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Dopamine Specifically Inhibits Forebrain Neural Stem Cell Proliferation, Suggesting a Novel Effect of Antipsychotic Drugs

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Neurogenesis has been implicated in antidepressant drug action and animal models of depression, suggesting that proliferating cells play a role in psychiatric disorders. Similar studies using antipsychotic drugs have yielded conflicting results, perhaps because of the lack of focus on specific cell types. We examine the effect of haloperidol on neural stem cells (NSCs), the ultimate precursors for adult cell genesis. We show that haloperidol increases NSC numbers, resulting in more progenitors and more new neurons and glia in the adult rat brain. The increase in NSCs by haloperidol is dependent on central dopamine D_2 receptors, and these receptors are expressed by NSCs. D_2 receptor stimulation *in vitro* inhibits NSC proliferation, which is reversed by haloperidol. Thus, haloperidol increases adult mammalian brain proliferation by antagonizing dopamine at D_2 receptors on NSCs. These findings demonstrate a direct link between neural activity and NSC proliferation and implicate cell genesis in antipsychotic drug effects.

Key words: **haloperidol; proliferation; dopamine receptor; dopamine; neuronal progenitor cell; neurogenesis; stem cells**

Introduction

Emerging evidence has linked the production of new cells in the adult forebrain to psychiatric disorders. Antidepressant drugs enhance neurogenesis in the adult rodent dentate gyrus of the hippocampus (Malberg et al., 2000; Duman et al., 2001; Santarelli et al., 2003), and animal models of depression are associated with decreased neurogenesis in the same brain structure (Malberg and Duman, 2003; Santarelli et al., 2003). Several studies have investigated the effects of antipsychotic drug on cell genesis in a number of brain regions; however, these studies have reported inconsistent findings (Dawirs et al., 1998; Wakade et al., 2002; Halim et al., 2004; Schmitt et al., 2004; Wang et al., 2004). This failure to establish a clear link between antipsychotic drug administration and cell genesis is likely attributable to not only administration of different antipsychotic drugs but also to the use of different protocols to detect cell genesis that are sensitive to mixed or different populations of proliferating and maturing cell types. Accordingly, we specifically examine the effect of the antipsychotic drug haloperidol on the ultimate precursor cell of adult forebrain cell genesis, the neural stem cell (NSC).

Adult forebrain NSCs reside in the subependyma of the ventricular system, proliferate slowly, and last for the lifetime of the organism (Gage, 2000; van der Kooy and Weiss, 2000). The NSCs in the subependyma of the lateral ventricle give rise to constitutively proliferating (CP) progenitor cells that transiently reside within the subependyma (Morshead et al., 1998). Some of these rapidly proliferating progeny of NSCs migrate out of the subependyma and differentiate into neurons in the olfactory bulbs (Lois and Alvarez-Buylla, 1994; Luskin, 1994) and mature cell types in the striatum and other brain regions under certain conditions (Craig et al., 1996; Benraiss et al., 2001; Mao and Wang, 2001; Pencea et al., 2001).

Here, we demonstrate that chronic antipsychotic drug treatment increases NSC proliferation in the adult forebrain by antagonism of dopamine D_2 receptors (D_2Rs). This primary effect on NSCs appears to have secondary indirect effects on the numbers of CP cells and new mature cells generated. These latter effects may contribute to the changes in brain morphology during antipsychotic drug treatment reported in previous clinical (Chakos et al., 1994; Gur et al., 1998) and animal (Chakos et al., 1998; Andersson et al., 2002) studies.

Materials and Methods

Subjects. Male Sprague Dawley rats (Charles River Canada, Montreal, Canada) weighing 250–300 g at the start of each experiment were housed under standard conditions with access to food and water *ad libitum*.

Male and female wild-type (C57BL/6), $D_1R^{-/-}$, $D_2R^{-/-}$, and $D_3R^{-/-}$ mice [all derived from The Jackson Laboratory (Bar Harbor, ME) stocks] weighed 25–30 g at the start of the experiment and were from heterozygous breeding of 10 times backcrossed C57BL/6 mice. Mice were housed in groups under standard conditions.

Drugs. Haloperidol (5 mg/ml; Sabex, Boucherville, Canada) was diluted in distilled water. Domperidone (Sigma, St. Louis, MO) was dissolved in low pH saline and then brought to pH >6.5 for administration. For *in vivo* drug treatments, haloperidol, domperidone, or vehicle was

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delivered via Alzet osmotic pumps (models 2ML2 or 2ML4 for rats and 1002 for mice; Durect Corporation, Cupertino, CA). Plasma levels were determined in the same manner as reported previously (Kapur et al., 2003). For *in vitro* drug treatments, dopamine, quinpirole, 2,3,4,5-tetrahydro-7,8-dihydroxy-1-phenyl-1*H*-3-benazepine HCl (SKF 38393), and haloperidol (all from Sigma) were dissolved in 0.01% glacial acetic acid at 1 mM, stored at −20°C, and diluted in media immediately before use.

Neurosphere assay. Neurosphere-forming cells were isolated from the adult forebrain lateral ventricle subependyma as described previously (Morshead et al., 1998). Tissue was digested with enzymes (1.33 mg/ml trypsin, 0.67 mg/ml hyaluronidase, and 0.2 mg/ml kynurenic acid) for 50 min at 37°C, isolated in serum-free media (SFM), and mechanically dissociated into a single cell suspension. Viable cell density was determined using trypan blue exclusion. Cells were cultured under clonal conditions (at 10 cells/μl in 0.25 ml of media in uncoated well plates; Nunclon, Copenhagen, Denmark) in SFM containing 20 ng/ml epidermal growth factor (EGF) (mouse submaxillary; Sigma), 10 ng/ml fibroblast growth factor-2 (FGF) (human recombinant; Sigma), and 2 μg/ml heparin (Sigma). The number of neurospheres (diameter of ≥100 μm) was counted after 7 d. Under these conditions, it has been shown that neurosphere colonies are derived from single cells and serve as an index of the number of *in vivo* neural stem cells (Morshead et al., 2003). To examine NSC proliferation *in vitro*, neurospheres were dissociated into single cells and cultured as above with additional pharmacologic agents as described. To specifically quantify NSC symmetric divisions *in vitro*, single neurospheres generated in vehicle or drug were repeatedly dissociated and cultured as above in the absence of drugs.

