

Effects of Chronic Haloperidol and Clozapine Treatment on Neurogenesis in the Adult Rat Hippocampus

Nader D Halim¹, Cynthia Shannon Weickert¹, Benjamin W McClintock¹, Daniel R Weinberger¹ and Barbara K Lipska^{*,1}

¹Clinical Brain Disorders Branch, Intramural Research Program, National Institute of Mental Health, NIH, Bethesda, MD, USA

It has been proposed that the therapeutic benefits of treatment with antidepressants and mood stabilizers may arise partially from their ability to stimulate neurogenesis. This study was designed to examine the effects of chronic antipsychotic treatment on cell proliferation and survival in the adult rat hippocampus. Haloperidol (0.05 and 2 mg/kg), clozapine (0.5 and 20 mg/kg), or vehicle were administered i.p. for 28 days, followed by bromodeoxyuridine (BrdU, 200 mg/kg, i.p.), a marker of DNA synthesis. One group of rats was killed 24 h following BrdU administration and BrdU-positive cells were quantified to assess the effects of drug treatment on cell proliferation. The remaining animals continued on antipsychotic medication for an additional 3 weeks following BrdU administration to assess the effects of antipsychotics on cell survival. Our results show that 24 h following BrdU, a low dose of clozapine (0.5 mg/kg) increased the number of BrdU-positive cells in the dentate gyrus (DG) by two-fold. Neither 20 mg/kg of clozapine nor haloperidol had any effect on cell proliferation in DG. Moreover, neither drug at either dose had an effect on the number of newly generated neurons surviving in the DG 3 weeks following BrdU administration. These preliminary findings suggest that clozapine may influence the number of cells which divide, but antipsychotics do not promote the survival of the newly generated neurons at 3 weeks after a BrdU injection.

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INTRODUCTION

Neurogenesis in the adult brain appears to occur throughout life in the subventricular zone of the lateral ventricle and the granule cell layer (GCL) of the hippocampus in rodents (Altman and Das, 1965; Markakis and Gage, 1999), non-human primates (Kaplan and Hinds, 1982; Gould *et al*, 1998, 1999; Kornack and Rakic, 1999, 2001), and humans (Eriksson *et al*, 1998). In the hippocampus, cells arise from progenitors within the border of the hilus and dentate gyrus (DG) and accumulate in the DG (Seaberg and van der Kooy, 2002; Palmer *et al*, 2000). Newly generated neurons in the DG are morphologically indistinguishable from other granule cell neurons (van Praag *et al*, 2002), may be long lived (Eriksson *et al*, 1998), may contact and receive appropriate targets from the existing hippocampal circuitry (Markakis and Gage, 1999; Hastings and Gould, 1999), generate action potentials and have functional synaptic inputs (van Praag *et al*, 2002), and may be important for

learning and/or memory formation (Shors *et al*, 2001; Gould *et al*, 1999).

Hippocampal dysfunction and/or atrophy are implicated in major neuropsychiatric disorders such as schizophrenia (for a review, see Weinberger, 1999) and mood disorders (Sheline *et al*, 1996; Duman *et al*, 1997; Benes *et al*, 1998). Antidepressant drugs, electroconvulsive therapy (ECT), and lithium used to treat mood disorders have been shown to increase neural proliferation and, perhaps, promote subsequent survival of neurons in the hippocampus (Malberg *et al*, 2000; Chen *et al*, 2000; Manev *et al*, 2001). Thus, it was postulated that some therapeutic properties of antidepressants and mood stabilizers may arise from their ability to increase hippocampal neurogenesis, which, in turn, subsequently improve hippocampal function. As atypical antipsychotics can be effective in the treatment of bipolar disorder and depression, it was hypothesized that these drugs may also regulate neural plasticity and, perhaps, stimulate neurogenesis (Duman, 2002). However, the extent to which atypical antipsychotics can modulate cell division and survival of newly generated cells within the hippocampus has not been established. The results of a few previous reports on the action of antipsychotic treatment on BrdU incorporation in the hippocampus are inconsistent (see Discussion) and have focused on estimates of cell proliferation shortly after BrdU injection rather than differentiation and survival of mature neurons. In other words, these

