Development/Plasticity/Repair

Ciliary Neurotrophic Factor Mediates Dopamine D_2 Receptor-Induced CNS Neurogenesis in Adult Mice

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Neurogenesis continues in the adult forebrain subventricular zone (SVZ) and the dentate gyrus of the hippocampal formation. Degeneration of dopaminergic projections in Parkinson's disease and animals reduces, whereas ciliary neurotrophic factor (CNTF) promotes, neurogenesis. We tested whether the dopaminergic system promotes neurogenesis through CNTF. Astrocytes of the SVZ and dentate gyrus expressed CNTF and were close to dopaminergic terminals. Dopaminergic denervation in adult mice reduced CNTF mRNA by ~60%, whereas systemic treatment with the D_2 agonist quinpirole increased CNTF mRNA in the SVZ and hippocampal formation, and in cultured astrocytes by 1.5–5 fold. The effect of quinpirole *in vitro* was blocked by the D_2 antagonist eticlopride and did not cause astroglial proliferation or hypertrophy. Systemic quinpirole injections increased proliferation in wild-type mice by ~25–75% but not in CNTF $^{-/-}$ littermates or in the SVZ of mice infused with CNTF antibodies. Quinpirole increased the number of neuroblasts in wild-type but not in CNTF $^{-/-}$ littermates. Neurogenesis was reduced by ~20% in CNTF $^{-/-}$ mice, confirming the endogenous role of CNTF. Nigrostriatal denervation did not affect SVZ proliferation in CNTF $^{-/-}$ mice, suggesting that the dopaminergic innervation normally regulates neurogenesis through CNTF. Quinpirole acted on postsynaptic receptors as it reversed the reduced proliferation seen after dopaminergic denervation in wild-type mice. Thus, CNTF mediates dopaminergic innervation- and D_2 receptor-induced neurogenesis in the adult forebrain. Because CNTF is predominantly expressed in the nervous system, this mechanism and the ability to pharmacologically modulate it have implications for Parkinson's disease and cell-replacement therapies for other disorders.

Key words: **astrocyte; dopaminergic; neuroblast; quinpirole; subgranular zone; subventricular zone**

Introduction

Prominent CNS neurogenesis occurs throughout adulthood in the subventricular zone (SVZ) of the anterior lateral ventricle and the dentate gyrus of the hippocampal formation (Alvarez-Buylla and Lim, 2004; Ming and Song, 2005). Neurodegenerative diseases are characterized by progressive neuronal loss, and replacement by newly generated neurons is under consideration (Lie et al., 2004; Snyder et al., 2004; Lindvall and Kokaia, 2006). One therapeutic approach might be the pharmacological modulation of molecular regulators within the adult CNS that normally control the fate of the resident neural stem cells and their progeny. This would solve problems of poor bioavailability in the CNS of systemically delivered proteins. Many endogenous molecular regulators of neurogenesis have been identified, cooperating to specify the niches of proliferation (Hagg, 2005). We investigated the potential role of ciliary neurotrophic factor (CNTF), because it is predominantly produced in the nervous system (Stockli et al., 1989; Ip, 1998), making it a potentially selective drug target. Intracerebral injection of CNTF antibodies and CNTF caused a decrease and increase, respectively, in neurogenesis in adult mice (Emsley and Hagg, 2003), suggesting that CNTF is an important endogenous regulator. CNTF promotes self-renewal or maintenance of neural precursors *in vitro* through the Notch pathway (Chojnacki et al., 2003; Hitoshi et al., 2004). CNTF can also maintain embryonic stem cell pluripotency *in vitro* (Wolf et al., 1994). Astrocytes, which produce CNTF, promote proliferation and neuronal specification of hippocampal precursors *in vitro* (Song et al., 2002).

Our recent studies raised the possibility that dopamine released by projections from the midbrain regulates CNS neurogenesis by modulating CNTF expression. The adult SVZ and dentate gyrus are innervated by dopaminergic fibers from the substantia nigra and ventral tegmental area in the midbrain, respectively (Swanson, 1982). Dopaminergic denervation in animals and in Parkinson's disease causes a dramatic reduction in the number of proliferating cells in the SVZ and dentate gyrus (Baker et al., 2004; Hoglinger et al., 2004). Conversely, D_2 agonists can increase neurogenesis in the adult mouse SVZ (Hoglinger et al., 2004) and in the explanted embryonic SVZ (Ohtani et al., 2003). In the CNS, CNTF is produced by subsets of astrocytes (Stockli et al., 1991; Dobrea et al., 1992; Ip, 1998). Expres-

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sion of CNTF in cultured astrocytes is under negative control of cAMP (Carroll et al., 1993; Rudge et al., 1994). Conversely, dopamine D_2 receptors are inhibitory G-protein-coupled receptors that after activation cause rapid reduction of the intracellular cAMP (Vallar and Meldolesi, 1989), suggesting that D_2 stimulation might increase CNTF expression. D_2 receptors are known to be present in astrocytes (Bal et al., 1994; Khan et al., 2001). Together, this raised the possibility that dopaminergic projections from the midbrain regulate forebrain neurogenesis via a D_2-CNTF pathway.

Here, we tested whether CNTF is produced by astrocytes in the neurogenic regions of the adult mouse, whether the midbrain projections and a D_2 agonist regulate CNTF in these regions, and whether CNTF mediates the D_2 dopaminergic regulation of adult neurogenesis, using knock-out mice and neutralizing CNTF antibodies.

Materials and Methods

All animal procedures were performed according to the guidelines of the University of Louisville Institutional Animal Care and Use Committee and the National Institutes of Health guidelines. All invasive procedures were performed under deep anesthesia obtained by an intraperitoneal injection of Avertin (0.4 mg 2,2,2-tribromoethanol in 0.02 ml of 1.25% 2-methyl-2-butanol in saline per gram body weight; Sigma-Aldrich, St. Louis, MO).

CNTF protein expression in the SVZ. Male C57BL/6 mice ($n = 3$; 10 weeks of age; 18–22 g; The Jackson Laboratory, Bar Harbor, ME) were anesthetized, and 1-mm-thick coronal slices through their brains freshly dissected to obtain a 0.5 mm wide, 1.5-mm-long strip of the lateral wall of the anterior lateral ventricle and medial part of the striatum containing the SVZ. The tissue strips were homogenized and lysed on ice for 30 min in 50 μl of lysis buffer containing 1% NP-40, 0.1% SDS, 300 mM NaCl, 1 mM $MgCl_2$, 2 mM EGTA, 20 mM Tris-HCl at pH7.5, 10% glycerol, and 1% deoxycholate as well as tissue protease/phosphatase inhibitor mixture (P8340; Sigma-Aldrich). Protein concentrations were determined using a Lowry protein assay kit (Sigma-Aldrich), and total proteins were separated in a 7.5% reducing protein gel, transferred onto a PVDF membrane, and probed with a chicken anti-CNTF antibody (AB5749, IgG; Chemicon, Temecula, CA), followed with incubations with horseradish peroxidase-conjugated goat anti-rabbit IgG and ECL plus (GE Healthcare, Pittsburgh, PA). Blotting signals were visualized on x-ray film.

CNTF mRNA measurement by real time reverse transcription-PCR. For reverse transcription (RT)-PCR, total RNA was isolated from freshly dissected SVZ strips or cultures using a commercial kit (Qiagen, Valencia, CA) and used as templates in RT, which runs with 1.0 μl total RNA (1.0 μg), 1.0 μl of 100 ng/μl random primers, 2.0 μl of 20 mM dNTP mix, 1.0 μl 10× RT buffer, 4.5 μl RNase-free water, and 0.5 μl of 20U/μl StrataScript reverse transcriptase (Stratagene, La Jolla, CA). Water was used in control reactions to replace the reverse transcriptase. RT reactions were performed at 25°C for 10 min, 42°C for 3 h, and 95°C for 3 min. Real time RT-PCR was performed using primer sets specific for mouse CNTF, 18S, and cyclophilin A gene sequences (designed by Vector NTI advanced 10; Invitrogen, Carlsbad, CA) (CNTF forward primer: TGGCTTTCGCAGAGCAATCAC, reverse primer: GCAGTCAGGTCTGAACGAATCTT, TaqMan probe: TTCACCGCCGGGACCTCTGTAGCC, product size: 97 bp; 18S RNA forward primer: CCCGAGTTCACGGTGGGTTC, reverse primer: CGAGAGAAGACACGCCAACG, TaqMan probe: CCTCCGCCTCCGCTTCTCGCCG, product size 101 bp, cyclophilin A forward primer: TCCAGGATTCATGTGCCAGGGTG, reverse primer: TGCCATGGACAAGATGCCAGGACC, TaqMan probe: TCTCTCCGTAGATGGACCTGC, product size: 132 bp). The TaqMan probes were prelabeled with FAM/BHQ (Biosearch Technologies, Novato, CA). PCRs were composed of 1.0 μl each of 2 mM forward and reverse primers, 1.0 μl of 10× PCR buffer, 1.0 μl of 50 mM magnesium chloride, 2.0 μl of 20 mM dNTP mix, 0.1 μl of 5 U/μl SureStart *Taq*DNA polymerase, 2.9 μl water, and 1.0 μl cDNA from the RT reaction and run for 40 cycles at 95°C for 30 s and 72°C for 60 s in an ABI 9700 Thermal Cycler (Applied Biosystems, Foster City, CA). Before experiments, pilot real-time RT-PCR tests were performed to ensure equal amplification efficiencies of the CNTF, 18S RNA, and cyclophilin A primer sets. PCR products of expected sizes were confirmed with electrophoresis on a 2% agarose gel. After real time RT-PCR, the numbers of cycles used to reach a given FAM fluorescence intensity for the CNTF fragment were subtracted from (normalized to) that for the 18S RNA or cyclophilin A fragment to calculate the relative abundance of CNTF mRNA.