Bromodeoxyuridine labeling and detection. Bromodeoxyuridine (BrdU) labeling of mitotic cells in S phase *in vivo* was used to index the number of neural stem cells in the subependyma of the lateral ventricle after long-term (30 d) retention, the number of progenitor cells in the subependyma of the lateral ventricle after short-term (1 h) retention (Morshead et al., 1998; Hitoshi et al., 2002), and the number of adult-born neurons and glia in the olfactory bulbs and striatum after long-term (30 d) retention. For determination of the effect of haloperidol on NSCs and differentiated cells, rats were implanted with pumps delivering haloperidol (2.0 $mg \cdot kg^{-1} \cdot d^{-1}$) for 30 d, injected intraperitoneally 48 h later with 60 mg/kg BrdU (Sigma) five times (once every 3 h), and killed 28 d after the final BrdU injection. For determination of the effect of haloperidol on CP cells, rats were implanted with pumps delivering haloperidol (2.0 $mg \cdot kg^{-1} \cdot d^{-1}$) for 14 d, received the same series of BrdU injections on the final day of haloperidol treatment, and were killed 1 h after the final BrdU injection. Animals were killed, tissue was prepared, and immunostaining for BrdU and neuronal-specific nuclear protein (NeuN) was performed as reported previously (Martens et al., 2002) using the following reagents: rat anti-BrdU antibody (1:100; Harlan Sera-Lab, Loughborough, UK), FITC donkey anti-rat antibody (1:200; Jackson ImmunoResearch, West Grove, PA), mouse anti-NeuN (1:200; Chemicon, Temecula, CA), and Alexa Fluor 555 goat anti-mouse (1:300; Molecular Probes, Eugene, OR). BrdU labeling in the subependyma of the lateral ventricles was quantified as described previously (Martens et al., 2002), and, in the granule zone of the olfactory bulbs and the striatum, the total number of positively stained cells was determined per section. All quantifications used optical dissector counts.

Ventricular and striatal volume estimates. Volumes were estimated based on surface area measurements made from coronal brain sections from rats treated with either vehicle or haloperidol (2.0 $mg \cdot kg^{-1} \cdot d^{-1}$) for 30 d. Tissue sections were prepared as for immunostaining. Surface area measurements of the lateral ventricles and striatum were made at ~300 μm intervals on sections corresponding to Paxinos and Watson (1998) plates 11 through 20 (~1.70 mm anterior to bregma to 0.40 mm posterior to bregma) based on major brain structure morphology. The average surface area for each structure was calculated and multiplied by the anteroposterior distance between the quantified sections. These measurements estimate the volumes of the lateral ventricles and adjacent striatum containing the areas of maximal ventricular cell proliferation.

D_2R immunocytochemistry and fluorescence-activated cell sorting. Adult rat brains (prepared as above) and lightly fixed neurospheres were cryosectioned at 14 μm thickness and incubated in rabbit anti-D_2R (1:1500; Research Diagnostics, Flanders, NJ) in PBS for 12 h at 4°C, followed by Alexa 488 or Alexa 568 goat anti-rabbit (1:300; Molecular Probes) in PBS for 2 h at 37°C.

For cell sorting, freshly dissected subependyma or neurosphere cultures were dissociated into single cell suspensions. Suspensions were incubated serially with rabbit anti-D_2R (1:1500) and Alexa 488 goat anti-rabbit (1:300) each for 1 h at 4°C. Suspensions were analyzed using forward and side scatter on an EPICS Elite Cell Sorter (Beckman Coulter, Fullerton, CA) and then sorted based on green fluorescence into $D_2R^{(+)}$ and $D_2R^{(-)}$ cell fractions. Because of the high background staining observed in the fluorescence-activated cell sorting (FACS), we selected as $D_2R^{(+)}$ cells only the cells with the highest expression that was clearly more than was seen by any of the cells in the control secondary antibody-only condition. Then, the separate $D_2R^{(+)}$ and $D_2R^{(-)}$ fractions were cultured as above under clonal conditions.

Reverse transcriptase-PCR. Total neurosphere RNA was isolated (RNeasy extraction kit; Qiagen, Hilden, Germany), and 1 μg of total RNA was used to synthesize cDNA with oligo-dT 12–18 primers and Moloney murine leukemia virus reverse transcriptase (Superscript II; Roche Products, Welwyn Garden City, UK) in a 25 μl reaction mixture at 42°C for 1 h. The PCR mixture (20 μl) consisted of 1 μl of cDNA, 16 pmol each of 5′ and 3′ primers, 0.2 mM dNTP, 1.5 mM Mg^{2+}, 2 μl of PCR buffer, and 0.8 U of *Taq* polymerase (Promega, Madison, WI). cDNA was amplified in a thermal cycler (PerkinElmer, Wellesley, MA) with 40 cycles of denaturation at 95°C for 30 s, annealing at 56°C for 40 s, and extension at 72°C for 40 s. The primers against D_2R cDNA used were as follows: sense, 5′-GTAGCAGCCGAGCTTTCAG-3′; and antisense, 3′-GGGATGTTGCAGTCACAGTG- 5′. This pair of primers was designed to encompass at least one intron to avoid false-positive amplification from contaminated genomic DNA.

Statistical analyses. Factorial design ANOVAs or *t* tests were used to analyze data as appropriate. Significant ANOVA values were followed by simple main effects analyses or *post hoc* comparisons of individual means using the Tukey's method when appropriate. The level of significance for all comparisons was 0.05.

Results

Haloperidol increases NSC proliferation

To assess the effect of haloperidol on NSC proliferation, adult male rats were implanted with a subcutaneous osmotic pump that delivered either vehicle or haloperidol (0.05, 0.25, or 2.00 $mg \cdot kg^{-1} \cdot d^{-1}$) for a period of 14 d, and the number of clonal neurospheres was determined to index the number of *in vivo* NSCs (Reynolds and Weiss, 1996; Morshead et al., 2003). Chronic haloperidol (0.25 or 2.00 $mg \cdot kg^{-1} \cdot d^{-1}$) produced a significant increase in the number of primary neurospheres derived from the subependyma of the lateral ventricles (Fig. 1*A*) ($F_{(3,90)} = 9.06$; $p < 0.05$, with the vehicle and 0.05 $mg \cdot kg^{-1} \cdot d^{-1}$ groups differing significantly from the 0.25 and 2.00 $mg \cdot kg^{-1} \cdot d^{-1}$ groups). The same pattern of results was seen when expressing the data as a function of numbers of neurospheres per forebrain or the proportion of plated cells that form neurospheres (data not shown).