*Correspondence: Dr BK Lipska, National Institute of Mental Health, NIH, Bldg. 10, Rm. 4N306, Bethesda, MD 20892-1385, USA, Tel: +1 301 496 9501, Fax: +1 301 402 2751, E-mail: lipskab@intram.nimh.nih.gov

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reports did not address the question of whether antipsychotics affect neurogenesis, as it takes weeks to generate a functionally integrated new neuron and each newly generated cell must be unambiguously identified as a neuron to conclude that neurogenesis has indeed been affected.

In this study, we asked whether chronic haloperidol or clozapine administration had an effect on cell proliferation or the survival of newly generated neurons. Using BrdU as a marker of DNA synthesis, we examined the effects of antipsychotic treatment on cell proliferation and subsequent survival. MK-801, an NMDA antagonist, has previously been shown to increase cell proliferation in the DG (Cameron *et al*, 1995) and was used in this study as a positive control. To confirm the phenotype of the newly generated cells, we used multiple labeling with antibodies against BrdU, neuronal (neuronal nuclear antigen—NeuN) and glial (glial fiber acid protein—GFAP) markers via immunohistochemistry, and confocal microscopy to determine the phenotype of cells that survive in the DG, 3 weeks after BrdU administration.

MATERIALS AND METHODS

Drug Preparation

A stock solution of haloperidol (Sigma Chemicals, St Louis, MO) (20 mg/ml) was prepared by heating 200 mg of haloperidol in 10 ml 1% lactic acid until dissolved. To obtain solutions of 0.05 and 2 mg/ml haloperidol, the stock solution was diluted with distilled water. NaOH (1 N) was added to adjust the final solutions to a pH of 5.1. Clozapine (Sigma Chemicals) was prepared daily by dissolving 140 mg of clozapine in 0.6 ml of 1 N HCl with gentle heating, then diluting the solution with distilled water to 20 and 0.5 mg/ml. Solutions were neutralized with 1 N NaOH to a pH of 5.1. Vehicle consisted of 0.1% lactic acid. MK-801 (Sigma Chemicals) was prepared by dissolving 10 mg MK-801 in 10 ml of saline. Bromodeoxyuridine (BrdU) was prepared by dissolving 10 mg BrdU in 1 ml of 0.007 N NaOH and 0.9% NaCl in distilled water.

Animals and Drug Administration

Adult male Sprague–Dawley rats (Harlan, Indianapolis, IN) ($n = 56$, weight 225–250 g) were housed two per cage with *ad libitum* access to food and water. All procedures were performed in accordance with the National Institutes of Health Guide for Use and Care of Laboratory Animals. After a 1-week habituation period, animals were administered haloperidol (2 mg/kg, $n = 13$ and 0.05 mg/kg, $n = 15$), clozapine (20 mg/kg, $n = 10$ and 0.5 mg/kg, $n = 12$) or vehicle ($n = 11$) daily via intraperitoneal (i.p.) injections. This dose regimen was chosen to emulate the therapeutic range of doses given to patients (Kapur *et al*, 2000), and was showed to be effective in our previous behavioral and biochemical studies (Al-Amin *et al*, 2000; Lipska *et al*, 2001, 2003). All animals were administered daily injections of drug or vehicle for 4 weeks prior to a single injection of BrdU (200 mg/kg, i.p.). This dose of BrdU has been shown to label nearly all dividing cells within the rat DG (Cameron and McKay, 2001). For determination of the effects of

antipsychotics on BrdU incorporation at an early time point after a BrdU injection (a 'proliferation' group), animals in each of the drug groups (haloperidol: 2 mg/kg, $n = 9$ and 0.05 mg/kg, $n = 9$; clozapine: 20 mg/kg, $n = 6$ and 0.5 mg/kg, $n = 6$; vehicle: $n = 6$) were transcardially perfused (phosphate buffered saline (PBS) 0.1 M, pH 7.4, followed by 4% paraformaldehyde in PBS) for 24 h following BrdU administration under sodium pentobarbital anesthesia (65 mg/kg, i.p.). Three additional rats were injected with vehicle daily for 4 weeks, who then received MK-801 (1 mg/kg, i.p.) 1 h prior to BrdU administration and were killed 24 h later. The remaining animals (a 'survival' group) continued receiving daily drug or vehicle treatment for an additional 21 days prior to perfusion.