LacZ reporter gene expression. CNTF $^{+/-}$ and $^{-/-}$ mice contain a lacZ gene inserted in the location of the deleted CNTF gene at the CNTF locus (Valenzuela et al., 2003). We indirectly assessed CNTF expression patterns by localizing the lacZ gene expression and β-galactosidase protein product. CNTF mice were bred from heterozygous parents and genotyped by the Velocigene mice genotyping protocol provided by Regeneron. CNTF $^{+/-}$ mice were transcardially perfused with 30 ml of ice-cold PBS, pH 7.4, and 30 ml of ice-cold 4% paraformaldehyde in 0.1 M phosphate buffer. Brains were postfixed in 4% paraformaldehyde overnight and cryoprotected in 30% sucrose in 0.1 M phosphate buffer overnight. Serial coronal brain sections of 30 μm thick were cut on a freezing stage microtome and stored in anatomical order in 24-well plates filled with Millonig's buffer. Sections were processed with 0.1% 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal), 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 1 mM magnesium chloride, 0.002% NP-40, and sodium deoxycholate in PBS, pH 7.0, at 37°C overnight, to identify cells expressing β-galactosidase activity in the SVZ ($n = 6$). In addition, coronal sections were processed for β-galactosidase immunostaining using a monoclonal antibody (Z3781, 1:500; Promega, Madison, WI) and fluorescent-labeled secondary antibodies (Alexa Fluor 488, 1:500; Invitrogen, Eugene, OR). Additional sections were double-stained with a rabbit antibody against GFAP (AB5804, 1:1,000; Chemicon) and followed with another fluorescent secondary antibody (Alexa Fluor 594, 1:500; Invitrogen). Sections were analyzed with laser scanning confocal microscopy (Nikon D-Eclipse C1; Nikon Instruments, Dallas, TX).

Astrocyte-neuron coculture. Forebrain tissues of newborn Fisher or Sprague Dawley rats (Charles River Laboratories, Wilmington, MA) were dissected on ice and cultured according to a published protocol (Carroll et al., 1993). After 14 d in culture, this astrocyte-enriched astrocyte-neuron coculture was treated with vehicle (growth media), 10 μM forskolin (Sigma), or the dopamine D_2 receptor agonist quinpirole (Q111; Sigma-Aldrich) (Cory-Slechta et al., 1996) at 10 μM for 3 d. In another experiment, astrocyte-neuron cocultures were treated after 10 d for 3 d with vehicle, quinpirole (10 μM), quinpirole (10 μM) plus the potent and selective D_2 dopamine receptor antagonist eticlopride (30 μM; E101; Sigma) (Hall et al., 1985), or eticlopride alone (30 μM). The total RNA was isolated after treatment and subjected for real time RT-PCR. To assess potential effects of quinpirole on proliferation, other cultures were treated with vehicle or 10 μM quinpirole for 3 d and with bromodeoxyuridine (BrdU) (10 μM) over the last 24 h. Afterward, the cells were fixed with 4% paraformaldehyde and immunostained for GFAP (1:1000; MAB3402; Chemicon). For BrdU staining, the wells were first incubated in 2N HCl at 37°C for 20 min, washed in 0.1 M PBS three times for 5 min, washed in 0.1 M PBS/0.1% Triton X-100 three times for 10 min, blocked with 3% rabbit serum in 0.1 M PBS/0.1% Triton X-100 for 30 min at room temperature, followed by BrdU (1:10,000; MAB3510, mouse IgG, clone BU-1, Chemicon) and appropriate secondary antibodies, and counterstaining with Hoechst 33258 nuclear dye (B2883; Sigma).

CNTF expression after dopaminergic denervation or D_2 stimulation. Male C57BL/6 mice (10 weeks of age; 18–22 g; The Jackson Laboratory) were anesthetized and unilaterally injected with 1.5 μg (per injection site) 6-hydroxydopamine (6-OHDA; Sigma-Aldrich) in 0.5 μl of saline containing 0.2% ascorbic acid ($n = 4$) or with saline containing 0.2% ascorbic acid ($n = 4$) into the right medial forebrain bundle and the substantia nigra at stereotaxic coordinates from Bregma: 1.1 mm caudal, 1.3 mm lateral, 5.3 mm from the dura mater and 3.3 mm caudal, 1.3 mm lateral, 4.8 mm from the dura mater, respectively. Dopaminergic neurons of the substantia nigra pars compacta project ipsilaterally to the neostriatum (Hagg and Varon, 1993), and the terminals are lost after the 6-OHDA lesion. One week after injury, the brains were freshly dissected

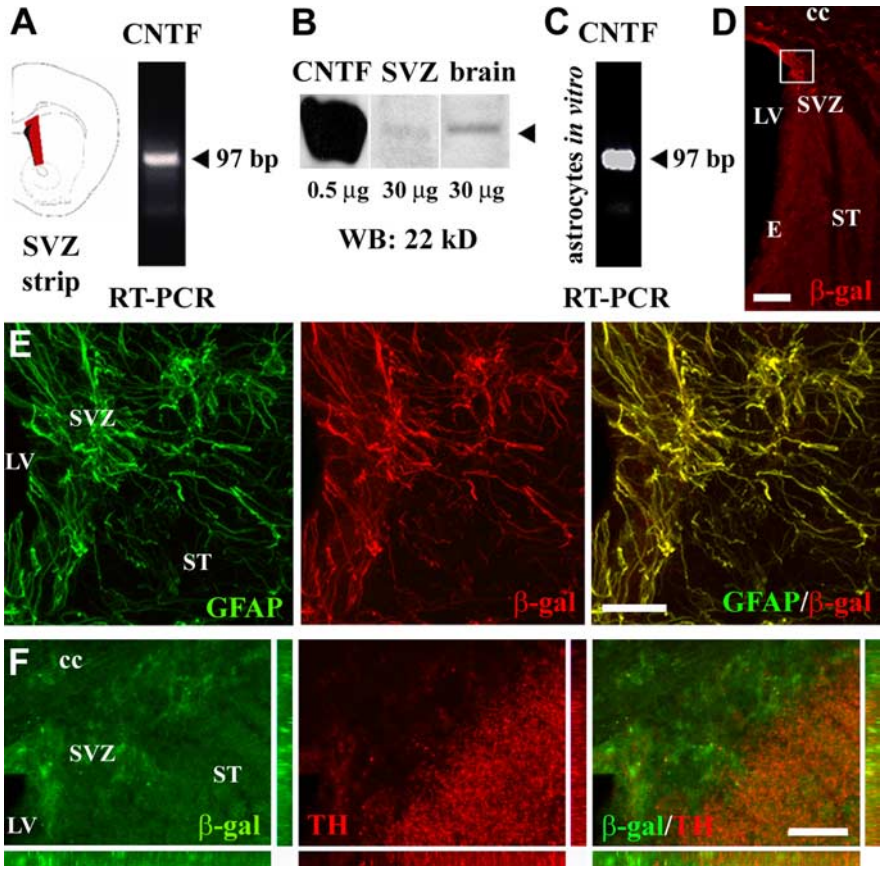

Figure 1. Astrocytes in the SVZ of the adult mouse forebrain produce CNTF. ***A***, RT-PCR analysis of a 0.5 mm strip of the striatum containing the SVZ reveals the expression of CNTF mRNA. ***B***, Western blotting assay shows the presence of CNTF protein in protein extracts of the SVZ/striatal strips. Recombinant human CNTF and whole-brain extracts are shown for comparison. The lower levels of CNTF in the SVZ/striatum compared with the whole brain probably reflect the low expression in the striatum (***E***) versus other regions of the brain. ***C***, Cultured neonatal forebrain astrocytes express CNTF mRNA. ***D***, β-Galactosidase immunostaining of a coronal section through the SVZ shows prominent CNTF reporter gene expression in the SVZ of a CNTF $^{+/-}$ mouse. ***E***, β-Galactosidase immunostaining was exclusively present in GFAP-positive cells in the SVZ. Note that CNTF expression is particularly abundant in the SVZ compared with the neighboring striatum. All these results indicate that the astrocytes produce CNTF in the SVZ. ***F***, TH-positive dopaminergic terminals are present in the SVZ and intermingled with β-galactosidase-expressing astrocytic processes. More densely stained dopaminergic fibers are observed in the adjacent striatum proper. CC, Corpus callosum; E, ependymal cell layer; LV, lateral ventricle; ST, striatum. Scale bars: ***D***, ***F***, 50 μm; ***E***, 20 μm.

to obtain the strip SVZ/striatal tissue for RNA isolation and CNTF mRNA measurements. To test the effect of the dopamine D_2 receptor agonist quinpirole on the expression of CNTF in the SVZ and hippocampal formation, adult male C57BL/6 mice were injected intraperitoneally with saline or 2 or 18 mg/kg quinpirole daily for 3 d ($n = 6$ each). To test the effects in another strain, male Friend virus B-type susceptibility (FVB) mice were injected with saline or 18 mg/kg quinpirole ($n = 6$ each). To test effects in our CNTF mice, sex-matched CNTF $^{+/+}$ and $^{+/-}$ littermates were injected with saline or 18 mg/kg quinpirole ($n = 6$ each). We use the 3 d injections to maximize our outcome measures, because we do not know when the systemically injected quinpirole would induce CNTF expression and how long before this would affect proliferation. SVZ-containing striatal tissue strips and whole hippocampal formation were obtained for subsequent RNA isolation and CNTF mRNA measurements.