Primary neurospheres derived from vehicle- or haloperidol-treated rats displayed almost identical *in vitro* self-renewal and multipotentiality characteristics. Individual primary neurospheres derived from rats treated with vehicle or haloperidol (2.00 $mg \cdot kg^{-1} \cdot d^{-1}$) *in vivo* were passaged or differentiated as described previously (Seaberg and van der Kooy, 2003). During dissociation into single cells and culturing in the presence of EGF, FGF, and heparin, individual neurospheres derived from rats treated with either vehicle and haloperidol gave rise to equivalent numbers of clonal secondary neurospheres (Fig. 1*B*, left) ($F < 1.0$; $p > 0.05$). Similarly, individual neurospheres derived from rats treated with either vehicle or haloperidol that were

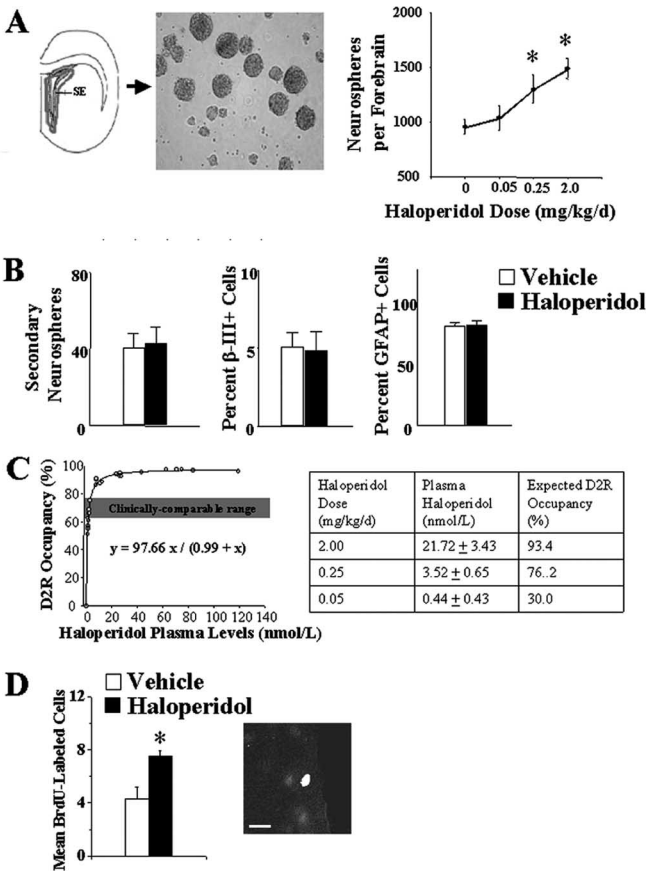

Figure 1. Haloperidol increases the number of NSCs in the subependyma and their progeny. ***A***, Left, Schematic of dissected subependyma (gray) and representative clonal neurospheres. Right, Haloperidol dose dependently increased neurosphere numbers derived from the adult lateral ventricle subependyma. ***B***, Neural stem cell self-renewal and multipotentiality *in vitro* after haloperidol treatment *in vivo*. Left, Passaging of single primary neurospheres derived from adult rats treated with vehicle or haloperidol (2.0 mg · kg^{-1} · d^{-1}) yields equivalent numbers of clonal secondary neurospheres. Middle, Right, Differentiation of single primary neurospheres derived from adult rats treated with vehicle or haloperidol (2.0 mg · kg^{-1} · d^{-1}) yields equivalent numbers of both neurons (middle) and astrocytes (right). All data are expressed as mean ± SEM. Scale bar, 100 μm. ***C***, Relationships between haloperidol dose, plasma levels, and striatal D_2R occupancy in the rat. Left, Relationship between haloperidol plasma levels and striatal D_2R occupancy in the rat. The formula depicts the level of D_2R occupancy (*y*) based on haloperidol plasma levels (*x*) in previously published data. Right, Haloperidol plasma level and expected D_2R occupancy at the end of 14 d of treatment with 2.00, 0.25, and 0.005 haloperidol. ***D***, Chronic haloperidol increased long-term retention (30 d) of BrdU labeling in the subependyma of the lateral ventricle; the photomicrograph displays typical labeling of a long-term BrdU-retaining cell. Scale bar, 20 μm. *$p < 0.05$.

cultured on Matrigel-coated plates in the presence of 1% serum were multipotential and gave rise to equivalent proportions of neurons (Fig. 1*B*, middle) ($F < 1.0$; $p > 0.05$) and astrocytes (Fig. 1*B*, right) ($F < 1.0$; $p > 0.05$). These data indicate that neurosphere-forming cells derived from rats treated with vehicle or haloperidol *in vivo* displayed the NSC characteristics of self-renewal and multipotentiality. Note that the effect of *in vitro* haloperidol treatment on neurosphere differentiation was not specifically assessed. Nevertheless, these data indicate that chronic haloperidol increases the proportion and absolute number of NSCs in the adult lateral ventricle subependyma.

Effective management of schizophrenia symptoms is produced by doses of antipsychotic drugs that produce 70–80% occupancy of D_2Rs in the striatum (Kapur et al., 2000; Seeman, 2002). Based on findings from Kapur et al. (2003) (Fig. 1*C* left), treatment with 0.05, 0.25, and 2.00 mg · kg^{-1} · d^{-1} haloperidol produced plasma haloperidol levels that correspond with 30, 76.2, and 93.4% D_2R occupancy (Fig. 1*C*, right). Thus, the 0.25 mg · kg^{-1} · d^{-1} haloperidol treatment achieves D_2R binding levels in the rat analogous to those necessary to produce clinical antipsychotic effects in patients with schizophrenia, demonstrating that haloperidol increases the number of NSCs at a clinically relevant dose.

Long-term (30 d) retention of BrdU labeling of NSCs in the subependyma *in vivo* confirmed the increase in NSC numbers during chronic haloperidol revealed by the neurosphere assay *in vitro*. Long-term retention of BrdU quantifies the proliferating portion of the relatively quiescent NSCs but not the rapidly dividing CP cells, which migrate out of the subependyma, undergo cell death, or dilute out the marker within 30 d (Morshead and van der Kooy, 1992; Lois and Alvarez-Buylla, 1994; Luskin, 1994; Morshead et al., 1998; Hitoshi et al., 2002). Rats were treated with haloperidol (2.0 mg · kg^{-1} · d^{-1}) for 30 d and received a series of five BrdU injections over 12 h on the third day of haloperidol treatment and were killed 28 d later. Chronic haloperidol increased the number of cells in the subependyma of the lateral ventricle that retained the BrdU label for 28 d (Fig. 1*D*) ($t_{(11)} = 3.02$; $p < 0.05$).

To assess the effect of haloperidol on the CP cell population, adult male rats were treated with vehicle or chronic haloperidol (2.00 mg · kg^{-1} · d^{-1}) and received a series of five BrdU injections over 12 h on the last day of treatment to label the progenitor population (Morshead et al., 1998; Hitoshi et al., 2002). Haloperidol increased the number of BrdU-labeled cells in the subependyma of the lateral ventricle (Fig. 2*A*) ($t_{(19)} = 3.49$; $p < 0.05$). In contrast, no increase in the number of progenitor cells was detected in the dentate gyrus of the hippocampus after haloperidol treatment in the same animals [mean ± SEM; BrdU-labeled cells (per square millimeter) for the vehicle group, 11.73 ± 3.54; and for the haloperidol group, 13.02 ± 3.29; $t_{(14)} = 0.57$; $p > 0.05$]. Furthermore, acute haloperidol (2.0 mg/kg, i.p.) either 6 or 24 h before the same series of BrdU injections did not significantly alter the number of BrdU-labeled cells in the subependyma of the lateral ventricle (Table 1): for haloperidol 6 h before BrdU, $t_{(12)} = 0.72$, $p > 0.05$; and for haloperidol 24 h before BrdU, $t_{(12)} = 0.29$, $p > 0.05$. Similarly, acute haloperidol (2.0 mg/kg, i.p.) either 6 or 24 h before the same series of BrdU injections did not significantly alter the number of BrdU-labeled cells in the dentate gyrus (Table 1): for haloperidol 6 h before BrdU, $t_{(12)} = 0.43$, $p > 0.05$; and for haloperidol 24 h before BrdU, $t_{(12)} = 0.89$, $p > 0.05$. Thus, chronic antipsychotic drug treatment increases the number of rapidly proliferating progeny of NSCs in the adult brain in a manner that is anatomically distinct with respect to the effects of antidepressant drugs (Malberg et al., 2000).