Tissue Processing

Following perfusion, the crania were removed and stored overnight in 4% paraformaldehyde in PBS at 4°C. Whole brains were then removed and immersion fixed for an additional 24 h, cryoprotected in 20% glycerol-PBS, then embedded into gelatin matrix blocks. Each matrix block contained up to 18 individual brains and two blocks were created per group ('proliferation' or 'survival', $n = 39$ and 25 brains, respectively). Gelatin matrix blocks were fixed in 4% paraformaldehyde with 30% sucrose for 72 h to preserve the integrity of the block. Serial 50 μ m thick composite coronal sections throughout the entire block were cut. Every 12th composite section was processed for stereological counts of BrdU-positive cells, and phenotypic determination using neuronal (NeuN) or glial markers (GFAP) via multiple fluorescent labeling. Sections were stored in a cryoprotective solution (20% glycerol and 20% ethylene glycol phosphate buffered to a pH of 7.4) at -20°C until processing.

Immunohistochemistry

Free-floating composite sections were thoroughly rinsed of cryoprotective solution with PBS and then post-fixed in 4% paraformaldehyde overnight at 4°C. DNA was denatured by incubating sections in 2 N HCl for 30 min at 37°C with agitation. The acid was then neutralized with 1 M Tris-HCl (pH 7.5) for 5 min, and then thoroughly rinsed in PBS. Endogenous peroxidase activity was blocked by treatment with 0.1% H_2O_2 and 10% CH_3OH in PBS for 30 min. Nonspecific binding was blocked by incubating sections in 5% normal horse serum (NHS) and 5% BSA in PBS + (PBS with 0.1% Triton X-100) for 2 h at room temperature with constant agitation. Sections were then incubated with a mouse anti-BrdU antibody (Becton-Dickinson, Franklin Lakes, NJ) at 1:300 dilution in 5% NHS-PBS + overnight at 4°C with agitation. After thorough rinsing in PBS, sections were incubated with a horse anti-mouse biotinylated secondary antibody (Vector Laboratories, Burlingame, CA) at 1:500 in 5% NHS-PBS + for 2 h at room temperature. Sections were then incubated in Vectastain elite ABC kit (Vector Laboratories) for 1 h, thoroughly rinsed in PBS, and developed with a standard diaminobenzidine kit (Vector Laboratories).

For multiple fluorescent labeling, sections were processed as above for DNA denaturation, then blocked in 10%

normal donkey serum (NDS) in PBS+ for 2 h, before incubation in a cocktail of primary antibodies in 10% NDS-PBS+ with rat anti-BrdU 1:200 (Accurate Chemicals, Westbury, NY), mouse anti-NeuN 1:300 (Chemicon, Temecula, CA), and rabbit anti-GFAP 1:500 (Chemicon) overnight at 4°C with constant agitation. After rinsing, sections were incubated for 2 h with anti-rat rhodamine-X, anti-rabbit Cy5, and anti-mouse Cy2 (Jackson Immuno) conjugated secondary antibodies at room temperature at a 1:300 dilution each in 10% NDS-PBS+. All secondary antibodies were produced in donkey and cross absorbed against multiple species. Sections were thoroughly rinsed, mounted on subbed slides, air dried, dehydrated, cleared in xylene, and coverslipped with DPX (Electron Microscopy Sciences, Ft. Washington, PA).