D_2 stimulation of proliferation in wild-type mice. To detect the effect of quinpirole on the most recent proliferative activity in the SVZ, mice (10 weeks of age; 18–22 g; The Jackson Laboratory) were injected intraperitoneally with saline or quinpirole ($n = 6$ each) at 2.0 mg/kg daily for 3 d and BrdU at 200 mg/kg 2 h immediately before perfusion [BrdU as in the study by Garcia et al. (2004)]. Another group of mice was injected with saline or quinpirole at 0.68, 2.0, 6.0, and 18.0 mg/kg ($n = 3–4$ each) and BrdU at 50 mg/kg daily for 3 d. The 50 mg/kg dose is widely used to investigate neurogenesis. The mice were perfused with 4% paraformaldehyde, and serial coronal sections of the brains were cut and stored in anatomical order.

D_2 stimulation in CNTF knock-out or CNTF antibody-treated mice. We used a CNTF-specific neutralizing antibody (AB-557-NA, Goat IgG; R&D Systems, Minneapolis, MN), which has an ED_{50} value of 7–15 μg/ml in the presence of 1.0 ng/ml recombinant rat CNTF in the survival assay of embryonic chick dorsal root ganglia neurons and is highly selective, because it has no cross reactivity to >150 other cytokines. Purified goat IgG served as a control (Chemicon). Antibody was infused at 10 μg/d into the right lateral ventricle of C57BL/6 mice ($n = 12$ CNTF antibody; 12 IgG) for 3 d using Alzet micro-osmotic pump (model 1003D; Durect Corporation, Cupertino, CA) and mouse brain infusion kits (Durect). The tip of the catheter was stereotactically placed in the lateral ventricle at the following coordinates from Bregma: rostrocaudal, −0.4 mm, mediolateral, 1.0 mm, dorsoventral, 2.3 mm. Half of each group was injected intraperitoneally daily with saline and the other half with 18.0 mg/kg quinpirole, and BrdU was injected at 50 mg/kg twice a day for 3 d. Age- and sex-matched CNTF $^{+/+}$, $^{+/-}$, and $^{-/-}$ littermates were injected daily with saline or 18.0 mg/kg quinpirole ($n = 6$ each per genotype) and BrdU at 50 mg/kg twice a day for 3 d. The mice were processed for histology as described above, and 30-μm-thick coronal sections through the brain were cut.

D_2 stimulation of SVZ proliferation after dopaminergic denervation in CNTF$^{-/-}$ and CNTF$^{+/+}$ mice. CNTF $^{-/-}$ and CNTF $^{+/+}$ mice ($n = 8$ each) were anesthetized and unilaterally injected with 6-OHDA as described above. Two weeks after injury, the mice were injected daily intraperitoneally with saline ($n = 4$ per genotype) or 18.0 mg/kg quinpirole ($n = 4$ per genotype) and twice a day with 50 mg/kg BrdU, over 3 d. They were processed for histology as described above.

Immunohistochemistry. Starting at a random point along the rostrocaudal axis of the brain, every sixth section through the SVZ and hippocampal formation was immunostained against BrdU (MAB3510, mouse IgG, clone BU-1, 1:30,000; Chemicon). Briefly, brain sections were incubated in 50% formamide in 2× SSC at 65°C for 2 h, rinsed in fresh 2× SSC, incubated in 2N HCl at 37°C for 30 min, neutralized in 0.1 M boric acid, pH 8.5, for 10 min, incubated in 10% normal serum for 30 min, primary antibodies overnight, biotinylated horse anti-mouse IgG (1:800; Vector Laboratories, Burlingame, CA) for 1 h, and avidin-biotin complex conjugated with peroxidase for 1 h (1:600; Vector Laboratories). Chromogen reaction was performed with 0.04% 3,3′-diaminobenzidine (Sigma-Aldrich) solution containing 0.06% nickel ammonium sulfate and 1% hydrogen peroxide in 0.05 M Tris buffer-HCl. Sections were then rinsed in 0.1 M phosphate buffer, mounted on glass slides, and coverslipped. Three adjacent sections from the rostral region were stained with goat anti-doublecortin antibody (goat IgG, 1:2000, catalog #s.c.-8067; Santa Cruz Biotechnologies, Santa Cruz, CA), which is a marker for neuroblasts. Selected sections were stained for double-fluorescence for β-galactosidase and an anti-tyrosine hydroxylase (TH) antibody (rabbit, 1:1000, AB152; Chemicon) to identify dopaminergic terminals innervating the SVZ.

Cell counting and statistics. The number of immunostained BrdU-positive nuclei in the SVZ or dentate gyrus of each brain was estimated

using a motorized Leica DMIRE2 microscope and an unbiased optical fractionator stereological method (Stereologer; Systems Planning and Analysis, Alexandria, VA) (Baker et al., 2004). For the SVZ, the reference space was defined as an ~50-μm-wide strip of the entire lateral of all the lateral ventricle, encompassing dorsoventrally the ventral tip and the dorsolateral triangular regions of the lateral ventricle where the rostral migratory stream forms, rostrocaudally from the genu of the corpus callosum to the caudal end of the decussation of the anterior commissure, and laterally the boundary between the SVZ and striatum. To calculate the number of $BrdU^+$ cells in the subgranular layer, the entire dentate gyrus was included for sampling, which contained 8–11 sections of 180 μm apart. Within the reference space, BrdU-positive nuclei were counted in the software-defined frames, and the total number of BrdU-positive cells in a brain was calculated by the software as: n = number of nuclei counted × 1/section sampling fraction × 1/area sampling fraction × 1/thickness sampling fraction. The area of GFAP-positive staining in the astrocyte cultures was calculated from 10 images per well taken with a 20× objective using Image-Pro Plus 6.2 (Media Cybernetics, Silver Springs, MD). The numbers of BrdU-positive and -negative nuclei were counted in seven images per well. Statistical analyses were performed with either the Student's unpaired t test and/or ANOVA followed by t tests between individual groups, using Excel software (Microsoft, Seattle, WA). One-tailed t tests were used when we had formulated a hypothesis before the experiment was performed and a two-tailed when a lack of difference was predicted or when there was no prediction. All data presented used the one-tailed t test, except where otherwise indicated. A value of $p < 0.05$ was considered to be statistically significant.