We next examined the effect of haloperidol on production of adult-born differentiated cells in the following: (1) the olfactory bulbs because it is the region in which the vast majority of surviving NSC progeny incorporate (Lois and Alvarez-Buylla, 1994; Luskin, 1994; Morshead et al., 1998); and (2) the striatum because it is the region in which antipsychotic drug-induced changes in tissue morphology are reported most consistently in both clinical (Chakos et al., 1994; Gur et al., 1998) and animal (Chakos et al., 1998; Andersson et al., 2002) studies. For this, adult male rats were treated with vehicle or chronic haloperidol (2 mg · kg^{-1} · d^{-1}) for a total period of 30 d. To label newly born cells, rats received a series of five BrdU injections over 12 h beginning 48 h after the start of drug treatment and were killed on

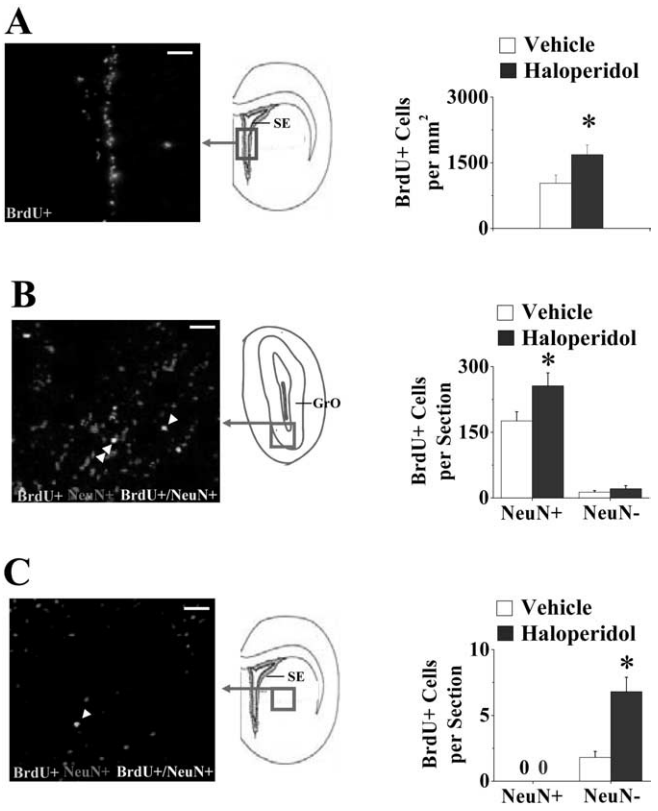

Figure 2. Haloperidol increases the number of CP cells in the subependyma, neurogenesis in the olfactory bulbs, and gliogenesis in the striatum. ***A***, Left, Photomicrograph of progenitor cells after haloperidol treatment. Right, Haloperidol increased BrdU$^{(+)}$ cell numbers in lateral ventricle subependyma. ***B***, Left, Photomicrograph of neurogenesis in the olfactory bulb. Right, Haloperidol increased BrdU$^{(+)}$/NeuN$^{(+)}$ cell numbers in the olfactory bulbs. ***C***, Left, Photomicrograph of non-neuronal cell genesis in the striatum. Right, Haloperidol increased BrdU$^{(+)}$/NeuN$^{(-)}$ cell numbers in the striatum. Single and double arrowheads indicate BrdU$^{(+)}$/NeuN$^{(-)}$ and BrdU$^{(+)}$/NeuN$^{(+)}$ cells, respectively. All data are presented as mean ± SEM. Scale bars, 50 μm. *$p < 0.05$.

the final day of drug treatment (i.e., 28 d later) to allow these cells to migrate and differentiate. Haloperidol produced a significant increase in the number of new neurons in the olfactory bulbs (Fig. 2*B*) ($t_{(10)} = 2.33$; $p < 0.05$) and a significant increase in the number of non-neuronal cells in the striatum (Fig. 2*C*) ($t_{(11)} = 6.18$; $p < 0.05$). Additionally, haloperidol-treated rats, relative to vehicle-treated rats, had both significantly smaller lateral ventricles ($t_{(12)} = 5.90$; $p < 0.05$; lateral ventricle volume estimates were 1.96 ± 0.25 mm^3 for haloperidol-treated rats and 3.67 ± 0.14 mm^3 for vehicle-treated rats) and significantly larger striatum ($t_{(12)} = 2.31$; $p < 0.05$; striatum volume estimates were 43.51 ± 1.87 mm^3 for haldoperidol-treated rats and 40.07 ± 1.41 mm^3 for vehicle-treated rats). This latter finding is consistent with the antipsychotic drug-induced increases in striatal volumes observed in other rat studies (Chakos et al., 1998; Andersson et al., 2002). Thus, antipsychotic drug treatments that alter brain morphology are associated with increases in both NSCs and their progeny.

The most parsimonious explanation for these results is that haloperidol has a single effect to increase NSC proliferation that in turn results in secondary increases in progenitor and newly differentiated cells. Alternatively, haloperidol may also have effects on progenitor proliferation and survival, which we examine more explicitly *in vitro* below. Furthermore, the increase in production of new cells suggests a novel mechanism for the changes in striatum morphology observed during antipsychotic drug treatment (for review, see Jeste et al., 1998; Harrison, 1999; Kapur and Remington, 2001; Konradi and Heckers, 2001).