Quantification of BrdU-positive cells

Every 12th coronal section throughout the entire brain was processed for BrdU immunohistochemistry. All BrdU-positive cells in both hemispheres of the DG granule cell layer (GCL—defined as the GCL proper and a 40 µm wide band immediately adjacent to the hilar surface of the GCL proper) for the proliferation and survival groups were counted through the rostral-caudal extent of the hippocampus (bregma −1.6 to −6.8 mm, according to the atlas of Paxinos and Watson, 1997) on coded slides, by an investigator blind to treatment. BrdU-positive cells were assessed using a Nikon E400 light microscope with an ×40 lens and an ×10 ocular (and in cases of clustered cells at ×1000 magnification). A total of eight sections per animal were counted for the assessment of the total number of BrdU-positive cells, according to a modified version of the fractionator method (Guillery and Herrup, 1997; Eisch *et al*, 2000; Cameron and McKay, 2001). The total number of BrdU-positive cells was multiplied by 12 in order to obtain the total number of BrdU-positive cells per bilateral hippocampus.

Phenotypic Determination

Triple-labeled fluorescent composite sections were analyzed using a Leica TCS confocal laser-scanning microscope. Images were taken using an oil immersion ×63 objective with an ×10 ocular. Approximately 50 BrdU-positive cells per subject were analyzed in Z-plane sections (0.5 µm step size) throughout the entire cell to determine if BrdU co-localized with either GFAP or NeuN.

Statistical Analysis

The numbers of BrdU-positive cells were compared between drug treatment groups using an analysis of variance (ANOVA). Proliferation and survival groups were analyzed separately as they were processed separately. Fisher's PLSD *post hoc* comparisons were performed when applicable. Analyses were performed using Statview statistical analysis software.

RESULTS

BrdU Immunohistochemistry

BrdU immunohistochemistry showed a similar staining distribution as reported previously (Cameron and McKay, 2001). BrdU-positive cells were located along the subependymal zone, the DG, and within major fiber tracts. Furthermore, confocal microscopy demonstrated that immunolabeling was present throughout the entire 50 µm section in the Z-plane (step size 0.5 µm), demonstrating that antibody penetration was adequate to label antigens within the entire thickness of the section.

Cell Proliferation

BrdU-positive cells, generally found in pairs or clusters, were distributed at the border of the GCL and hilus (Figure 1). These cells had an elliptical shape and a reduced size compared with adjacent Nissl-stained granule cells in

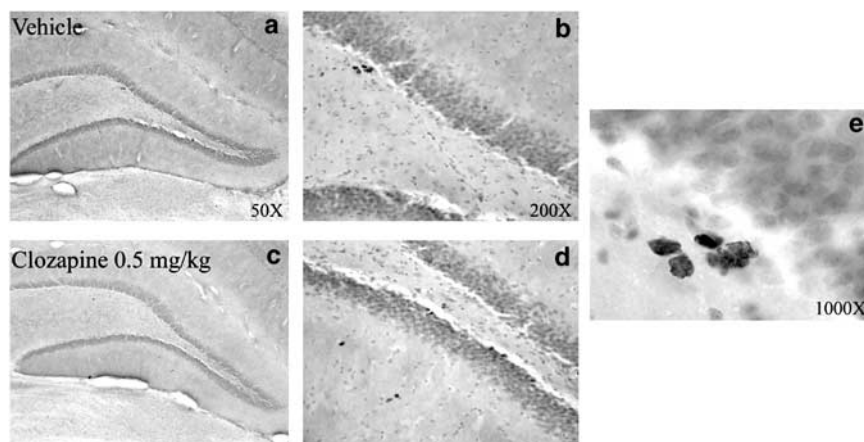


Figure 1 Representative photomicrographs of BrdU-positive cells in the dentate gyrus of adult rats. Chronic clozapine (0.5 mg/kg) significantly increased the number of BrdU immunopositive cells (c, d) relative to vehicle-treated animals (a, b, e) 24 h following BrdU administration (200 mg/kg). BrdU-positive cells, generally in pairs or clusters, were distributed at the border of the granule cell layer and hilus (a–d). These cells had an elliptical shape and a reduced size compared with adjacent Nissl-stained granule cells (e).

the GCL. Analysis of the number of BrdU-positive cells in the DG 24 h following BrdU administration demonstrated a significant effect of drug treatment ($F_{5,38} = 26.9$, $p < 0.0001$).

