IL-6 inhibiting pathway(s) downstream from FAK (stippled line in Fig. 10).

Conclusion

Collectively, these results indicate that CNTF plays a key role in promoting SVZ neurogenesis and is uniquely regulated by FAK-JNK signaling in astrocytes. JNK inhibition represents a novel method for promoting adult SVZ neurogenesis. Our data also reveal differential regulation mechanisms that may provide opportunities to individually regulate these cytokines in glial cells as a potential therapeutic approach for a variety of neurological disorders such as stroke, by increasing neurogenesis as well as neuronal and glial protection, and/or reducing excessive detrimental inflammation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Main points:

FAK-JNK signaling has a unique role in inhibiting CNTF expression in astrocytes and CNTF-mediated neurogenesis of the adult mouse SVZ compared to the differential regulation by FAK, P38 and ERK signaling of closely related anti-neurogenic LIF and IL-6.

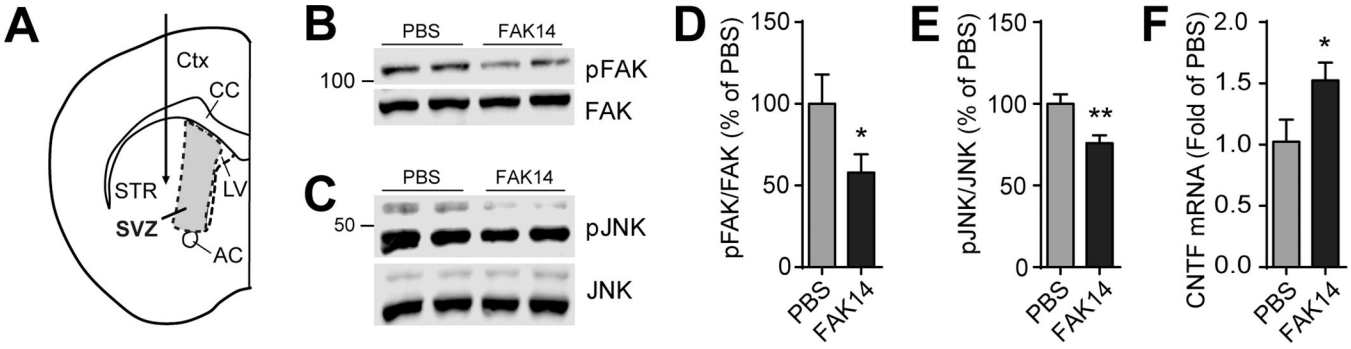

Figure 1. Intrastriatal FAK inhibition reduces JNK phosphorylation and increases CNTF expression in the adult mouse periventricular region.

A) Schematic showing the intrastriatal injection site (arrow) and collected tissue of periventricular region containing the SVZ (0.5 mm gray area). AC=anterior commissure, CC=corpus callosum, Ctx=cortex, LV=lateral ventricle, STR=striatum. **B**) Intrastriatal injection of the water soluble FAK inhibitor, FAK14 (1 μg/μl), reduced FAK activation in the periventricular region of adult C57BL/6 mice at 24 h, as shown by reduced pFAK compared to total FAK in representative western blots of individual mice. **C**) FAK14 injection also reduced phosphorylation of JNK (pJNK). **D,E**) Quantification by densitometry. **F)** FAK14 increased CNTF mRNA expression in the periventricular region in the same mice. Data are mean + SEM, PBS, n=5 and FAK14, 5 mice, Student t test, * $p<0.05$, ** $p<0.01$.

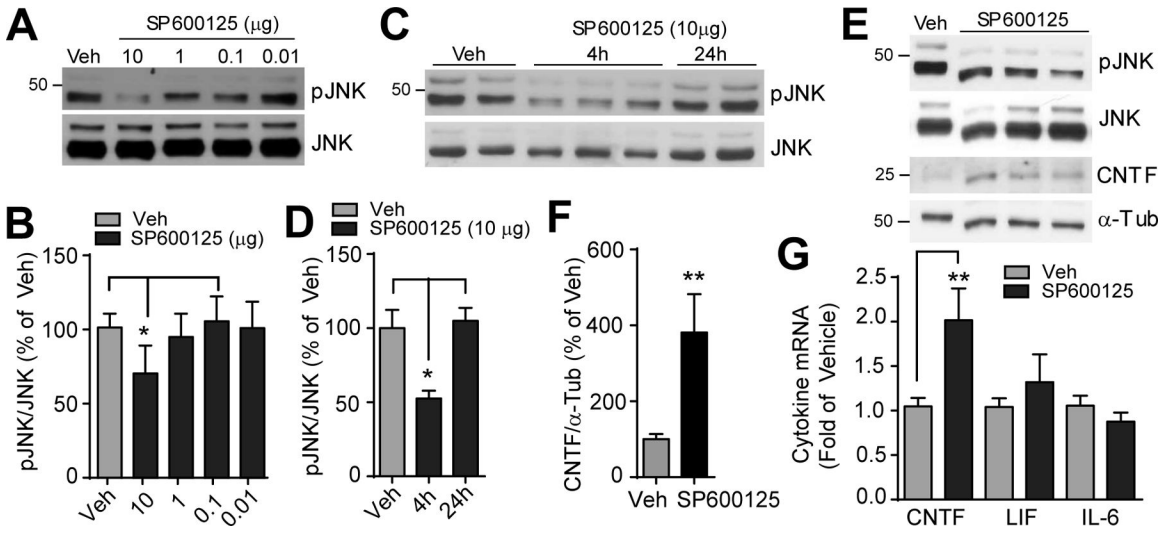

Figure 2. Intrastriatal JNK inhibition increases CNTF without affecting LIF and IL-6 expression in the adult mouse periventricular region.