Central D_2Rs mediate NSC expansion by haloperidol

Haloperidol has high binding affinity for D_2Rs, and D2Rs have been widely implicated in both the clinically beneficial and adverse effects of antipsychotic drugs (Nyberg and Farde, 2000; Kapur and Remington, 2001; Kapur and Seeman, 2001; Kapur et al., 2003). Accordingly, we examined the role of D_2Rs in haloperidol regulation of NSCs by treating $D_2R^{+/+}$ and $D_2R^{-/-}$ mice with vehicle or chronic haloperidol (2 mg · kg^{-1} · d^{-1}) by subcutaneous osmotic pumps for 14 d and then determined the number of neurospheres derived from the subependyma of the lateral ventricle to index NSC numbers. As in the rat (Fig. 1*A*), $D_2R^{+/+}$ mice that received haloperidol had increased numbers of neurospheres compared with $D_2R^{+/+}$ mice that received vehicle (Fig. 3*A*). In contrast, $D_2R^{-/-}$ mice that received haloperidol did not show an increase in the numbers of NSCs compared with $D_2R^{-/-}$ mice that received vehicle (Fig. 3*A*) (genotype by drug treatment interaction, $F_{(1,32)} = 3.97$, $p < 0.05$, with the haloperidol-treated $D_2R^{+/+}$ mice yielding significantly more neurospheres than all other groups). Notably, there was no difference between the number of neurospheres derived from untreated $D_2R^{+/+}$ and $D_2R^{-/-}$ mice, suggesting that loss of D_2Rs throughout development does not alter that number of NSCs present in adulthood. We also asked whether central or peripheral D_2Rs control NSC proliferation. Chronic domperidone (2.0 mg · kg^{-1} · d^{-1}), a potent D_2R antagonist that does not cross the blood–brain barrier, did not alter the number of neurospheres derived from the lateral ventricle subependyma of the adult rat (Fig. 3*B*) ($t_{(14)} = 0.03$; $p > 0.05$). Together, these findings demonstrate that NSCs are regulated by antagonism of dopamine at central D_2Rs.

NSCs express D_2Rs

To determine whether NSCs potentially express D_2Rs, we examined protein and mRNA expression in the adult subependyma and neurospheres. High D_2R protein expression is apparent in the adult rat lateral ventricle subependyma and striatum (Fig. 4*A*), as well as in neurospheres derived from adult lateral ventricle subependyma (Fig. 4*B*). D_2R mRNA was detected in neurospheres derived from the adult lateral ventricle subependyma by reverse transcriptase-PCR (Fig. 4*C*). These findings indicate that D_2Rs are present in the *in vivo* anatomical location of NSCs and in *in vitro* cell populations containing NSCs.

We prospectively determined whether neurosphere-forming cells express D_2Rs using a D_2R antibody for FACS of cells into $D_2R^{(+)}$ and $D_2R^{(-)}$ fractions and then cultured the cells under clonal conditions. FACS sorting of both dissociated lateral ventricle subependyma and passaged neurospheres yielded $D_2R^{(+)}$ cells (Fig. 4*D*); $D_2R^{(+)}$ cells comprised 3.4–4.4 and 1.6–3.6% of the total cells from lateral ventricle subependyma (three analyzed samples) and passaged neurospheres (five analyzed samples), respectively. Neurosphere-forming cells were present in all $D_2R^{(+)}$ samples, and, in fact, a modest enrichment of neurosphere-forming cells was produced by D_2R FACS in both dissociated subependyma (2.1-fold enrichment) and passaged neurosphere (4.4-fold enrichment) cultures (Fig. 4*E*) ($F_{(1,125)} = 12.28$; $p < 0.05$). The only modest enrichment of neurosphere-forming cells after D_2R FACS is likely attributable to D_2R expression on heterogeneous cell types, which is consistent with the dense pattern of D_2R immunoreactivity observed in the subependyma and neurospheres and suggests that both NSCs and progenitor cells express

Table 1. Lack of effects of acute haloperidol (2 mg/kg, i.p.) on the number of $BrdU^{(+)}$ cells in the subependyma of the lateral ventricle and dentate gyrus

	Subependyma		Dentate gyrus	
Treatment	6 h after injection	24 h after injection	6 h after injection	24 h after injection
Vehicle	1167 ± 209	1091 ± 194	9.8 ± 1.9	9.7 ± 1.7
Acute haloperidol	880 ± 242	1150 ± 240	8.8 ± 1.8	11.0 ± 2.4

All values are expressed as mean ± SEM per square millimeter.

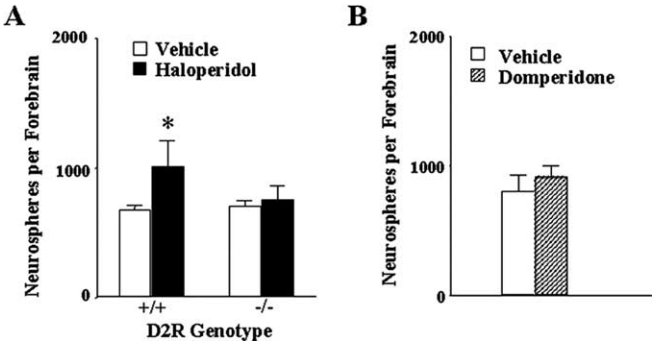

Figure 3. Haloperidol increases NSC number via central D_2Rs. ***A***, Haloperidol increases neurosphere number in $D_2R^{+/+}$ mice but not in $D_2R^{-/-}$ mice. ***B***, Peripheral D_2R antagonism by domperidone does not alter neurosphere number. All data are presented as mean ± SEM. *$p < 0.05$.

D_2Rs. Moreover, together, the expression of D_2Rs on neurosphere-forming cells and the D_2R-mediated regulation of NSCs *in vivo* suggests that the proliferation of NSCs may be directly influenced by these receptors.

D_2Rs inhibit NSC proliferation

Given that D_2R antagonism by haloperidol *in vivo* increases the proliferation of NSCs (Fig. 3*A*), we hypothesized that the direct effect of stimulating D_2Rs is inhibition of NSC proliferation. To test this hypothesis, we examined the effects of dopaminergic agents on NSC proliferation *in vitro*. The addition of dopamine or quinpirole (a selective $D_{2/3}$R agonist), but not SKF 38393 (a selective D_1R agonist), to neurosphere cultures derived from wild-type mice produced a dose-dependent (and approaching complete) inhibition of neurosphere formation (Fig. 5*A*). Statistical analyses confirmed an interaction between drug and dose ($F_{(10,205)} = 12.40$; $p < 0.05$). For the effects of dopamine dose ($F_{(5,60)} = 63.02$; $p < 0.05$) and quinpirole dose ($F_{(5,60)} = 88.63$; $p < 0.05$), follow-up analyses revealed that doses of 10 nM or higher of dopamine or quinpirole significantly decreased the number of neurospheres with the maximal effects at 100 nM or higher doses of dopamine or quinpirole. Doses of 1 nM or lower (1–100 pM; data not shown) did not significantly alter neurosphere formation. In contrast, no significant effect of SKF 38393 dose was found ($F_{(5,85)} = 0.58$; $p > 0.05$). These findings demonstrates that dopamine or a $D_{2/3}$R agonist inhibit neurosphere formation.