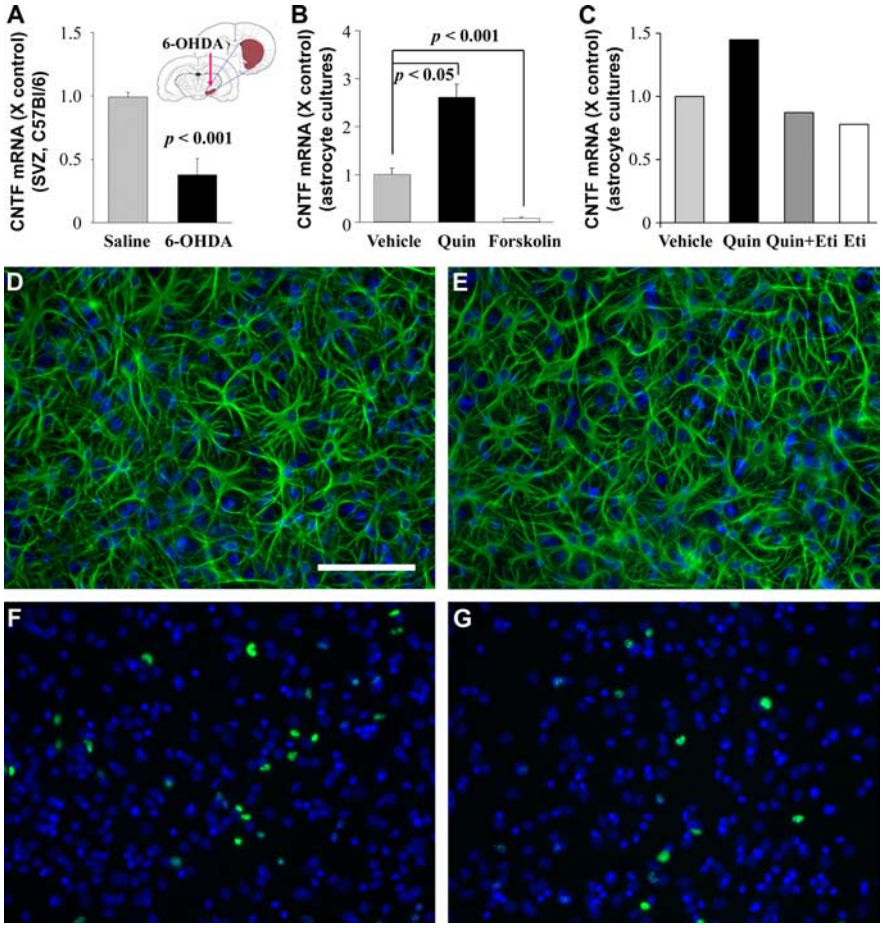

Figure 2. CNTF expression is regulated by dopaminergic innervation in adult mice and through D_2 receptors in cultured astrocytes. ***A***, A unilateral dopaminergic denervation of the striatum 1 week after 6-OHDA injection reduces CNTF mRNA expression in the SVZ of adult mice ($n = 4$ each; +SEM). ***B***, Stimulation of the dopamine D_2 receptor by 10 μM quinpirole (Quin) in a mixed culture of astrocytes and neurons results in increased expression of CNTF mRNA. As expected, CNTF expression was reduced in 10 μM forskolin-treated cultures. Vehicle control consisted of media. ***C***, Quinpirole (10 μM) stimulates astroglial CNTF mRNA levels through D_2 receptors as shown by cotreatment with the D_2-selective antagonist eticlopride (Eti; 30 μM). Data in ***A–C*** were obtained by real-time RT-PCR. Cultured astrocytes treated with vehicle (***D***) or quinpirole (***E***) had a similar morphology as shown by GFAP staining. Proliferation as shown by BrdU labeling appeared reduced between astrocytes treated with vehicle (***F***) or quinpirole (***G***). Counts showed that quinpirole caused a 24% reduction in the number of BrdU-labeled nuclei. Together, this suggests that increased CNTF is not caused by hypertrophy or an increase in the number of astrocytes. Nuclei were stained with blue Hoechst dye. Scale bar, 100 μm.

Results

CNTF is expressed by SVZ astrocytes

RT-PCR and Western blot analyses of freshly dissected 0.5 mm strips of the striatum containing the wall of the anterior lateral ventricle, including the SVZ, of adult male C57BL/6 mice showed that CNTF mRNA (Fig. 1*A*) and protein (Fig. 1*B*) were present. As expected, cultured neonatal forebrain astrocytes also expressed CNTF mRNA (Fig. 1*C*). Adult $CNTF^{+/-}$ mice with a LacZ reporter gene inserted in the deleted allele (Valenzuela et al., 2003), showed clear β-galactosidase signal in sections containing the SVZ (Fig. 1*D*). Immunostaining was exclusively present in GFAP-positive cells which, judged by their multipolar morphology and abundance, were mainly astrocytes (Fig. 1*E*). TH-positive dopaminergic terminals were intermingled with β-galactosidase-positive cells and processes in the SVZ (Fig. 1*F*), suggesting that dopamine could be released among the CNTF-synthesizing astrocytes. Together, this suggests that SVZ astrocytes produce CNTF *in vivo* and could be affected by dopamine.

The dopaminergic nigrostriatal pathway regulates CNTF mRNA in the SVZ

Dopaminergic neurons of the substantia nigra pars compacta project their axons to the neostriatum. Dopaminergic denervation reduces precursor proliferation in the SVZ of animal models and in humans with Parkinson's disease (Baker et al., 2004; Hoglinger et al., 2004). Endogenous CNTF is known to promote SVZ neurogenesis (Emsley and Hagg, 2003). Here, we investigated whether there might be a link between the two findings. C57BL/6 mice were injected unilaterally with the dopaminergic toxin 6-OHDA into the midbrain part of the medial forebrain bundle. One week later, CNTF mRNA levels in a strip of ipsilateral SVZ/striatal tissue were decreased to 38% of saline-treated control mice, as measured by quantitative real-time RT-PCR using FAM/BHQ-labeled amplicon probes ($p < 0.001$) (Fig. 2*A*). This suggests that the dopaminergic pathway regulates CNTF expression in the SVZ, possibly through dopamine.

D_2 receptor stimulation increases astroglial CNTF mRNA expression *in vitro*

To test whether D_2 dopamine receptor activation could promote CNTF expression, neonatal forebrain astrocytes were cultured for 14 d and in the presence of forebrain neurons to reduce CNTF

expression (Rudge et al., 1995). This makes it easier to detect increases in CNTF mRNA. The cultures were grown in the absence of epidermal growth factor (EGF) and FGF2 to remove neural stem cells and their progeny. The D_2-selective agonist quinpirole induced CNTF mRNA levels to 2.6-fold higher than that in the control group (quinpirole vs saline; $p < 0.05$) (Fig. 2*B*). In contrast, forskolin, which increases cAMP, dramatically reduced the CNTF mRNA levels (Fig. 2*B*) as also described by others (Carroll et al., 1993; Rudge et al., 1994). To confirm that the quinpirole effect was through the D_2 receptor, other cultures were treated with quinpirole with or without the selective D_2 antagonist eticlopride. The increase in CNTF mRNA was completely blocked in the presence of eticlopride (Fig. 2*C*). The increase in CNTF mRNA levels could reflect increased expression per cell, increases in cell volume, or increases in cell number. Analyses of the GFAP-positive area in cultures treated with quinpirole showed no change compared with those treated with vehicle (both 33% of total area), suggesting that increased CNTF is not caused by hypertrophy. The morphology of the astrocytes also appeared similar between the two treatments (Fig. 2*D*,*E*). The number of BrdU-positive nuclei was ~24% lower after the quinpirole treatment than without treatment (Fig. 2*F*,*G*), suggesting that increased CNTF is not caused by an increase in the number of astrocytes. Thus, activation of D_2 dopamine receptors can increase expression of CNTF in astrocytes.

D_2 stimulation increases CNTF mRNA in adult mice

We next tested whether D_2 stimulation in naive mice would increase CNTF expression. Adult male C57BL/5 mice received daily intraperitoneal injections of saline or quinpirole over 3 d. Quinpirole dosed at 18 mg/kg caused acute hypolocomotion because of its effects on extrapyramidal motor systems as expected (Dall'olio et al., 1997). CNTF mRNA levels in freshly dissected SVZ/striatal tissue strips were significantly increased with 2 or 18 mg/kg by 2.8- and 1.9-fold, respectively ($p < 0.001$; $p < 0.01$) (Fig. 3*A*). Quinpirole injections (18 mg/kg) also increased CNTF expression in male FVB mice ($p < 0.05$) (Fig. 3*B*). Our CNTF mouse colony has a mixed C57BL/6×129Sv background. The 3 d, 18 mg/kg quinpirole treatment was also effective in inducing SVZ/striatal CNTF in $CNTF^{+/+}$ wild-type and $CNTF^{+/-}$ littermates by 2.4- and 5.6-fold, respectively ($p < 0.001$; $p < 0.01$) (Fig. 3*C*,*D*).

Together, the results thus far suggest that the dopaminergic pathway normally promotes CNTF expression in the astrocytes of the SVZ by stimulating D_2 receptors.

Dopamine D_2 receptor agonist increases proliferation of neural precursor cells in the SVZ

Others have shown in adult mice that the D_2 preferential agonist ropinirole can induce proliferation in naive mice and restore neurogenesis in mice after a nigrostriatal dopaminergic denervation by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (Hoglinger et al., 2004). To verify that quinpirole would promote proliferation, C57BL/6 mice were injected daily for 3 d intraperitoneally with 2 mg/kg quinpirole. To identify the proliferative cells in the SVZ, 200 mg/kg BrdU was injected 2 h before perfusion with 4% paraformaldehyde (Garcia et al., 2004). Quinpirole-treated mice had 20% more $BrdU^+$ nuclei in coronal tissue sections through the SVZ as counted by unbiased stereology using the optical fractionator method (3697 ± 147, SEM, vs 3087 ± 252; $p < 0.01$) (Fig. 4*A*–*E*). Next, we determined the dosing effects of quinpirole on proliferation throughout the 3 d of treatment of C57BL/6 mice. Saline or quinpirole was injected daily at different doses from 0.68–18 mg/kg (i.p.), with 50 mg/kg BrdU injected twice daily. Quinpirole caused a significant increase in the number of $BrdU^+$ nuclei in the SVZ ($p < 0.05$; $F_{(4,14)} = 3.92$; one-factor ANOVA) (Fig. 4*F*). *Post hoc* analysis showed that significance was reached at 0.68 mg/kg ($p < 0.05$). No inhibitory effects on proliferation were observed. The 18 mg/kg dose caused clear hypolocomotion in the mice over the first few hours after the injection. Therefore, this dose was used in all the following experiments to ensure that we had a behavioral readout for efficacy of the quinpirole treatments. These findings confirm that the dopamine D_2 receptor plays an important role in the regulation of the SVZ neural precursor proliferation.

