Chronic atypical antipsychotics, but not haloperidol, increase neurogenesis in the hippocampus of adult mouse

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Abstract

It is suggested that altered neuroplasticity contributes to the pathophysiology of schizophrenia and antipsychotics may exhibit some of their therapeutic efficacies by improving neurogenesis and/or proliferation of neural progenitors. The aim of this study is to investigate whether chronic antipsychotics treatment affect neurogenesis in adult mouse hippocampus. Animals were administered olanzapine, quetiapine, clozapine, risperidone, aripiprazole, or haloperidol via the osmotic minipump for 21 days and then injected with 5-bromo-2'-deoxyuridine (BrdU) to label mitotic cells. BrdU-positive cells in the hippocampus were quantified by stereology. Aripiprazole, quetiapine, clozapine, and olanzapine significantly increased density of BrdU-positive cells in the hippocampus. Interestingly, other antipsychotic drugs had tendency to increasing BrdU-positive cells, whereas haloperidol had propensity to decrease with a marginal significance. These results suggest that differences of neurogenesis among these drugs may, at least in part, account for their pharmacological profiles.

Key words

neurogenesis, 5-bromo-2'-deoxyuridine (BrdU), hippocampus, antipsychotics, schizophrenia, mouse

1. Introduction

Neurogenesis appears to occur throughout the life in the subventricular zone (SVZ) of the lateral ventricle and the subgranule zone (SGZ) of the hippocampus in rodents (Altman and Das, 1965; Kaplan and Hinds, 1977; Markakis and Gage, 1999), non-human primates (Gould et al., 1999; Kornack and Rakic, 1999), and humans (Eriksson et al., 1998). In the SVZ of adult newborn neurons migrate anteriorly into the olfactory bulb (OB), where they mature into local interneurons (Alvarez-Buylla and Garcia-Verdugo, 2002). In the meanwhile, in the hippocampus, neurogenesis in the adult dentate gyrus (DG) originates from a precursor population that resides in the SGZ, a thin band of tissue between the granule cell layer (GCL) and the hilus (Ehninger and Kempermann, 2008). The proliferating cells in the SGZ give rise to mature neurons that migrate into the GCL (Hastings and Gould, 1999) and these new neurons differentiate and appear to have morphological and physiological characteristics that are similar to adult granule cells (van Praag et al., 2002; Malberg and Duman, 2003).

The integration of adult-born neurons into the circuitry of the adult hippocampus has been implicated in physiological brain function such as spatial pattern separation (McHugh et al., 2007; Clelland et al., 2009; Sahay et al., 2011), memory reconsolidation (Kitamura et al., 2009), and stress responses (Snyder et al., 2011). Moreover, alteration of this process has been associated with a number of neuropsychiatric diseases including depression (Gould et al., 1997; Malberg et al., 2000), drug addiction (Nixon and Crews, 2002; Noonan et al., 2008), epilepsy (Parent et al., 2006), and schizophrenia (Keilhoff et al., 2004; Flagstad et al., 2005). Recent studies aimed at understanding the role of neurogenesis in such illnesses have focused more on hippocampal neurogenesis, partially owing to the low numbers of neurons born in the human OB under physiological conditions (Kempermann, 2013). These facts raised new questions on what neurogenesis in this area really means in both pathophysiology of mental diseases and their therapeutic process.

Although the principal brain target that conventional antipsychotic drugs act on is the dopamine D2 receptor, and many typical antipsychotics induce extrapyramidal symptoms (EPS) or hyperprolactinemia by acting on it, atypical antipsychotics given in dosages within the clinically effective range do not bring about these adverse clinical effects. To understand how atypical antipsychotics work, it is important to examine their mechanism of action (Seeman, 2002). In fact, the effects of antipsychotics on proliferation and neurogenesis have been investigated in the neurogenetic region of adult animal brain and these studies are closely reviewed elsewhere (Toro and Deakin, 2007). Dawirs et al. found that acute treatment of haloperidol stimulated proliferation of 5-bromo-2'-deoxyuridine (BrdU)-positive cells in the gerbil hippocampus (Dawirs et al., 1998). However, results of subsequent studies in rats differ from the previous one. Wakade et al. reported that repeated treatment of haloperidol for 21 days had no effect on the cell proliferation either in the DG or the SVZ (Wakade et al., 2002). Likewise, Halim et al. found that there was no change in number of BrdU-positive cells in the DG after acute or chronic haloperidol treatment (Halim et al., 2004). Moreover, it was shown by Wang et al. that chronic haloperidol treatment had no changes in proliferation in