Although the preceding results demonstrate that dopamine inhibits the proliferation of cells that comprise clonally derived neurospheres, it does not indicate whether this effect is on NSCs or their progeny, which comprise <1% and >99% of neurospheres cells, respectively (Morshead et al., 2003), or both. To address this issue, we took advantage of the finding that some residual clonal neurospheres remain at all doses of dopamine or quinpirole. We selectively passaged individual neurospheres of equivalent size (and therefore equivalent cell numbers) generated in vehicle, 100 nM dopamine, 100 nM quinpirole, or 100 nM SKF 38393 to determine the numbers of neurosphere-forming cells within each primary neurosphere (all of the passaging was done in the absence of dopamine agonists). Passaging of individual neurospheres generated in 100 nM dopamine or 100 nM quinpirole (but not 100 nM SKF 38393) produced ~65% fewer secondary (i.e., subsequent culture) clonal neurospheres compared with those generated in vehicle (Fig. 5*B*) ($F_{(3,36)} = 6.00$; $p < 0.05$). These findings demonstrate inhibition of proliferation of individual NSCs by dopamine or a D_2R agonist. The reduction in secondary neurospheres derived from neurospheres generated in 100 nM dopamine or quinpirole was not attributable to prolonged decreases in NSC survival or selection of NSCs with intrinsically lower proliferation kinetics, because subsequent clonal passaging of individual secondary neurospheres (again in the absence of dopamine agonists) did not result in differences in tertiary neurosphere numbers from any of the conditions (Fig. 5*B*) ($F_{(3,49)} = 0.71$; $p > 0.05$).

In contrast to the dramatic effects of dopamine and quinpirole on neurosphere formation, there were no significant effect of either drug on neurosphere diameters ($F_{(3,146)} = 1.49$; $p > 0.05$; mean ± SEM, for vehicle, 232 ± 16.0 μm; 100 nM dopamine, 208 ± 26.0 μm; 100 nM quinpirole, 202 ± 18 μm; 100 nM SKF, 222 ± 40.0 μm), suggesting that the number of cells in each neurospheres that did form was not altered by D_2R stimulation. These findings demonstrate that stimulation of D_2Rs on NCSs directly inhibits their proliferation (but not their long-term survival), whereas the proliferation of progenitors is not directly inhibited (given that most of the cells in each neurosphere are progenitor cells and the neurospheres that did form were not decreased in size).

Loss or antagonism of D_2Rs prevents the inhibition of NSC proliferation by dopamine

The above pharmacologic data along with the expression of D_2R on NSCs implicate D_2Rs in the control of NSC proliferation. To confirm this role, we determined the ability of dopamine to inhibit neurosphere formation in NSCs lacking functional dopamine D_1Rs, D_2Rs, or D_3Rs. Dopamine dose dependently inhibited neurosphere formation in cultures derived from $D_1R^{-/-}$ and $D_3R^{-/-}$, but not $D_2R^{-/-}$, mice (Fig. 5*C*). Statistical analyses revealed a significant interaction between gene deletion and dopamine dose ($F_{(10,198)} = 21.54$; $p < 0.05$); follow-up analyses revealed that 10 nM and higher doses of dopamine significantly inhibited neurosphere formation in $D_1R^{-/-}$ and $D_3R^{-/-}$ cultures, whereas neurosphere formation was not inhibited in $D_2R^{-/-}$ cultures. These results, together with our pharmacologic data (Fig. 5*A*), implicate D_2Rs, but not D_1Rs or D_3Rs, in the effects of dopamine on NSC proliferation.

The above findings suggest that the effects of haloperidol *in vivo* (Fig. 1) are mediated by haloperidol antagonism of dopamine at D_2Rs on NSCs. We tested this hypothesis by culturing NSCs in the presence of both dopamine and haloperidol. Haloperidol dose dependently blocked dopamine inhibition of neurosphere formation (Fig. 5*D*); haloperidol alone did not alter neurosphere formation. Statistical analyses revealed a significant interaction between dopamine dose and haloperidol dose ($F_{(8,170)} = 2.87$; $p < 0.05$). Follow-up analyses revealed significant effects of haloperidol dose in the presence of 10 nM dopamine ($F_{(4,67)} = 4.65$; $p < 0.05$) and 100 nM dopamine ($F_{(4,29)} = 15.46$; $p < 0.05$), with 100 and 1000 nM haloperidol differing

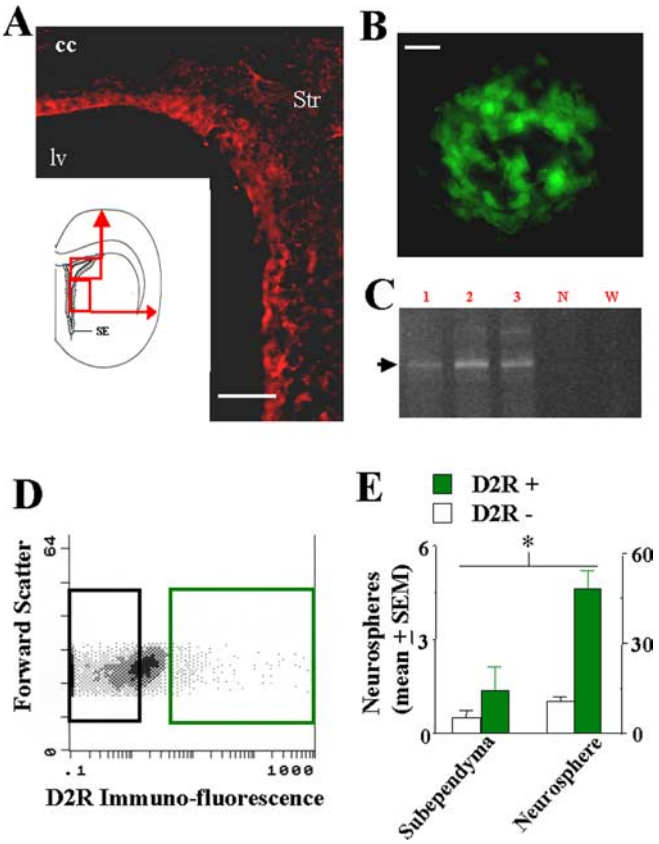

Figure 4. D_2Rs are expressed by NSCs. ***A***, D_2R immunoreactivity in the adult lateral ventricle subependyma. cc, Corpus callosum; Str, striatum; LV, lateral ventricle. ***B***, D_2R immunoreactivity in neurosphere derived from the adult lateral ventricle subependyma. ***C***, Reverse transcriptase-PCR detection of D_2R mRNA (442 bp PCR product) in adult lateral ventricle subependymal neurospheres (lanes 1–3); no product was observed without mRNA (lane W) or reverse transcription (lane N). ***D***, FACS analysis of D_2R immunoreactivity in dissociated neurospheres. ***E***, Enrichment of neurosphere-forming cells by FACS for D_2R immunoreactivity. Scale bars, 50 μm. $^*p < 0.05$.