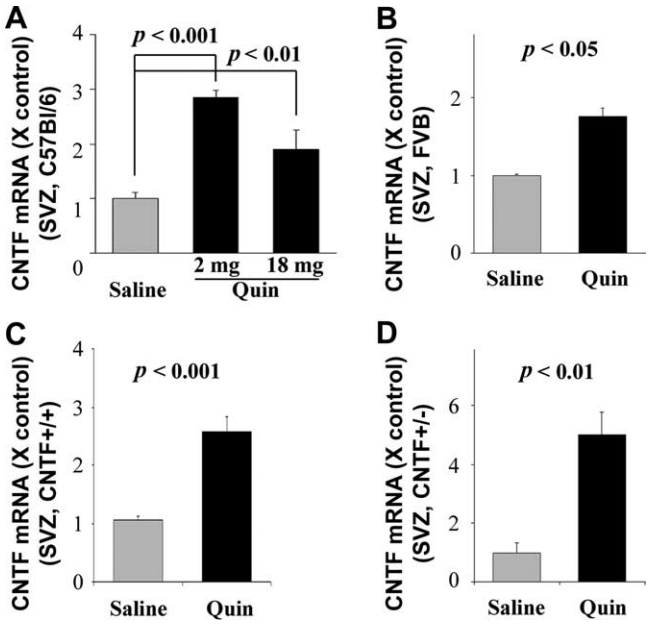

Figure 3. CNTF expression is regulated by D_2 receptors in adult mice. Daily quinpirole injections over 3 d increases CNTF mRNA expression (real time RT-PCR) in C57BL/6 mice (***A***; 2 and 18 mg/kg), FVB mice (***B***; 18 mg/kg), wild-type $CNTF^{+/+}$ mice (***C***; 18 mg/kg), and $CNTF^{+/-}$ mice (***D***; 18 mg/kg). $n = 6$ per saline and quinpirole groups. Values represent the fold change compared with the control saline group + SEM.

D_2-stimulated SVZ neurogenesis is dependent on CNTF

We identified CNTF as an important endogenous regulator in promoting adult mouse SVZ neurogenesis (Emsley and Hagg, 2003). Our current studies revealed that dopaminergic projections regulate astroglial CNTF in the SVZ, and D_2 receptors regulate astroglial CNTF expression *in vitro* and CNTF and neurogenesis *in vivo*. Next, we tested whether the D_2 regulation of neurogenesis was directly dependent on, and thus mediated by, CNTF in the adult mouse SVZ, using CNTF knock-out mice and their wild-type and heterozygous littermates. Daily intraperitoneal injections of 18 mg/kg quinpirole over 3 d were accompanied by twice daily intraperitoneal injections of 50 mg/kg BrdU. Two-factor ANOVA indicated that the CNTF genotypes significantly affected the effectiveness of quinpirole treatment ($p < 0.01$; $F_{(2,35)} = 6.07$) (Fig. 5*A*). Quinpirole increased the number of $BrdU^+$ nuclei in the SVZ of wild-type mice by 35% compared with wild-type littermates injected with saline (9586 ± 1016 vs 7109 ± 550; $p < 0.05$). In sharp contrast, quinpirole treatment did not alter the number of $BrdU^+$ SVZ nuclei in $CNTF^{-/-}$ littermate mice compared with saline injections (5473 ± 747 vs 5605 ± 406; $p > 0.05$). Notably, $CNTF^{-/-}$ mice had 20% fewer BrdU-labeled nuclei compared with their wild-type littermates in

either saline (5605 ± 406 vs 7109 ± 550; $p < 0.05$) or quinpirole-treated groups (5473 ± 747 vs 9586 ± 1016; $p < 0.005$).

Because the knock-out mice had less proliferation in the SVZ, they potentially could have undergone alterations during development in response to the lack of CNTF. Therefore, we confirmed that the neurogenic effect of D_2 stimulation is mediated by CNTF by injecting a CNTF-specific neutralizing antibody into the lateral ventricle close to the SVZ of naive C57BL/6 mice. A 3 d quinpirole treatment increased the number of $BrdU^+$ nuclei in the SVZ (twice daily BrdU injections) by 24% compared with saline-treated mice, whereas both groups were injected into the ventricle with purified IgG (7919 ± 657 vs 6386 ± 476; $p < 0.05$) (Fig. 5*B*). However, in mice injected with CNTF antibody, 3 d daily intraperitoneal injections of 18 mg/kg quinpirole failed to cause a statistically significant increase in $BrdU^+$ nuclei in the SVZ compared with the saline treatment (6139 ± 559 vs 5110 ± 483; $p > 0.05$). In the mice treated with saline, the CNTF antibody treatment resulted in a 20% reduction of $BrdU^+$ nuclei in the SVZ compared with the IgG-injected group (5110 ± 483 vs 6386 ± 476; $p < 0.05$), consistent with our previous study (Emsley and Hagg, 2003).

These results show that D_2-stimulated proliferation in the adult mouse SVZ is mediated by CNTF and support the idea that the nigrostriatal dopaminergic pathway regulates neurogenesis by modulating CNTF expression.

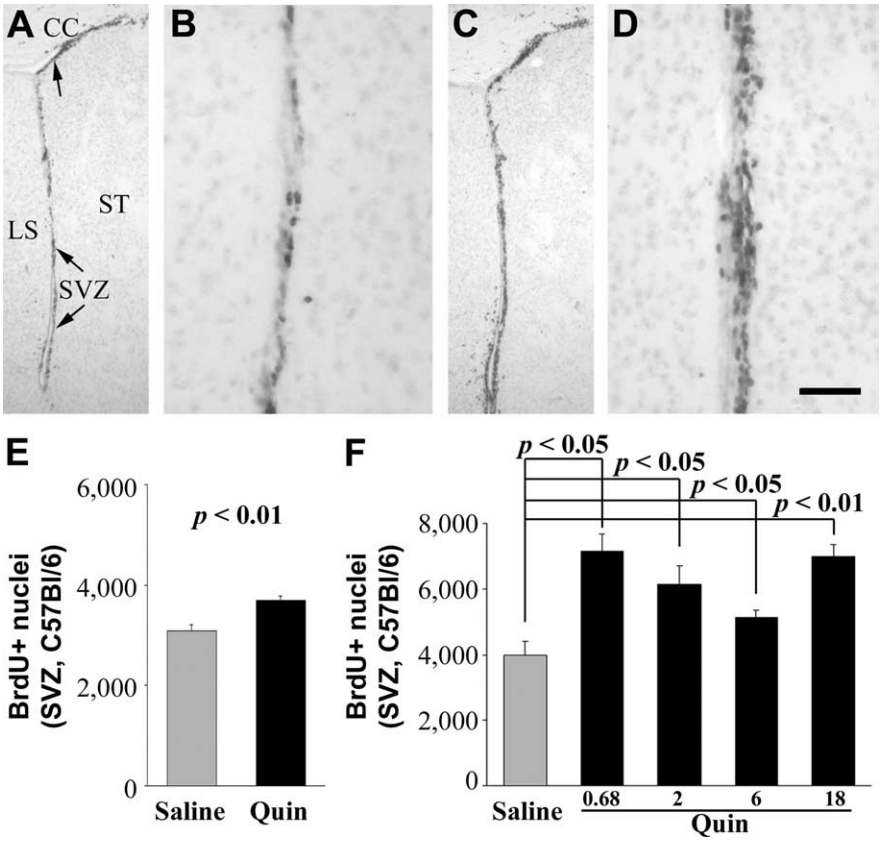

Figure 4. Dopamine D_2 receptor promotes neural precursor proliferation in the adult mouse forebrain SVZ. Compared with the number of BrdU-labeled nuclei in coronal sections through the SVZ of C57BL/6 mice injected intraperitoneally with saline (***A***, ***B***), that of mice injected with quinpirole was increased (***C***, ***D***). The newly generated cells remain within their normal boundaries, suggesting their migration route is not affected by quinpirole treatment (***C***, ***D***) compared with the saline group (***A***, ***B***). ***E***, The quinpirole treatment (2 mg/kg per day) induced proliferation, as a single BrdU injection 2 h before perfusion, resulted in a 20% increase of BrdU+ nuclei in the SVZ ($n = 6$ each; +SEM). ***F***, A 3 d daily treatment with quinpirole increases the number of BrdU+ nuclei (BrdU injected twice daily) over a wide range of doses ($n = 3–4$ each). No inhibitory effects were observed. The 18 mg/kg dose causes significant behavioral change and was used in following experiments as a sign of pharmacological effectiveness. CC, Corpus callosum; LV, lateral ventricle; LS, lateral septum; ST, striatum. Scale bar (in ***D***): ***A***, ***C***, 200 μm; ***B***, ***D***, 40 μm.

D_2-stimulated CNTF also increases SVZ-derived neuroblast numbers without affecting cell fate choice

We next investigated whether the increased proliferation after dopamine D_2 receptor stimulation would affect the fate choice of the newly generated cells in the SVZ, using doublecortin as a marker for immature neuroblasts (Fig. 6*A–F*). Three anatomically similar sections across the SVZ of each mouse were selected for comparison. In $CNTF^{+/+}$ mice, the 3 d, 18 mg/kg per day quinpirole treatment resulted in a 25% increase of doublecortin-positive cells in the SVZ compared with saline-treated littermates (1173 ± 81 vs 938 ± 40; $p < 0.05$) (Fig. 6*G*). In $CNTF^{-/-}$ mice, quinpirole treatment did not cause a significant change in the number of doublecortin-positive cells in the SVZ compared with saline-treated littermates (843 ± 77 vs 777 ± 35; $p > 0.05$). The saline-treated $CNTF^{-/-}$ mice had 34% fewer doublecortin-positive neuroblasts than their $CNTF^{+/+}$ littermates (777 ± 35 vs 1173 ± 81; $p < 0.01$). The ratio of doublecortin-positive neuroblasts and $BrdU^+$ nuclei remained at ~13% after the quinpirole treatment in wild-type mice, and the ratio in $CNTF^{-/-}$ mice was ~14%, regardless of the treatment (Fig. 6*H*). These findings indicate that the quinpirole-induced and CNTF-mediated changes in proliferation do not alter the fate choice of the newly generated cells in the SVZ, consistent with our previous results using CNTF and CNTF antibodies in naive mice (Emsley and Hagg, 2003). Also consistent with our previous findings that CNTF does not alter migration in the rostral migratory stream (Emsley and Hagg, 2003), we did not see an effect of the CNTF gene deletion on the normal migration patterns of the new neuroblasts (Fig. 6*A–F*).