A,B) Intrastriatal injection of JNK inhibitor, SP600125, at 10 μg, decreased phosphorylation of JNK in the periventricular region at 4 h compared to vehicle (veh) injections as shown by reduced pJNK in representative western blots of individual mice and densitometry quantification (n=7, 4, 4, 3 and 4 mice, One-way ANOVA followed by *post hoc* Bonferroni multiple comparison tests, * $p<0.05$). **C,D)** At 24 h following intrastriatal injection of SP600125, the levels of pJNK in the periventricular region returned to control levels as shown by a representative blot and densitometry quantification (n=10, 5 and 5 mice, One-way ANOVA followed by *post hoc* Bonferroni multiple comparison tests). The intrastriatal JNK inhibition increased CNTF protein **E,F)** and mRNA **G)** at 4 h, while LIF and IL-6 **G)** expression was not affected (n=10 veh and 5 SP600125, Student t test, ** $p<0.01$).

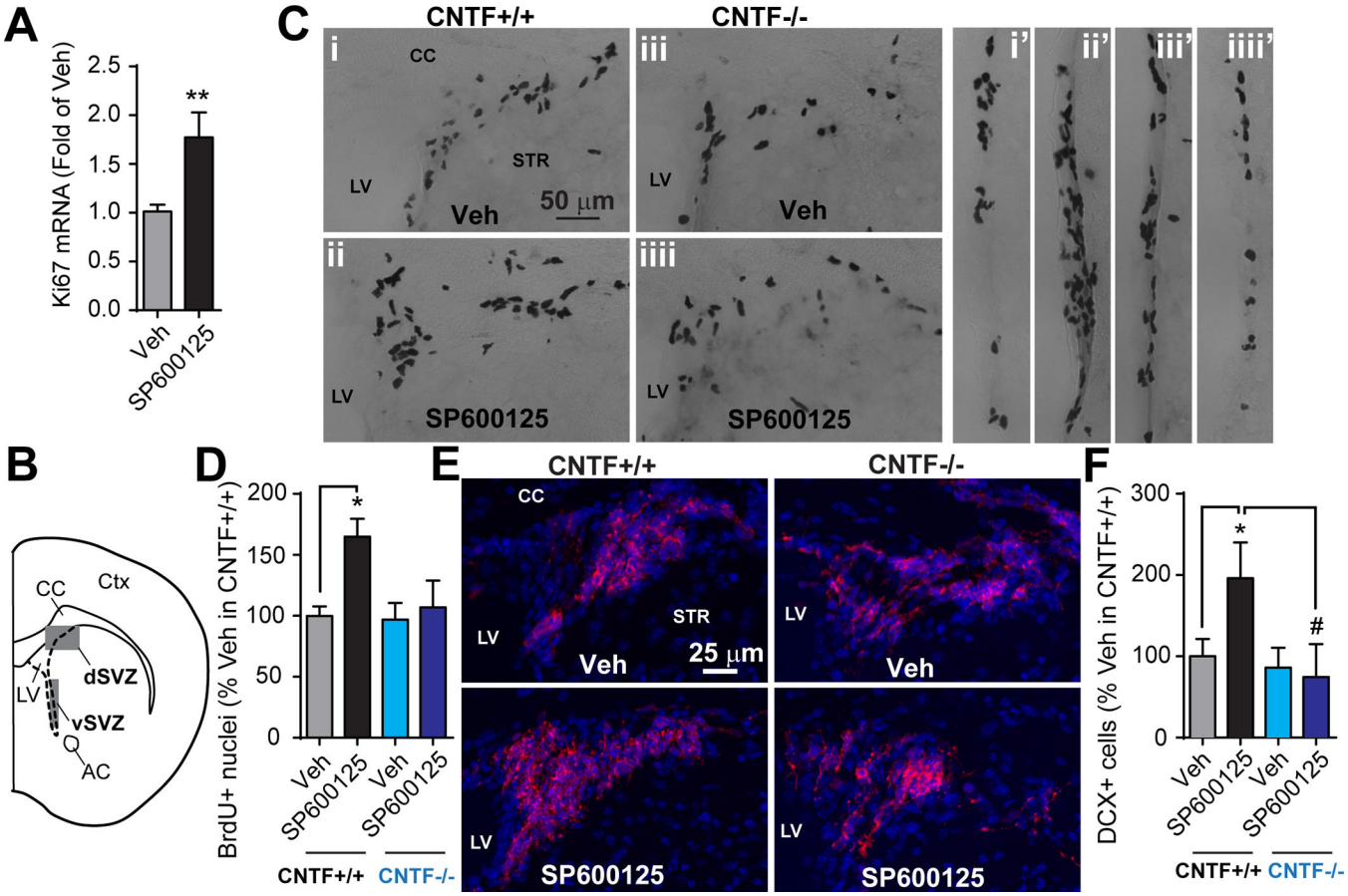

Figure 3. Intrastriatal JNK inhibition increases SVZ neurogenesis through CNTF.