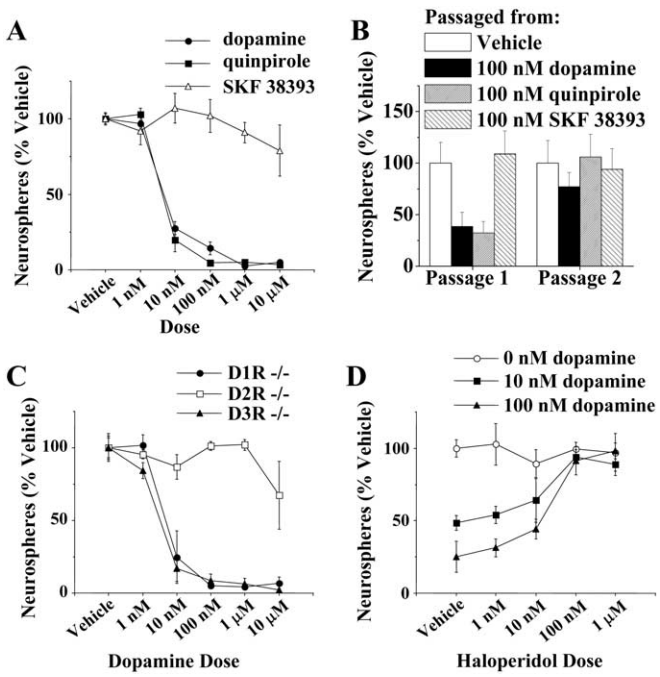

Figure 5. D_2R stimulation inhibits NSC proliferation *in vitro*. ***A***, Dopamine or quinpirole dose dependently inhibits neurosphere formation. ***B***, Passaging (in the absence of dopamine agonists) of individual neurospheres generated in 100 nM dopamine or 100 nM quinpirole yields fewer subsequent neurospheres (reflecting decreased symmetrical divisions in primary neurospheres). However, neurosphere numbers are restored by a second passage (in the absence of dopamine agonists), demonstrating no long-term effects on either symmetrical or asymmetrical NSC divisions. ***C***, Dopamine dose dependently inhibits neurosphere formation in $D_1R^{-/-}$ and $D_3R^{-/-}$ cultures but not in $D_2R^{-/-}$ cultures. ***D***, Haloperidol dose dependently antagonizes dopamine (10 or 100 nM) inhibition of neurosphere formation but has no effect in the absence of dopamine. All data are presented as mean ± SEM. For statistics, see Results.

significantly from haloperidol vehicle for both dopamine doses (p values <0.05). Conversely, in the absence of dopamine, there was no significant effect of haloperidol dose ($F_{(4,74)} = 0.29$; $p > 0.05$). In addition, haloperidol (in the absence of dopamine agonists) did not increase neurosphere formation when the exogenous growth factors (FGF2 and EGF) were absent or in very low concentration (data not shown), indicating that haloperidol by itself does not stimulate NSC proliferation. In summary, these findings demonstrate that haloperidol antagonism of D_2Rs prevents the ability of dopamine to inhibit NSC proliferation by a D_2R-specific mechanism. Our findings suggest that antagonism of endogenous dopamine at D_2Rs on NSCs mediates their expansion *in vivo* by haloperidol.

Discussion

D_2R stimulation specifically inhibits NSC proliferation

The present study provides extensive *in vivo* and *in vitro* evidence for a novel role of the neurotransmitter, dopamine, in the regulation of NSC proliferation. Our data indicate that dopamine stimulation of D_2Rs inhibits NSC proliferation and suggest that tonic endogenous dopamine inhibits the proliferation of NSCs. Furthermore, chronic D_2R antagonism by haloperidol produces increases in NSCs and their progeny *in vivo*. On the one hand, these data can be explained by a specific D_2R effect on NSC proliferation with secondary effects on progenitors and differentiating cells. Conversely, D_2Rs may also have distinct direct effects on progenitor cell proliferation and differentiation. However, the present findings are not able to specifically distinguish between these possibilities. The present findings are consistent with evidence for similar D_2R regulation of proliferation of other dividing precursor cells, for example, pituitary lacotrophs (Kelley et al., 1997; Saiardi et al., 1997; Asa et al., 1999; Iaccarino et al., 2002).

The function of D_2R regulation of NSCs is unclear but may contribute to induction of the relative quiescence displayed by adult NSCs (Morshead et al., 1994). Consistent with this hypothesis, dopamine innervation of the forebrain striatal embryonic germinal zone (Ohtani et al., 2003) coincides temporally with the progressive extension of cell cycle time in embryonic NSCs (Martens et al., 2000). However, we did not observe any increase in NSC numbers in adult mice lacking D_2Rs, suggesting that there is sufficient redundancy in the developmental control of NSC proliferation to compensate for loss of D_2R signaling. In contrast to our finding that chronic blockade of D_2R increases the numbers of NSC and their progeny, other recent reports suggest that the numbers of CP progenitors in the subependyma *in vivo* are increased by D_2R (Hoglinger et al., 2004) or D_3R (Van Kampen et al., 2004) activation. However, it is not clear whether these effects are attributable to alterations in proliferation or survival of these CP cells *in vivo*, and indeed even whether the effects are direct effects on NSCs or CP progenitor cells. In an attempt to assess the effects of dopamine function directly on NSCs and progenitor cells *in vitro*, Hoglinger et al. (2004) report that dopamine in-

creases BrdU incorporation in dissociated neurosphere cells in a 12 h assay. However, this facilitatory effect of dopamine on BrdU incorporation was only seen at doses of dopamine two orders of magnitude greater than the doses that inhibited the proliferation of neural stem cells in the present study. Moreover, given that neural precursor cells rarely proliferate in the 24 h immediately after sphere dissociation, BrdU uptake observed during this period may represent abortive DNA synthesis preceding cell death in their cultures. In the study by Van Kampen et al. (2004), BrdU was administered daily for a period of 14 d, with increased numbers of BrdU-labeled cells in the subependyma after concurrent intracranial administration of a D_3R agonist. Again, however, it is unclear whether the D_3R agonist altered proliferation, survival, or migration of the progenitor cells. In contrast to the above studies and consistent with the present findings, amphetamine, presumably attributable to increased dopamine levels, decreases the number of CP cells (Mao and Wang, 2001). After dopamine denervation by 6-hydroxy-dopamine (6-OHDA) or 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), proliferation is decreased in the subependyma of the lateral ventricles (Baker et al., 2004; Hoglinger et al., 2004), whereas gliogenesis is increased in the striatum (Kay and Blum, 2000; Mao et al., 2001; Mao and Wang, 2003) and substantia nigra (Kay and Blum, 2000, Mao et al., 2001; Zhao et al., 2003; Frielingsdorf et al., 2004) and neurogenesis is increased in the olfactory bulb (Yamada et al., 2004). Although 6-OHDA or MPTP result in diminished dopamine levels in the striatum, they also cause degeneration of the dopaminergic neurons in the midbrain and reorganization of synaptic circuitry in the striatum (Ingham et al., 1993, 1998; Nitsch and Riesenberg, 1995). Other reports have demonstrated that the levels of several neurotrophic factors are altered after 6-OHDA or MPTP lesions (Nagatsu et al., 2000; Nakajima et al., 2001; Yurek and Fletcher-Turner, 2001), suggesting that there maybe both direct and indirect effects of dopamine denervation on neural stem and progenitor cells. Thus, the proliferation and survival of NSC progeny (the CP progenitor cells) *in vivo* appears to be regulated by dopamine in a complex manner that depends on whether the dopamine effects are direct effects on proliferating neural precursor cells or indirect by way of growth factor release by nearby postmitotic cells, on whether NSCs or progenitor cells are primarily affected, on the dopamine receptor subtype involved, and on neural insults. Nevertheless, our findings indicate that neurotransmitters are capable of directly regulating not only the differentiation of neuronal progenitors (Deisseroth et al., 2004) but also the proliferation of NSCs.