Dopaminergic innervation regulates SVZ neurogenesis mainly through CNTF and postsynaptic D_2 receptors

We next determined to which extent CNTF mediates the regulation of SVZ neurogenesis by the dopaminergic projections from the midbrain. The striatum was denervated by an injection of 6-OHDA in the midbrain, and saline and BrdU injected 14 d later over a 3 d period. Injured $CNTF^{+/+}$ littermates showed the expected 30% reduction in BrdU+ nuclei (Fig. 7) ($p < 0.001$) (Baker et al., 2004). In contrast, the number of BrdU+ nuclei was not affected by the injury in $CNTF^{-/-}$ mice compared with uninjured $CNTF^{-/-}$ mice, both being ~18% less than in uninjured $CNTF^{+/+}$ mice. The contralateral side of the unilaterally injured $CNTF^{-/-}$ mice had ~24% fewer BrdU+ nuclei than normal ($p < 0.005$) (data not shown). These data suggest that the nigrostriatal dopaminergic pathway regulates SVZ neurogenesis predominantly by modulating CNTF.

We also determined whether quinpirole could reverse the reduced neurogenesis seen after the denervating 6-OHDA lesions.

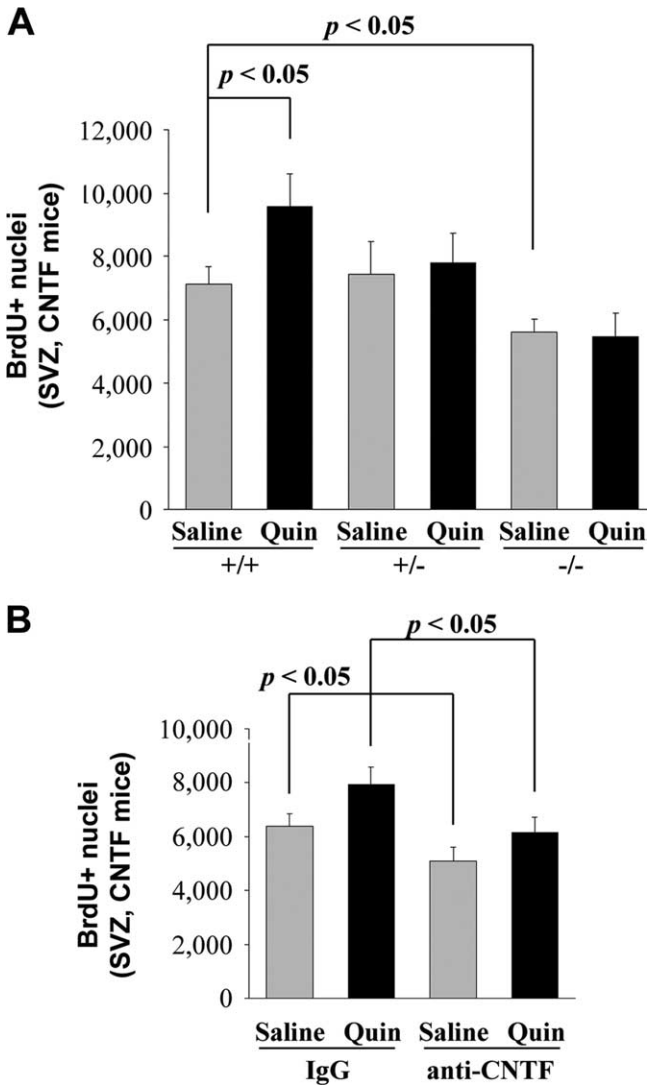

Figure 5. D_2-induced SVZ proliferation is dependent on CNTF. ***A***, A 3 d, 18 mg/kg/d quinpirole treatment increases the number of $BrdU^+$ nuclei in the SVZ of adult $CNTF^{+/+}$ mice but not in their $CNTF^{-/-}$ littermates, suggesting endogenous CNTF mediates the dopaminergic regulation of neurogenesis. $CNTF^{+/+}$ mice injected with saline treatment have more $BrdU^+$ nuclei compared with their saline-injected $CNTF^{-/-}$ littermates, confirming the endogenous role of CNTF in neurogenesis ($n = 6$ each group; +SEM). ***B***, The 3 d, 18 mg/kg/d quinpirole treatment was not effective in increasing the number of $BrdU^+$ SVZ nuclei when CNTF-specific neutralizing antibodies were simultaneously infused into the lateral ventricle of C57BL/6 mice. Quinpirole induced a significant increase in the presence of a purified IgG control ($n = 6$ each group).

Others have shown that another D_2 agonist can reverse reduced neurogenesis after an MPTP injury (Hoglinger et al., 2004). Starting 14 d after the injury, mice received daily injections of 18 mg/kg quinpirole (with twice daily BrdU) over a 3 d period. The number of BrdU+ nuclei was higher in quinpirole-treated injured $CNTF^{+/+}$ mice than in saline-treated injured mice (Fig. 7) ($p = 0.016$) and not significantly different from normal mice ($p = 0.16$; two-tailed t test). Quinpirole had no significant effect in injured $CNTF^{-/-}$ mice ($p = 0.99$; two-tailed t test). These results suggest that the quinpirole effects are mediated by postsynaptic D_2 receptors and not by presynaptic D_2 receptors known to be present on the dopaminergic terminals (Sesack et al., 1994).

Dopamine D_2 receptor activation increases CNTF mRNA and precursor proliferation in the dentate gyrus

The dopaminergic neurons of the ventral tegmental area innervate the hippocampal formation (Swanson, 1982) and midbrain MPTP lesions that involve the ventral tegmental area result in reduced hippocampal neurogenesis (Hoglinger et al., 2004). CNTF is produced in the granular neurons and in the subgranular zone of the dentate gyrus (Fig. 8*A*,*B*), where proliferation occurs. As in the SVZ, multipolar GFAP-positive astrocytes in the dentate gyrus also expressed CNTF (Fig. 8*B*). Therefore, we tested whether dopamine D_2 stimulation would increase CNTF mRNA levels and subsequently proliferation of neural precursor cells in the dentate gyrus. Three days after daily 18 mg/kg quinpirole injections in adult C57BL/6 mice, there was a 5.3-fold increase in CNTF mRNA in freshly dissected hippocampal formation compared with saline injections ($p < 0.005$) (Fig. 8*C*). Spinal cord tissue from the same mice did not show a significant change in CNTF mRNA after quinpirole treatment (Fig. 8*D*).

The effect of quinpirole on the proliferation of hippocampal neural precursor cells was evaluated by the unbiased stereological counting of $BrdU^+$ nuclei in the entire dentate gyrus. The 3 d injections of 18 mg/kg quinpirole showed an increase that was significantly related with the CNTF genotypes ($p = 0.005$; $F_{(2,35)} = 6.80$; two-factor ANOVA) (Fig. 8*E*). Quinpirole injections resulted in a 25% increase of $BrdU^+$ nuclei (twice daily BrdU injections) in the $CNTF^{+/+}$ mice compared with saline control injections (2569 ± 217 vs 2062 ± 122; $p < 0.05$). As in the SVZ, quinpirole treatment failed to increase the number of $BrdU^+$ nuclei in $CNTF^{-/-}$ littermates compared with saline treatment (1515 ± 197 vs 1564 ± 237). $CNTF^{-/-}$ mice had 24% fewer $BrdU^+$ nuclei compared with their $CNTF^{+/+}$ littermates (2062 ± 122 vs 1564 ± 237; $p < 0.05$), indicating that endogenous CNTF promotes neural precursor proliferation in the DG. These data show that dopamine D_2 receptor-induced hippocampal neural precursor proliferation is also mediated by CNTF.

Discussion

The main findings are, first, that astroglial CNTF expression is regulated by dopaminergic innervation and increased by systemic administration of a D_2 agonist. This provides novel insight into normal and pharmacological regulation mechanisms of this nervous system-selective neurotrophic factor. Second, D_2 dopamine receptor activation promotes neurogenesis in the SVZ and dentate gyrus and is mediated by CNTF, an endogenous molecular regulator that promotes normal patterns of neurogenesis. These findings are relevant to the understanding and treatment of some symptoms of Parkinson's disease and for cell-replacement therapies in other disorders.

Dopaminergic pathways regulate CNTF expression in astrocytes through D_2 receptors

CNTF expression is increased in the CNS after injuries (Ip et al., 1993; Lee et al., 1997; Park et al., 2000; Albrecht et al., 2003) and in the PNS by aldose reductase inhibition (Mizisin et al., 1997). Identifying the molecular mechanisms regulating CNTF expression *in vivo* and their pharmacological modulators is important, because CNTF is neuroprotective (Hagg and Varon, 1993; Thoenen and Sendtner, 2002) and enhances adult CNS neurogenesis (Emsley and Hagg, 2003). Dopaminergic projections (Baker et al., 2004; Hoglinger et al., 2004) and a D_2 agonist (Hoglinger et al., 2004) also promote neurogenesis, suggesting that dopamine might stimulate CNTF expression in the neurogenic

regions. In fact, we show that CNTF expression is abundant in the SVZ and close to dopaminergic terminals.