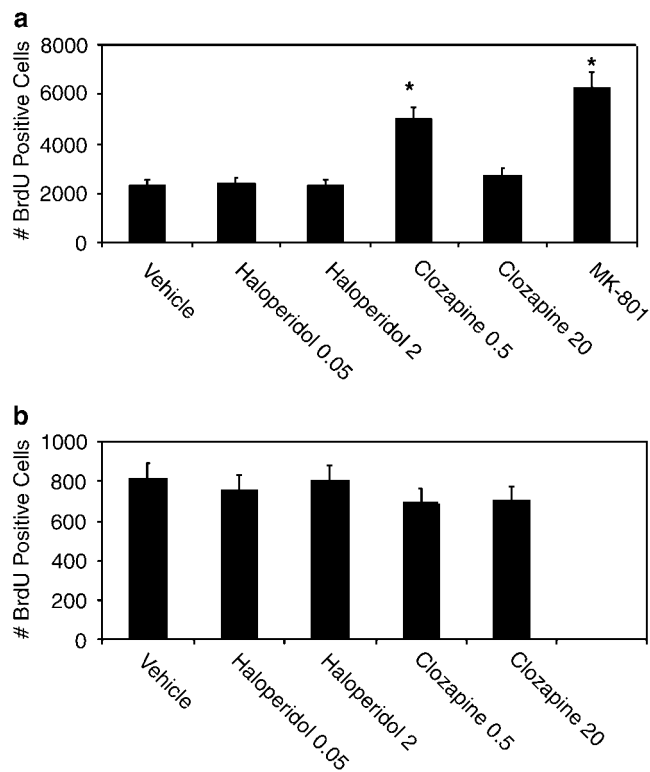


Figure 2 The numbers of BrdU-positive cells (mean \pm SD) in the dentate gyrus of rats treated with clozapine (0.5 or 20 mg/kg), haloperidol (0.05 or 2 mg/kg), or vehicle (0.1% lactic acid) for 28 days, followed by a single BrdU injection 24 h (a) or 21 days (b) prior to perfusion (see the text for details). Immunopositive cells were assessed throughout the entire bilateral dentate gyrus. (a) Chronic clozapine (0.5 mg/kg) treatment and a single injection of MK-801 (1 mg/kg) 1 h prior to BrdU administration (200 mg/kg) significantly increased the number of BrdU immunopositive cells relative to vehicle-treated animals 24 h following BrdU administration within the adult rat dentate gyrus ($*P < 0.0001$ significantly different from vehicle control). (b) Chronic treatment with either clozapine or haloperidol had no effect on the number of BrdU immunopositive cells surviving in the dentate gyrus 21 days after BrdU administration.

Post hoc analysis revealed that treatment with the lower dose of clozapine (0.5 mg/kg) and MK-801 (1 mg/kg) significantly increased the number of BrdU-positive cells as compared to all other treatments ($p < 0.0001$). The lower dose of clozapine (0.5 mg/kg) and MK-801 increased the number of BrdU-positive cells by 216 or 268%, respectively, relative to vehicle (Figure 2a).

Cell Survival

BrdU-positive cells were located throughout the DG GCL from basal to apical portions of the GCL, though the majority of cells were located at the edge of GCL on the hilar surface. These cells had similar morphology and size to adjacent Nissl-stained GCL granule neurons (Figure 3). There was no effect of treatment ($F_{4,24} = 0.57$, $p = 0.68$) on the number of BrdU-positive cells in the DG 3 weeks following BrdU administration (Figure 2b).