Antipsychotic drug regulation of NSCs and their progeny

Although the present study is the first to specifically assess the effects of an antipsychotic drug on NSCs, several previous attempts have been made to elucidate the relationship between antipsychotic drugs and cell proliferation in the adult brain, but they have yielded a confusing pattern of results. In the subependyma, we report that chronic haloperidol (2.0 $mg \cdot kg^{-1} \cdot d^{-1}$ via osmotic pump) increases the number of BrdU-labeled cells 1 h after BrdU administration, whereas Wakade et al. (2002) reported that chronic administration via drinking water of risperidone (0.5 $mg \cdot kg^{-1} \cdot d^{-1}$) or olanzapine (10.0 $mg \cdot kg^{-1} \cdot d^{-1}$), but not haloperidol (0.4 $mg \cdot kg^{-1} \cdot d^{-1}$), increased BrdU labeling in the subependyma at 24 h after BrdU administration. Olfactory bulb neurogenesis was increased by chronic haloperidol in the present study but has not been examined following other antipsychotic drugs. In the striatum, we report that chronic haloperidol increased the number of BrdU-labeled cells at 28 d after BrdU labeling, whereas Wang et al. (2004) reported that chronic olanzapine (10.0 $mg \cdot kg^{-1} \cdot d^{-1}$ in drinking water) increased the number of labeled cells at 14 d after labeling (these authors also report that, in the same protocol, 2.0 $mg \cdot kg^{-1} \cdot d^{-1}$ haloperidol failed to significantly increase BrdU labeling, but inspection of their data reveals an approximate twofold increase in labeled cells). Neither typical nor atypical antipsychotic drugs at a variety of doses and chronic administration regimens appear to alter BrdU labeling in the dentate gyrus after either short (2–24 h) or long (14- 21 d) retention of BrdU (Malberg et al., 2000; Wakade et al., 2002; Schmitt et al., 2004; Wang et al., 2004) (but see Dawirs et al., 1998; Halim et al., 2004). Finally, chronic olanzapine (10.0 $mg \cdot kg^{-1} \cdot d^{-1}$ in drinking water) increased the number of BrdU-labeled cells in the prefrontal cortex at 14 d after BrdU administration (again, these authors report that 2.0 $mg \cdot kg^{-1} \cdot d^{-1}$ haloperidol under the same conditions did not increase the number of labeled cells, but inspection of their data show an approximate twofold increase). Furthermore, acute injections of antipsychotic drugs do not increase BrdU labeling in either the subependyma or dentate gyrus (Schmitt et al., 2004).

Resolution of these apparently contradictory reports of the effects of antipsychotic drugs requires consideration of the known biological characteristics of proliferating and maturing cell types in the adult nervous system (Morshead et al., 2003). NSCs divide slowly (Morshead et al., 1994) and thus DNA synthesis marker assays (e.g., BrdU labeling) are relatively insensitive to these cells, especially after short retention times. However, NSCs have unique properties that allow them to be identified and quantified using the clonal *in vitro* neurosphere assay (Reynolds and Weiss, 1996; Morshead et al., 2003). CP cells proliferate rapidly and DNA synthesis assays are highly sensitive to these cells; however, to quantify the entire population, BrdU must be administered across the cycle period of the cells (Morshead et al., 1998) and the retention period must be relatively short (i.e., no more than a few hours) to minimize migration (Morshead and van der Kooy, 1992; Lois and Alvarez-Buylla, 1994; Luskin, 1994) or cell death (Morshead and van der Kooy, 1992). Thus, examination of BrdU labeling in the subependyma at 24 h after BrdU administration does not accurately quantify either the CP or NSC populations. Less is known about the kinetics of progenitor differentiation; however, colabeling of mature cell markers and DNA synthesis assays allows their unambiguous detection. Accordingly, our study used these various assays along with *in vitro* pharmacology to identify a specific effect and mechanism of antipsychotic drug action on NSCs. The discrepancies between studies are likely attributed to the use of inappropriate detection strategies (i.e., those that do not target specific cell types) or antipsychotic dosing regimens. For instance, given the long cycle time of NSCs, altering the proliferation of these cells may require constant drug levels that are readily achieved by use of osmotic pumps rather than the peaks and trough pattern of drug levels produced by daily injection or *ad libitum* oral administration.

Clinical significance of haloperidol-induced expansion of NSCs

In humans and animals models, chronic antipsychotic drug treatment induces morphologic changes in the brain, primarily decreases the volume of the ventricles, and increases in the volumes of the striatum and other basal ganglia structures (for review, see Jeste et al., 1998; Harrison, 1999; Konradi and Heckers, 2001). Conversely, drug-naive patients with schizophrenia display reduced striatal volume, and these morphological alterations are ameliorated or abolished by antipsychotic drug treatment

(Chakos et al., 1994; Keshavan et al., 1994). Although previous studies have implicated increased cell volume (Benes et al., 1985; Kerns et al., 1992) and synapse numbers (Meshul and Casey, 1989; Uranova et al., 1991) in the mediation of these changes in brain morphology, our findings suggest that NSC activity also contributes to these effects. Haloperidol increases NSC symmetric divisions (increasing NSC number) and, perhaps indirectly, asymmetric divisions (increasing progenitor cell number), leading to an increased pool of undifferentiated cells that are able to incorporate into the adult brain as neurons and glia in the olfactory bulb and striatum, respectively. Thus, it appears that an increase in cell number and changes in cell differentiation contribute to the changes in brain morphology observed with typical antipsychotic drug treatments. Similarly, atypical antipsychotic drug treatment increases CP proliferation in the subependyma of the lateral ventricle (Wakade et al., 2002) and the striatum (Wang et al., 2004), suggesting that regulation of NSCs or their progeny (either directly or indirectly) is common across classes of antipsychotic drugs. In contrast, the relationships between NSC proliferation or changes in brain morphology and the clinical effects observed during antipsychotic drug treatments remain unclear.

The present study adds to our understanding of the forebrain NSC niche by extending the known regulators of NSC proliferation beyond growth factors to include neurotransmitters. These findings indicate that cell genesis may contribute to the clinical effects of antipsychotic drugs and more generally that neural activity may directly influence adult neural stem cells.

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