A) Intrastriatal injection of the JNK inhibitor, SP600125, increased the expression of the cell proliferation marker Ki67 at 4 h in the periventricular region of the same C57BL/6 mice as shown in Fig. 2E,F (n=10 vehicle (veh) and 5 SP600125, ** $p<0.01$, Student t test). **B)** Schematic showing the location of the images taken from the dorsal (dSVZ) and ventral (vSVZ) SVZ in C and E. AC=anterior commissure, CC=corpus callosum, Ctx=cortex, LV=lateral ventricle. **C)** Representative images of BrdU immunostaining in the SVZ of CNTF+/+ and CNTF−/− mice show that intrastriatal injection of JNK inhibitor increases cell proliferation at 48 h in the SVZ of CNTF+/+ but not CNTF−/− littermate mice. BrdU was given by i.p. injection at 21, 24 and 27 h following intrastriatal injection. STR=striatum. Images i-iiii are from the dorsal SVZ and images i'-iiii' from the corresponding ventral SVZ. **D)** This was confirmed by unbiased stereological counts of the BrdU-positive nuclei in the entire SVZ at 48 h. Data were calculated as a percentage of the group average of vehicle injected CNTF+/+ mice (n=5 mice/group, two-way ANOVA followed by Tukey multiple comparison tests, * $p<0.05$). **E)** Representative images of DCX immunostaining in the SVZ show that intrastriatal injection of SP600125 increases neurogenesis at 48 h in the SVZ of CNTF+/+ but not CNTF−/− mice. Images are from the dorsolateral SVZ. **F)** Quantification of the DCX-positive neuroblasts in the SVZ at 48 h. Data were calculated as a percentage of the group average of vehicle injected CNTF+/+ mice (Two-way ANOVA followed by Tukey multiple comparison tests, * or # $p<0.05$).

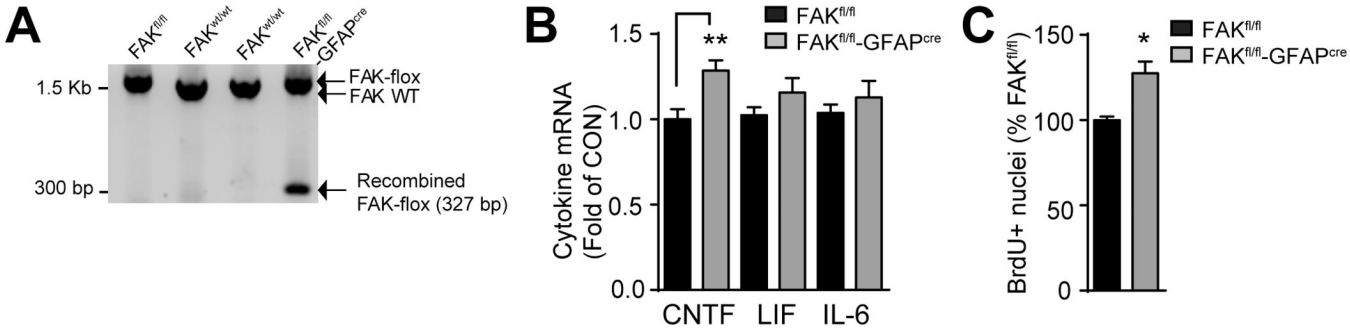

Figure 4. Inducible conditional knockout of FAK in GFAP-positive astrocytes increases CNTF and neurogenesis in the SVZ.

A) PCR of DNA isolated from the periventricular region of adult Wildtype (WT), $FAK^{fl/fl}$ and $FAK^{fl/fl}$-$GFAP^{cre}$ mice treated with tamoxifen, using genotyping primers showed an expected 1.4 kb wildtype allele in wildtype mice, the expected 1.6 kb FAK-flox allele in $FAK^{fl/fl}$ and $FAK^{fl/fl}$-$GFAP^{cre}$ mice and an expected 327 bp recombined locus only in $FAK^{fl/fl}$-$GFAP^{cre}$ mice. The conditional knockdown of FAK in GFAP+ astrocytes increased CNTF mRNA **B)** in the periventricular region and BrdU-positive nuclei **C)** in the SVZ (n=8 and 7 mice for mRNA analysis, N=9 and 7 mice for BrdU analysis, Student t test, * $p<0.05$, ** $p<0.01$).