Phenotypic Evaluation

Using confocal microscopy, we evaluated the phenotypes of BrdU-positive cells in the DG 3 weeks following BrdU administration (Figure 4). Only cells that were located in the same anatomical region as used for counting were analyzed for phenotype (see Materials and methods). Triple-labeling of BrdU-positive cells revealed that the percentage of cells double-labeled for neurons and glia did not differ between the treatment groups ($F_{4,24} = 0.377$, $p > 0.8$). The majority of all BrdU-positive cells examined co-expressed NeuN ($> 75\%$), a marker of mature differentiated neurons. A small percentage of cells were BrdU and GFAP positive ($< 10\%$). BrdU/GFAP-positive cells were generally located at the outer edge of the subgranular zone and in the hilus proper. A minor percentage of BrdU-positive cells ($< 10\%$) were GFAP and NeuN negative, and may represent a quiescent undifferentiated cell type (van Praag *et al*, 1999a) or an endothelial cell type (Palmer *et al*, 2000).

We were unable to perform a similar unambiguous analysis of the phenotype of BrdU-positive cells during proliferation (1 day after BrdU injection) using various pro-neural, glial or endothelial cell markers (NeuroD1,

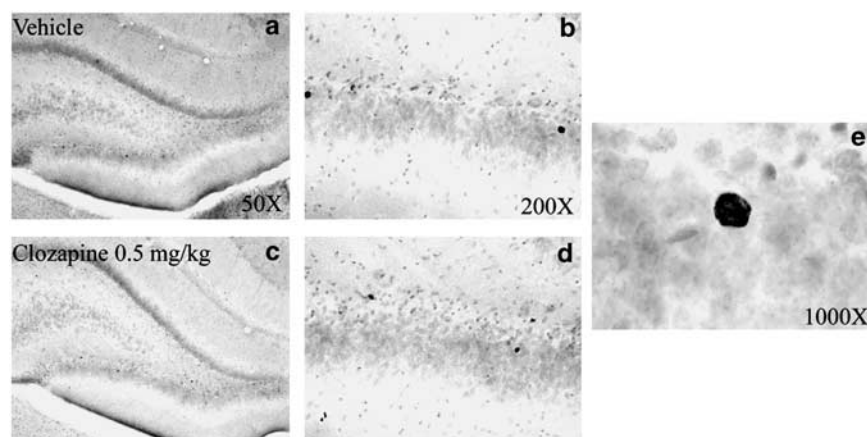


Figure 3 Representative photomicrographs of BrdU-positive cells surviving in the dentate gyrus for 21 days following BrdU (200 mg/kg i.p.) administration and 49-day treatment with clozapine (0.5 mg/kg; c, d) or vehicle (0.1% lactic acid (a, b, e)). BrdU-positive cells were located throughout the granule cell layer of the dentate gyrus (a–d). BrdU-positive cells had similar morphology and size to adjacent Nissl-stained GCL granule neurons (e).