Astrocytes are the predominant CNTF-producing cell type in the CNS (Stockli et al., 1991; Dobrea et al., 1992; Ip, 1998; Park et al., 2000). Here, the majority of CNTF-producing cells in the SVZ and dentate gyrus had a multipolar morphology typical for regular astrocytes. Moreover, our astrocytes, cultured under conditions that select against neural stem cells (no EGF or FGF2), produce CNTF. It is possible that the less abundant unipolar or bipolar GFAP-positive neural stem cells (Doetsch et al., 1997; Alvarez-Buylla and Lim, 2004; Garcia et al., 2004) can also produce CNTF and increase production in response to quinpirole. In contrast, CNTF mRNA was not found in gene expression analyses of neural stem cells (Suslov et al., 2002; Wright et al., 2003).

CNTF-producing SVZ astrocytes are close to dopaminergic fibers (Hoglinger et al., 2004). We found that dopaminergic denervation of the striatum results in a 60% reduction in CNTF mRNA in SVZ/striatal tissue. Dopamine is one of the main agents released by these terminals and could reach the astrocytes by diffusing through the extracellular space (Agnati et al., 1995). CNTF expression in cultured astrocytes is decreased by intracellular cAMP (Carroll et al., 1993; Rudge et al., 1994). This suggested that reduced cAMP could increase CNTF expression. Dopamine D_2 receptors are G-protein-coupled inhibitory receptors that reduce cAMP (Vallar and Meldolesi, 1989) and are present in astrocytes (Bal et al., 1994; Khan et al., 2001). In the SVZ, the D_2 type is the most abundant dopamine receptor (Araki et al., 2007). In fact, the D_2 agonist quinpirole increased CNTF mRNA in the SVZ/striatum and hippocampal formation *in vivo* and in cultured astrocytes. The D_2-specific effect was confirmed *in vitro* by the blocking effect of the D_2-selective antagonist eticlopride. Quinpirole and ropinerole (Hoglinger et al., 2004) reversed the reduced neurogenesis seen after dopaminergic denervation, suggesting that postsynaptic D_2 receptors regulate CNTF and not autoreceptors on the dopaminergic terminals (Sesack et al., 1994). D_2 receptors are abundant in neurons of the neighboring striatum (Brock et al., 1992), but their presence on SVZ and hippocampal astrocytes *in vivo* remains to be confirmed.

These results identify dopamine as a novel regulator of CNTF expression in the CNS and a strategy to pharmacologically regulate CNTF. This is relevant for diseases that would benefit from increased or decreased CNTF levels. The advantage of CNTF as a drug target is its nervous system-selective expression (Stockli et al., 1991; Ip, 1998), such that indirect stimulation would reduce the systemic side effects and low CNS bioavailability seen after peripheral administration of CNTF. CNTF expression is particularly abundant in the SVZ compared with the neighboring striatum, but dopaminergic innervation is dense throughout the striatum, suggesting that additional regulators exist. Elucidating such overlapping mechanisms would help to develop additional CNTF- and neurogenesis-regulating drugs. Ultimately, combining low doses of two or more of such drugs might reduce side

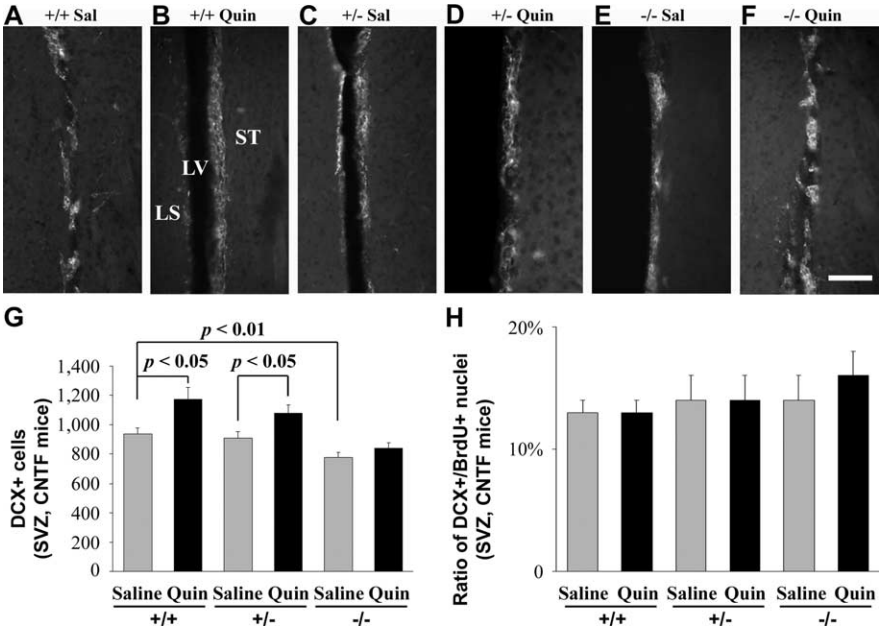

Figure 6. D_2 stimulation increases the number of SVZ neuroblasts. More doublecortin-positive cells are seen in the wild-type (***B***) and heterozygous CNTF (***D***) mice receiving quinpirole treatment compared with saline-treated littermate controls (***A*** and ***C***, respectively). ***E***, ***F***, In contrast, quinpirole treatment fails to increase the number of doublecortin-positive cells in the SVZ in the CNTF $^{-/-}$ mice. Coronal sections through the vertical part of the SVZ are shown. LS, Lateral septum; LV, lateral ventricle; ST, striatum. ***G***, These observations were confirmed by cell count ($n = 6$ each group; +SEM). Note that the CNTF $^{-/-}$ littermates have fewer doublecortin-positive cells. ***H***, The ratio of doublecortin- versus BrdU-positive nuclei (data from Fig. 5*A*) remains constant after quinpirole treatment and is similar between genotypes, suggesting CNTF does not affect cell fate choice but increases normal neurogenesis. Scale bar: (in ***F***) ***A–F***, 50 μm.

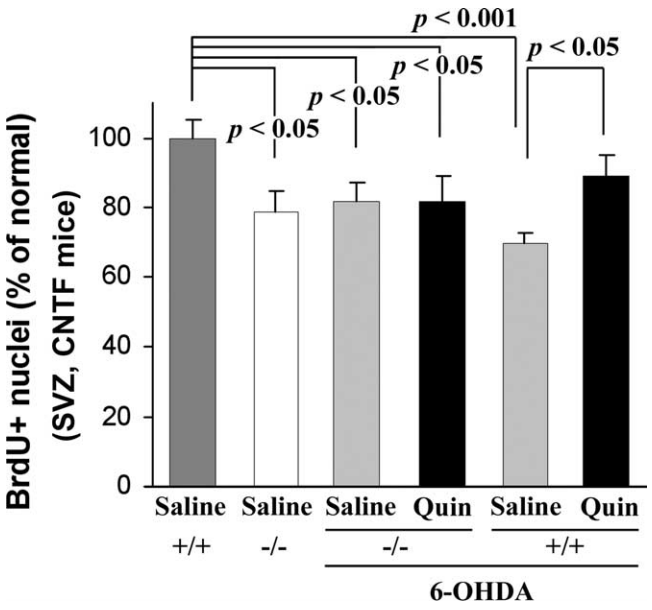

Figure 7. Dopaminergic innervation regulates SVZ neurogenesis mainly through CNTF and postsynaptic D_2 receptors. Mice received a unilateral injection of 6-OHDA in the midbrain to cause dopaminergic denervation of the striatum. Saline or quinpirole was injected intraperitoneally 14 d later together with BrdU for 3 d. Saline- or quinpirole-injected injured CNTF $^{-/-}$ mice ($n = 4$ each; +SEM) had a similar number of BrdU+ nuclei as uninjured saline-injected CNTF $^{+/+}$ mice, all being ~20% lower than normal, as expected for CNTF $^{-/-}$ mice. Data for the normal and uninjured CNTF $^{-/-}$ mice are from Figure 5*A*. In contrast, quinpirole reversed the reduced number of BrdU+ nuclei seen in saline-treated mice after the 6-OHDA injury ($n = 4$ each), suggestive of a postsynaptic D_2 receptor mechanism regulating neurogenesis.