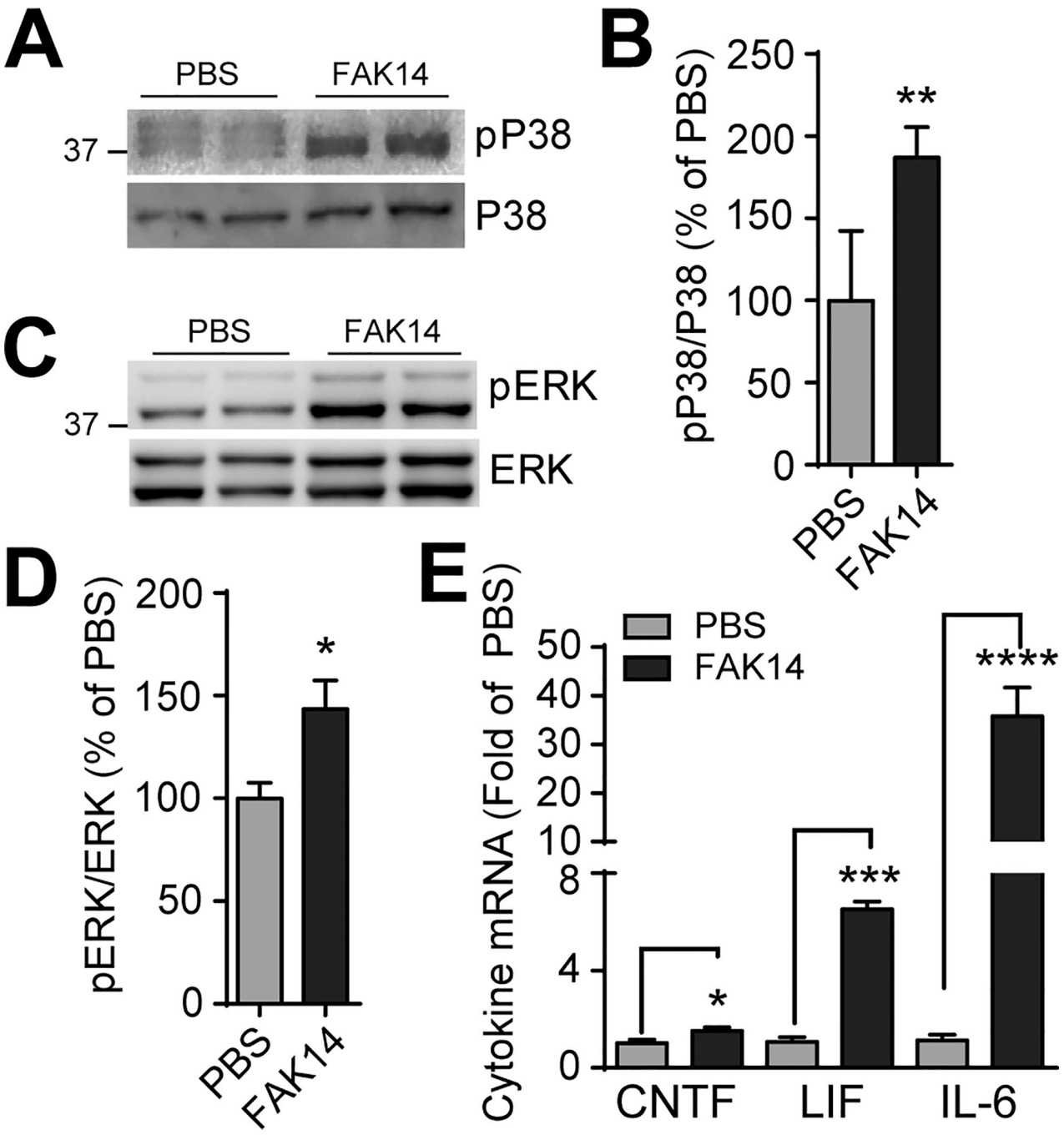

Figure 5. Intrastriatal FAK inhibition activates P38 and ERK and increased LIF and IL-6 expression in the adult mouse periventricular region.

Intrastriatal injection of FAK14 increased phosphorylation of P38 **A,B)** and ERK **C,D)** in the periventricular region of adult C57BL/6 mice at 24 h compared to control PBS injections, as shown by increased pP38 and pERK in representative western blots of individual mice (A,C) and densitometry quantification (B,D, n=5 and 5 mice). **E)** In the same mice, FAK14 increased CNTF (same data as in Fig. 1F), LIF and IL-6 mRNA

expression in the periventricular region (Note the different scale for the IL-6 values; Student t test, *, **, *** or **** $p<0.05$, 0.01, 0.001 or 0.0001).