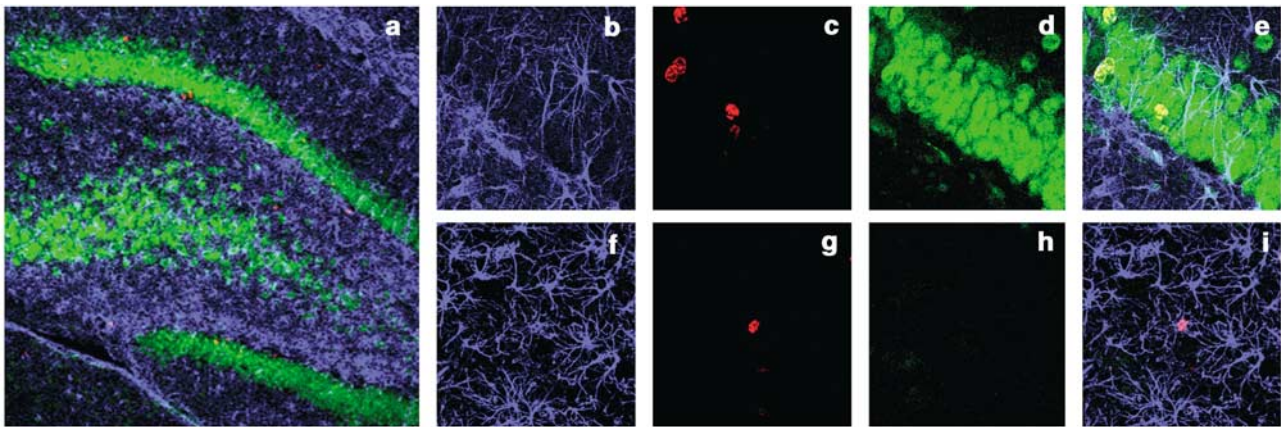


Figure 4 A representative photomicrograph illustrating that a majority of BrdU-positive cells surviving in the dentate gyrus 21 days following BrdU administration are of a neuronal phenotype. Triple label immuno-fluorescence for GFAP (b,f), BrdU (c,g), and NeuN (d,h), and merged images (a,e,i) obtained via confocal microscopy. Triple-labeling confirmed that the majority of all BrdU-positive cells examined (red) co-expressed the marker of mature neurons NeuN (green) (a, e) (> 75%). A small percentage of cells were BrdU positive and GFAP positive (blue) (< 10%) (a, i). A minor percentage of BrdU-positive cells (< 10%) were GFAP and NeuN negative (not shown). Magnification (a) $\times 100$ and (b–i) $\times 660$.

β -tubulin III, GFAP, RECA-1), either because the pattern of staining made co-localization difficult to confirm (NeroD1 and RECA) or that there were very low incidence of co-localization with BrdU (β -tubulin III, GFAP).

DISCUSSION

The results of this study demonstrate that chronic treatment with clozapine (0.5 mg/kg) increased the number of BrdU-positive cells in the DG 24 h following BrdU administration, whereas treatment with haloperidol (0.05 or 2 mg/kg) or 20 mg/kg clozapine had no effect. However, there was no change in the number of BrdU-positive cells in response to antipsychotic treatment 3 weeks following BrdU administration. These findings suggest that although a low dose of clozapine may upregulate cell proliferation in the DG, these newly generated cells do not survive and subsequently are not integrated into the existing hippocampal circuitry. Furthermore, antipsychotic treatment did not influence the phenotype of newly generated cells, as demonstrated by confocal microscopy using immunohistochemical co-localization for neuronal and glial markers. Confocal analysis revealed that, similar to previous reports, a majority of BrdU-labeled cells in the GCL were neurons (Liu *et al*, 1998; Malberg *et al*, 2000). Thus, the results of this study demonstrate that neither haloperidol nor clozapine have an effect on the survival of newly generated neurons. Although it is still unclear what type of cells increased proliferation after a low dose of clozapine, as a quantitative analysis of co-localization with glial or pro-neural markers proved to be very difficult (see methods), our preliminary data suggest that these cells may be of endothelial type (eg, RECA-1 positive).

Our findings on cell proliferation are in agreement with some, but not all, previous reports. Malberg *et al* (2000) reported that subchronic haloperidol treatment (1 mg/kg for 7 days and 2 mg/kg for an additional 7 days) had no effect on cell proliferation in the DG. Similarly, Wakade *et al* (2002) found that chronic haloperidol (0.4 mg/kg for 21 days) had no effect on cell proliferation in the DG. They also reported that the atypical antipsychotics, risperidone and

olanzapine (0.5 and 2 mg/kg, respectively, for 21 days), similarly had no effect. Kippin *et al* (2002) reported that neither haloperidol nor clozapine (2 and 20 mg/kg, respectively, for 14 days) affected BrdU-positive cells in the DG. Yet, Dawirs *et al* (1998) reported that acute treatment (4 injections over 24 h at 5 mg/kg each) with haloperidol significantly increased BrdU-positive cells in the DG 7 days after BrdU administration in gerbils, whereas Backhouse *et al* (1982) reported that treatment with haloperidol (a single injection of 20 mg/kg in 11-day-old rat pups) decreased the number of BrdU-positive cells. However, the latter two studies examined the effects of antipsychotics in an acute rather than chronic paradigm and used either different species (gerbils rather than rats in Dawirs *et al*, 1998) or preadolescent rats (Backhouse *et al*, 1982).