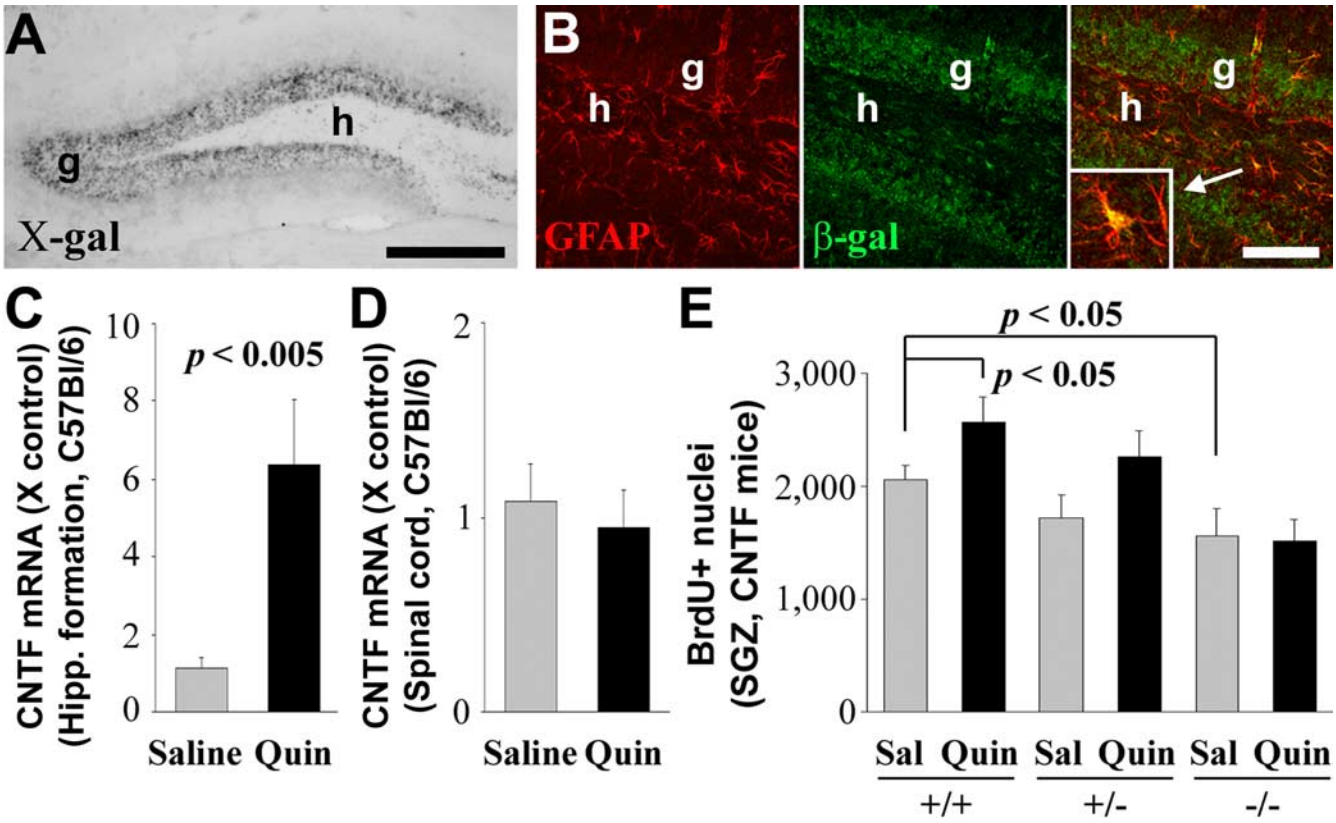

Figure 8. D_2-induced hippocampal proliferation is also dependent on CNTF. CNTF is expressed in astrocytes, and hilar (h) and granule (g) cells of the dentate gyrus as shown by X-gal histochemical staining (***A***) and β-gal immunostaining (***B***) in heterozygous CNTF reporter mice. Double fluorescence immunostaining revealed the coexpression of β-galactosidase and GFAP in the astrocytes, including those of the subgranular zone (***B***). A 3 d, 18 mg/kg/d quinpirole treatment increases CNTF mRNA expression in the hippocampal formation (***C***) but not spinal cord (***D***) as measured by real time RT-PCR. Values represent the fold change compared with the control saline group + SEM. ***E***, Stereological analysis of the $BrdU^+$ nuclei in the entire dentate gyrus reveals that the 3 d quinpirole treatment increases proliferation in $CNTF^{+/+}$ but not in $CNTF^{-/-}$ littermates ($n = 6$ each group). Note that the numbers of $BrdU^+$ cells in the $CNTF^{-/-}$ mice are significantly lower than $CNTF^{+/+}$ littermates, as evidence for an endogenous role of CNTF. Scale bars: ***A***, 200 μm; ***B***, 50 μm.

effects of the regular doses of agents such as D_2 agonists. CNTF-regulating mechanisms probably differ in different regions of the CNS, because quinpirole did not increase CNTF in the spinal cord.

Midbrain dopaminergic projections enhance normal patterns of adult forebrain neurogenesis primarily through a direct D_2–CNTF pathway

The dopaminergic projections regulate neurogenesis in the SVZ and dentate gyrus as suggested by the reduced proliferation in animal models and Parkinson's disease (Baker et al., 2004; Hoglinger et al., 2004) and stimulation by different D_2 agonists in naive and injured mice (Hoglinger et al., 2004; our study). Quinpirole has some D_3 activity, but despite the presence of D_3 receptors in the adult SVZ (Araki et al., 2007), they do not regulate SVZ proliferation in adult mice, although they do in rats (Baker et al., 2005). Systemic quinpirole injections increased proliferation, as shown by a 2 h pulse or by daily injections of BrdU, and increased the number of SVZ neuroblasts. Quinpirole also stimulates proliferation in the embryonic SVZ (Ohtani et al., 2003). In apparent contrast, a D_2 preferential antagonist increases proliferation in the adult rat SVZ (Kippin et al., 2005). One difference is the delivery via chronic 14–30 d infusions, whereas we and others used intermittent injections over shorter times. It is possible that chronic treatments induce adaptive changes in D_2 receptors.

Our data suggest that CNTF mediates D_2-induced neurogenesis in the SVZ and dentate gyrus. Systemic quinpirole treatments increased neurogenesis in $CNTF^{+/+}$ and $CNTF^{+/-}$ but not $CNTF^{-/-}$ littermates. Because CNTF is only produced in GFAP+ cells of the SVZ, the lack of an effect of quinpirole in the $CNTF^{-/-}$ mice also suggest that only astrocytes mediate the D_2-induced neurogenesis. Moroever, the nigrostriatal dopaminergic pathway appears to regulate forebrain neurogenesis predominantly by regulating CNTF in the SVZ, because the denervation failed to reduce neurogenesis in $CNTF^{-/-}$ mice. Together, our data suggest that midbrain dopaminergic neurons regulate forebrain neurogenesis exclusively through a D_2–CNTF pathway.

CNTF clearly is an endogenous regulator of neurogenesis. $CNTF^{-/-}$ mice have ~20% less neurogenesis, and intracerebral CNTF knockdown in normal mice also reduces SVZ neurogenesis, here using a different antibody than before (Emsley and Hagg, 2003). The antibodies did not affect hippocampal neurogenesis, possibly because of poor tissue penetration. However, $CNTF^{-/-}$ mice also have reduced hippocampal proliferation. The neuroblasts/BrdU ratio remains constant after quinpirole treatment and in different CNTF genotypes, and migration appears not to be affected by CNTF or quinpirole. Thus, CNTF enhances normal patterns of adult CNS neurogenesis, confirming our previous findings with intracerebral injections of recombinant CNTF (Emsley and Hagg, 2003).

CNTF can promote self-renewal or maintenance of neural precursors *in vitro* (Chojnacki et al., 2003; Hitoshi et al., 2004) and maintain embryonic stem cell pluripotency *in vitro* (Wolf et al., 1994). Astrocytes, which produce CNTF, promote prolifera-

tion and neuronal specification of hippocampal precursors *in vitro* (Song et al., 2002). Thus, CNTF most likely promotes adult neurogenesis by activating CNTF receptors on the neural stem cells. The CNTF receptor complex consists of the CNTF-specific receptor α, the leukemia inhibitory factor β (LIFβ) receptor, and gp130. LIFβ receptor $^{-/-}$ mice have fewer neural stem cells in the SVZ (Shimazaki et al., 2001). Knocking out suppressor of cytokine signaling-3, which negatively regulates gp130 signaling, increases neural precursor proliferation *in vitro* (Emery et al., 2006). The impact of CNTF receptor α knock-out on CNS neurogenesis is unknown. The CNTF receptor α is exclusively present in GFAP+ cells in the SVZ (Emsley and Hagg, 2003), which could include the neural stem cells (Doetsch et al., 1997; Alvarez-Buylla and Lim, 2004; Garcia et al., 2004). It remains to be determined whether D_2 stimulation increases neurogenesis by increasing CNTF in the regular astrocytes and/or the GFAP+ neural stem cells. CNTF and its receptor are produced in the much more numerous astrocytes, suggesting that CNTF also has an autocrine/paracrine role in astroglial functions, as also proposed by others (Lee et al., 1997). However, CNTF does not appear to stimulate proliferation of regular SVZ astrocytes *in vivo* (Emsley and Hagg, 2003).

Reduced dopamine levels are central to Parkinson's disease, and it is possible that reduced neurogenesis in the SVZ and dentate gyrus contributes to the clinical symptoms of loss of olfactory function and depression (Klockgether, 2004), respectively. In animal models, reduced SVZ neurogenesis can cause reduced olfactory function (Enwere et al., 2004), and the effects of antidepressants are dependent on neurogenesis in the dentate gyrus (Santarelli et al., 2003). Our finding that CNTF $^{-/-}$ mice have a reduced neurogenesis may also have implications for the common CNTF null mutation in humans (Takahashi et al., 1994).

In summary, this study identifies a neurotransmitter-related mechanism that directly regulates the expression of the normal nervous system-selective CNTF and that can be pharmacologically modulated. Moreover, this increased CNTF expression is functionally important, because it induces adult neurogenesis, pointing to novel CNTF-targeted approaches for cell replacement therapies.

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