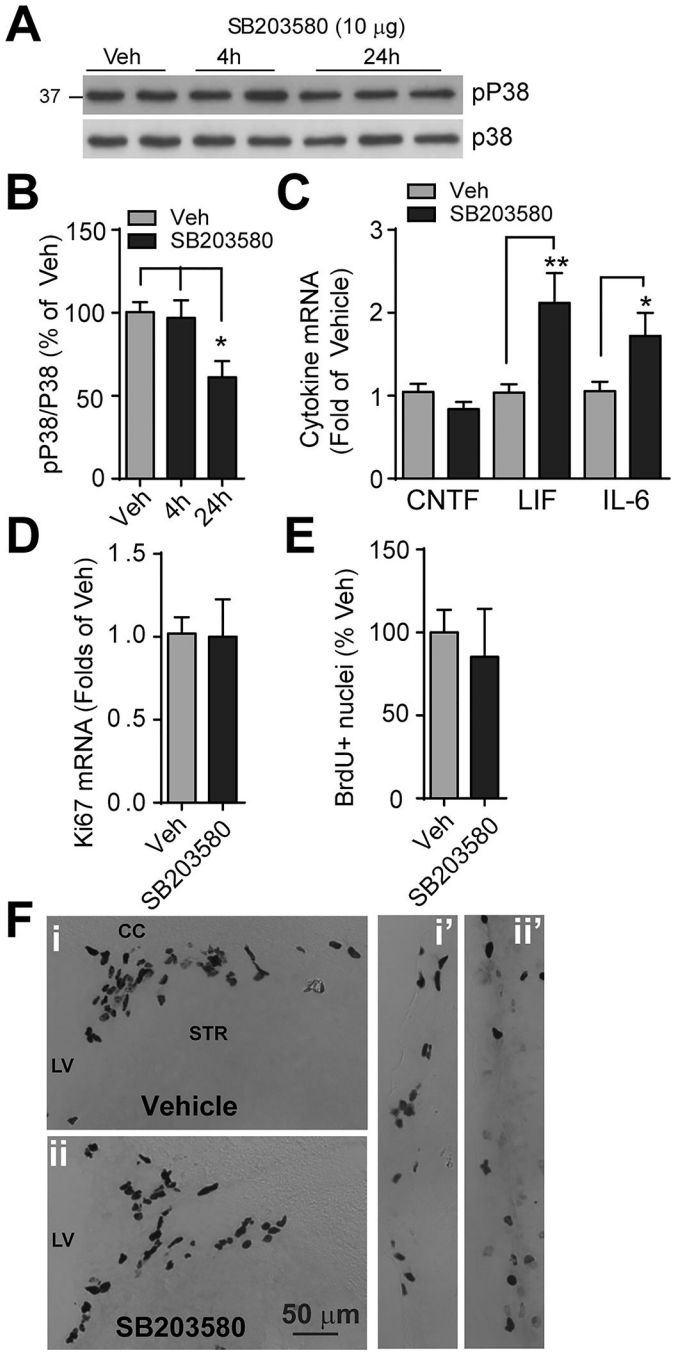

Figure 6. Intrastriatal P38 inhibition increases LIF and IL-6 expression without affecting neurogenesis in the adult mouse SVZ.

Intrastriatal injection of the P38 inhibitor, SB203580, reduced phosphorylation of P38 in the periventricular region of adult C57BL/6 mice at 24 h compared to injection of control vehicle, as shown by reduced pP38 in a representative western blot of individual mice (**A**) and densitometry quantification (**B**, n=9, 5 and 5 mice, one-way ANOVA followed by Bonferroni multiple comparison tests, * $p<0.05$). **C**) In the same mice, P38 inhibition increased LIF and IL-6 expression at 24 h, without altering CNTF, in the periventricular

region (Student t test, * or ** p<0.05 or 0.01). **D)** Intrastriatal P38 inhibition did not change expression of Ki67, a marker for cell proliferation, in the same mice as C. **E)** Quantification of the BrdU-positive nuclei in the entire SVZ using unbiased stereology shows that intrastriatal P38 inhibition does not affect cell proliferation in the SVZ at 48 h. Data were calculated as a percentage of the group average of Veh (n=5 and 5 mice, Student t test). **F)** Representative images of BrdU immunostaining in the SVZ of C57BL/6 mice at 48 h following intrastriatal injection. CC= corpus callosum, LV=lateral ventricle, STR=striatum. Images i-ii are from dorsal SVZ and images i'-ii' from the ventral SVZ.

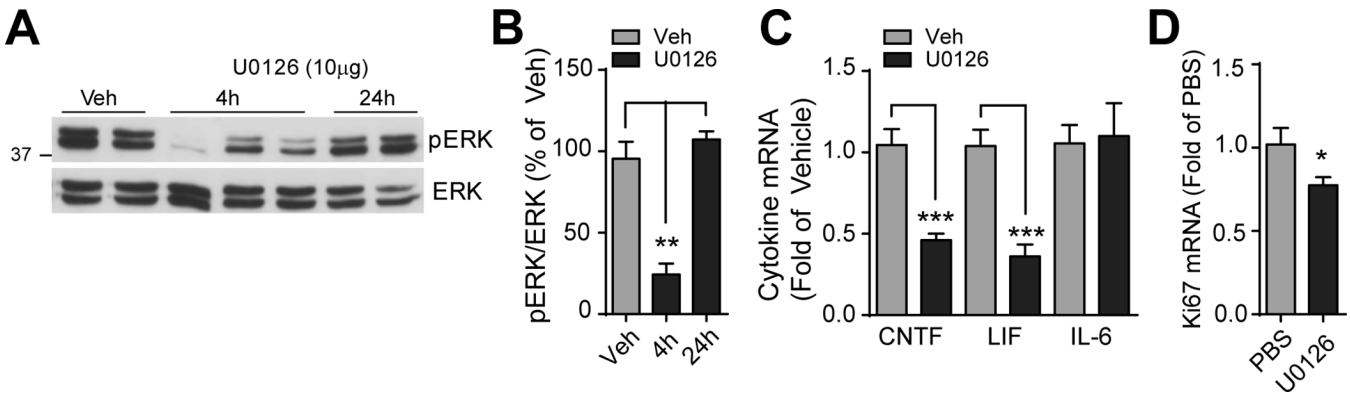

Figure 7. Intrastriatal ERK inhibition decreases CNTF, LIF and cell proliferation in the adult mouse SVZ.