Proliferation and survival of newly generated neurons are regulated by different factors (see Prickaerts *et al* (2004) for a review), and while previous reports have examined the effects of antipsychotics on proliferation, none has examined whether antipsychotics affect the survival of newly generated neurons. Our current data show that neuronal survival is either unaffected or perhaps (after clozapine) adversely affected by chronic antipsychotic treatment.

Various trophic factors have been implicated in the modulation of cell survival in neurogenic regions of the brain. For instance, BDNF was shown to be a survival and/or proliferative factor for neurons, and to increase cell proliferation *in vivo* via intraventricular BDNF infusions (Zigova *et al*, 1998). Antidepressant treatments upregulate BDNF expression in the DG (Chen *et al*, 2001; Nibuya *et al*, 1995). In contrast, BDNF expression is reduced in the hippocampus of rats chronically treated with haloperidol or clozapine (Dawson *et al*, 2001; Lipska *et al*, 2001; Chlan-Fourney *et al*, 2002).

It is possible that the increase in the number of BrdU-positive cells seen after chronic treatment with clozapine 0.5 mg/kg may not represent an increase in the number of newly generated neurons, but another cell lineage. For instance, Palmer *et al* (2000) demonstrated that up to 37% of proliferating cells within the DG are of an endothelial cell lineage. These cells die, migrate away, or continue dividing,

whereby diluting BrdU to undetectable levels, within 1 month of their genesis (Palmer *et al*, 2000). Hence, the increase seen after treatment with a lower dose of clozapine may represent proliferation of endothelial cells or glia, which have a transient existence or migrate away from the GCL and thus may have little functional implications within the hippocampus. Our preliminary findings support this possibility.

Though neurogenesis continues in the DG throughout life, little is known of the function of the newly generated neurons. Neurogenesis is positively correlated with learning and memory in some hippocampal-dependent tasks in rats (van Praag *et al*, 1999b; Shors *et al*, 2001; Gould *et al*, 1999), suggesting a possible role in memory formation. Yet others have failed to find a correlation between neurogenesis and performance on other cognitive tasks, such as contextual fear conditioning or Morris water maze performance (Shors *et al*, 2001). Since chronic antipsychotic treatment may decrease the performance on memory tasks (Levin *et al*, 1996; Levin, 1997) and increased learning and memory may be associated with an increase in neurogenesis, it may not be surprising that antipsychotics have no long-term influence on neurogenesis in the DG. Alternatively, as the cause-effect relationship between memory function and neurogenesis is unclear, it is also possible that no improvements in memory are seen following antipsychotic treatment because these drugs do not stimulate neurogenesis, in agreement with the results of our findings.

Finally, it remains possible that the increase in BrdU incorporation seen here may represent methodological confounds rather than increased cell proliferation. Increased BrdU incorporation after high doses of BrdU and/or experimental manipulations may represent DNA repair rather than DNA replication (Cooper-Kuhn and Kuhn, 2002). Yet several recent reports have demonstrated that BrdU-positive cells express proteins associated with proliferation (KI-67, proliferating cell nuclear antigen), migration (doublecortin), and differentiation (NeuN, nuclear specific enolase, and calbindin) in the temporal pattern that would be expected of newly generated neurons (Cooper-Kuhn and Kuhn, 2002; Seki, 2002; Palmer *et al*, 2000). It has also been demonstrated that BrdU incorporation was undetectable in cultured fibroblasts undergoing DNA repair initiated by UV irradiation (Palmer *et al*, 2000). In our study, the increase in the number of BrdU-positive cells was seen in response to a relatively low dose of clozapine but not the higher dose, arguing against drug-induced neurotoxicity. Finally, it is possible that the low dose of clozapine altered the bioavailability of BrdU in the DG and thus contributed to the increased BrdU incorporation.

In summary, our findings suggest that a low dose of clozapine may promote cell division but both haloperidol and clozapine have no effect on neuronal survival in the DG at 3 weeks after a BrdU injection. Perhaps, these newly generated cells lack appropriate targets, cues or trophic factors to survive.

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