A,B) Intrastriatal injection of the ERK inhibitor, U0126 (10 μg), reduced activation of ERK in the periventricular region of C57BL/6 mice at 4 h, but not 24 h, as shown by decreased pERK in a representative western blot of individual mice (A) and densitometry quantification (B, n=9, 5 and 5 mice, one-way ANOVA followed by Bonferroni multiple comparison tests, ** $p<0.01$). **C**) Intrastriatal injection of U0126 reduced CNTF and LIF, but not IL-6, expression in the periventricular region of the same mice at 4 h (student t test, *** $p<0.001$). **D**) Intrastriatal ERK inhibition reduced SVZ cell proliferation measured by Ki67 at 4 h (same extracts as in C, student t test, * $p<0.05$).

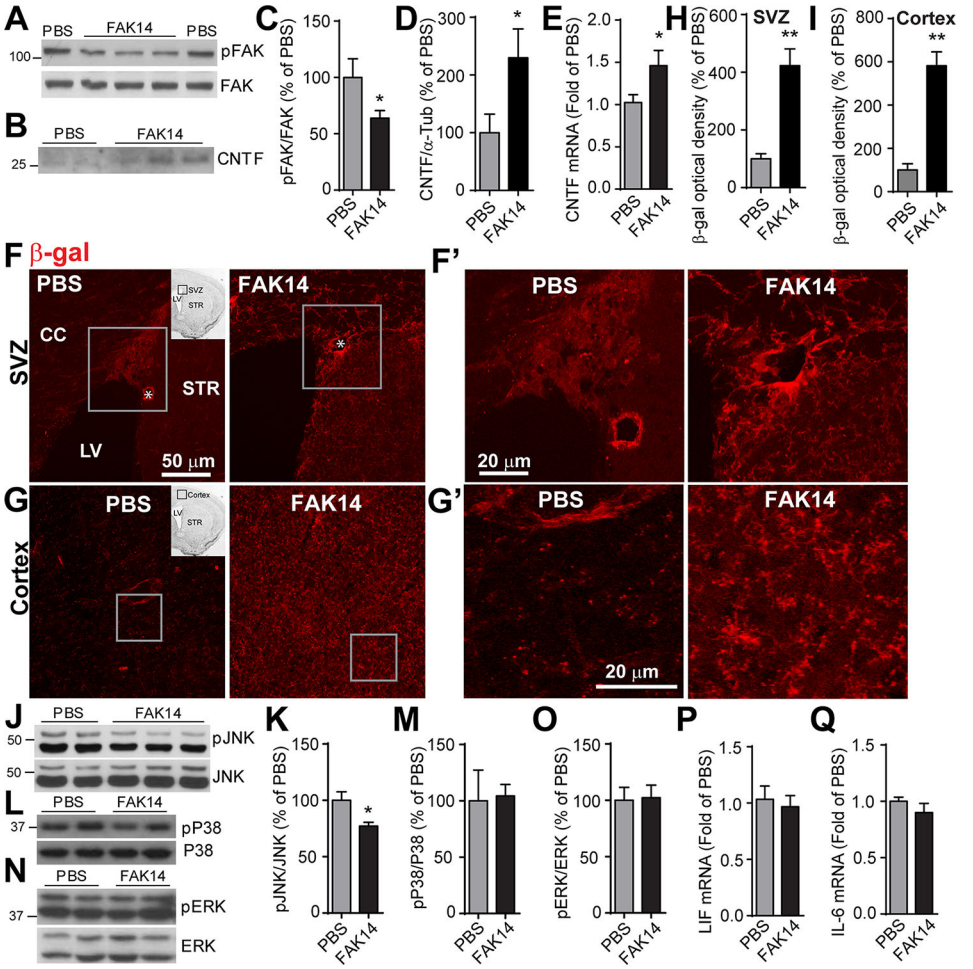

Figure 8. Systemic FAK inhibition reduces pJNK and increases CNTF expression in adult mouse periventricular region.

A,C) Systemic i.p. injection of FAK14 for 3 days in C57BL/6 mice reduced phosphorylation of FAK in the periventricular region at 2 h following last injection as shown by a representative western blot of individual mice (A) and densitometry quantification (C, n=6 and 6 mice, Student t test, * $p<0.05$). Systemic FAK14 treatment also increased CNTF protein **B,D)** and mRNA **E**) expression in the periventricular region of the same mice. **F,G)** Representative images of β-gal immunostaining in the SVZ and cortex of heterozygous CNTF+/− mice with a lacZ gene inserted in one of the deleted CNTF loci, at 2 h following 3 days of systemic treatment with PBS or FAK14. FAK14 treatment increased β-galactosidase expression in the SVZ, striatum (STR), corpus callosum (CC) and overlaying cortex. The black boxes in the half brain insets indicate the location of the SVZ and cortex images. LV=lateral ventricle, * blood vessel. The white boxes indicate the localization of the higher magnification images in F' and G'. FAK14 increased 4 fold of β-gal expression in the periventricular region (**H)** and 6 fold in the cortex (**I)** compared to PBS (n=5 and 4 For FAK14 and PBS group, Student t test, ** $p<0.01$). Three days of systemic FAK inhibition also reduced pJNK (**J,K**) but did not affect phosphorylation of P38 **L,M)** and ERK (**N,O**), or expression of LIF (**P)** and IL-6 (**Q)** in the periventricular region at 2 h following last systemic treatment (n=6 mice/group, same mice as in A-D, Student t test, * $p<0.05$).

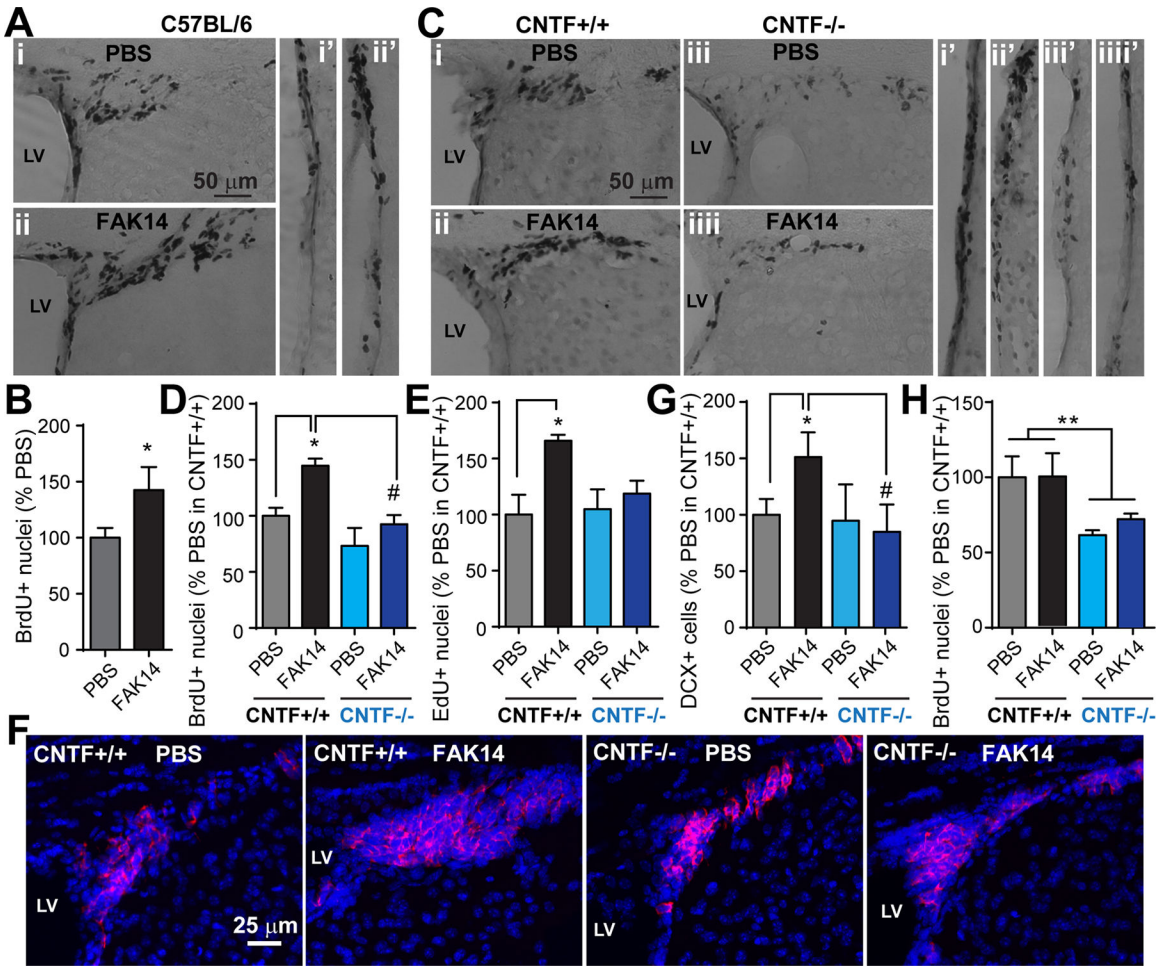

Figure 9. Systemic FAK inhibition increases SVZ neurogenesis through CNTF.

A) Representative images of BrdU immunostaining in the SVZ of C57BL/6 mice show that 3 days of systemic FAK14 treatment leads to an increase in cell proliferation at 6 h following last systemic treatment. BrdU was given 4 h after PBS or FAK14 each day and tissue collection was 2 h following last BrdU injection. LV=lateral ventricle. Images Ai-Aii are from dorsal SVZ and images Ai'-Aii' from the corresponding ventral SVZ. **B**) Quantification of the BrdU-positive nuclei in the SVZ using unbiased stereology. Data were calculated as a percentage of the group average of PBS (n=6 and 5, Student t test, * $p<0.05$). **C**) BrdU immunostaining in the SVZ of CNTF+/+ and CNTF−/− mice at 6 h following 3 days of systemic treatment with PBS or FAK14. BrdU was given 4 h after PBS or FAK14 each day and tissue collection was 2 h following last BrdU injection. Images Ci-Ciiii are from dorsal SVZ and images Ci'-Ciiii' from the corresponding ventral SVZ. LV=lateral ventricle. **D**) Quantification shows that FAK14 increases the number of BrdU-positive cells in the SVZ of CNTF+/+, but not CNTF−/−, littermate mice. There was no significant difference between PBS and FAK14 in the numbers of BrdU-positive cells in the SVZ of CNTF−/− mice. Data were calculated as a percentage of the group average of PBS in the CNTF+/+ mice (n=8, 3, 4 and 5 mice/group, Two-way ANOVA followed by Tukey multiple comparison tests, * or # $p<0.05$). **E**) In a separate experiment, FAK14 increased the number

of EdU-positive nuclei in the SVZ of CNTF+/+, but not CNTF−/−, littermates at 6 h following 3 days of systemic treatment with PBS or FAK14 (n=4, 4, 3 and 3 mice/group, Two-way ANOVA followed by Tukey multiple comparison tests, * P<0.05). **F**) Representative images of DCX immunostaining in the SVZ of CNTF+/+ and CNTF−/− mice show that 3 days of systemic FAK inhibition increases the number of neuroblasts in the SVZ of CNTF+/+, but not CNTF−/− mice at 6 h following last systemic treatment. LV=lateral ventricle. Images are from dorsal SVZ with most populated neuroblasts. Nuclei were stained with DAPI (blue) and neuroblasts for DCX (red). **G**) Quantification of the DCX-positive cells in the SVZ. Data were calculated as a percentage of the group average of PBS in CNTF+/+ mice (same mice as in D, two-way ANOVA followed by Tukey multiple comparison tests, * or # p<0.05). **H**) Systemic FAK14 treatment did not affect slowly proliferating cells, such as neural stem cells and post-mitotic cells in the SVZ, as measured by a BrdU pulse-chase method. BrdU was given 4 h after PBS or FAK14 each day and tissue collection was 20 days following last BrdU injection. CNTF−/− mice had fewer BrdU-retaining nuclei irrespective of the treatment (n=3, 3, 3 and 4 mice, Two-way ANOVA followed by Tukey multiple comparison tests, ** p<0.01).

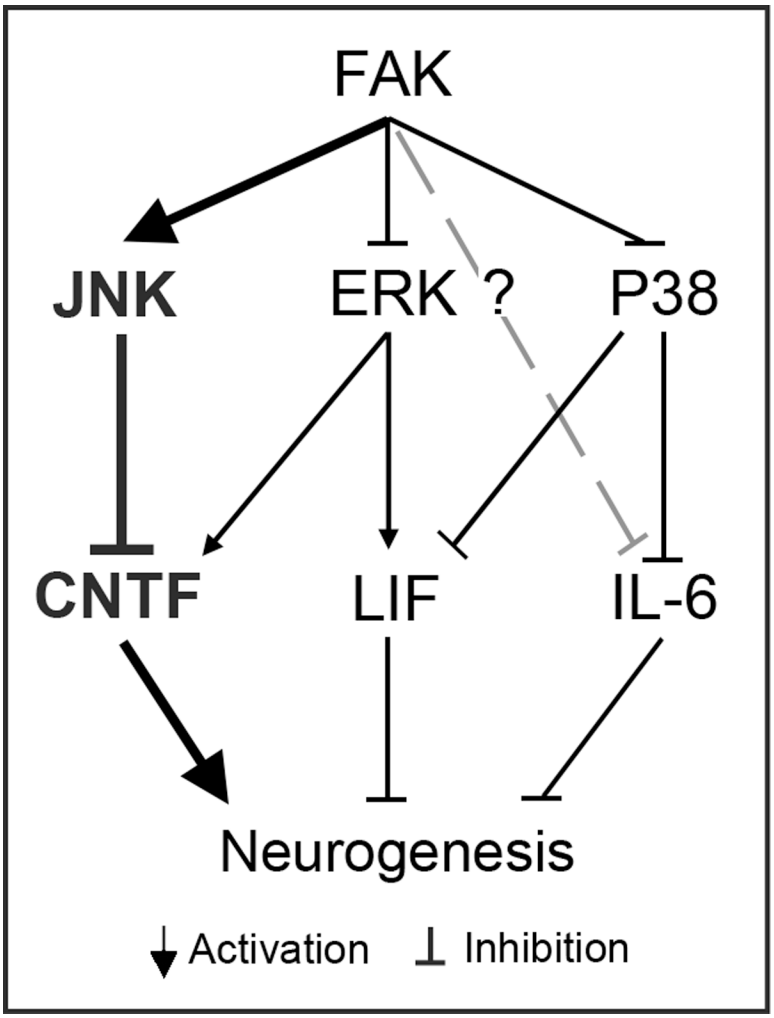

Figure 10. Summary of differential regulation of CNTF, LIF and IL-6 by MAPK in the periventricular region of adult mouse brain.

FAK represses CNTF expression specifically through JNK. Inhibition of FAK or JNK increases SVZ neurogenesis through CNTF. FAK also inhibits P38 and ERK signaling in the SVZ. ERK activates LIF and CNTF expression, while P38 reduces LIF and IL-6 expression. FAK inhibits CNTF expression via JNK activation. FAK inhibits LIF expression, possibly by inhibiting ERK. FAK inhibits IL-6 expression through unknown pathway(s) (stippled line). CNTF promotes and LIF and IL-6 counteract SVZ neuroblast formation, i.e., neurogenesis.