the medial prefrontal cortex (PFC), striatum (STR), DG, and nucleus accumbens (NAc) (Wang et al., 2004). These results suggest that neurogenesis may play little role in the therapeutic efficacy of haloperidol.

With regard to atypical antipsychotics, results have been more complicated; experimental methods such as types, dosage or periods of drugs used lead to completely different results. It is reported by Wang et al. that olanzapine significantly increased both total number and density of BrdU-labeled cells in the PFC and dorsal striatum (Wang et al., 2004). Similarly, Kodama et al. found that chronic administration of olanzapine increased the number of newborn cells in the DG of hippocampus (Kodama et al., 2004). On the other hand, acute and chronic treatment of clozapine had no effects in another study (Halim et al., 2004). Additionally, Wakade et al. found that chronic risperidone and olanzapine administration induced a significant increase in BrdU-positive cells in SVZ, however, this effect was not seen in the hippocampus (Wakade et al., 2002).

As described above, it remains inconsistent whether antipsychotics, especially atypical antipsychotics, may enhance neurogenesis and proliferation in contrast to antidepressant drugs. Further work is required to resolve these discrepancies because they may be due to diversity not only in the types of antipsychotics used in studies but in their dosages, application regimens, or animal species.

The aim of this study is to investigate effects of antipsychotics on the neurogenesis in the hippocampus. To uncover the effects exhaustively, we examined six of the most commonly used antipsychotics. We reported here that atypical antipsychotics, but not haloperidol, enhance neurogenesis in the mouse hippocampus, which may reflect beneficial properties of these drugs.

2. Result

2.1 BrdU immunohistochemistry

BrdU immunohistochemistry revealed uniformly-stained nuclei which were predominantly distributed in the SGZ throughout the hippocampal structure in all animals examined as we reported previously (Yutsudo et al., 2013), thus, demonstrating that both BrdU administration and the antibodies used in this study were adequate to label newborn cells in this area (Fig. 1).

2.2 Chronic atypical antipsychotics administration increase the number of BrdU-positive cells

We then counted the number of BrdU-positive cells in SGZ using unbiased stereology. We found that chronic atypical antipsychotics administration increased density of BrdU-positive cells in the hippocampus by 30-70% (vehicle, 10182.6± 1132.3 cells/mm^3; aripiprazole,17606 ± 1132.3 cells/mm^3; clozapine, 16265.4 ± 1200.9 cells/mm^3; olanzapine, 14400.8±1074.2 cells/mm^3; quetiapine, 16804.8± 1200.9 cells/mm^3; risperidone, 13036.9±1024.2 cells/mm^3; mean±SEM) (Fig. 2). Statistical analysis revealed that aripiprazole, clozapine, olanzapine, and

quetiapine administration significantly increased BrdU-positive cells in the hippocampus ($F_{6,58}$=10.5; $p < 0.0001$).
Whereas, chronic administration of haloperidol showed a 23% reduction of BrdU-positive cells compared to the vehicle group (7871.8±1074.2 cells/mm^3; mean± SEM; $p < 0.05$). This indicates that an increase in density of BrdU-positive cells may be specific to atypical antipsychotics.

3. Discussion

This is the first study that examines the effect of antipsychotics on the hippocampal neurogenesis exhaustively. We revealed in this study that chronic atypical antipsychotics administration increased the density of BrdU-positive cells in SGZ by 30-70%, and this effect was not observed in haloperidol treatment. The fact that upregulation of neurogenesis was only observed in atypical antipsychotics may reflect these drugs share pharmacologically similar mechanism of action.

Previous studies have demonstrated that haloperidol had no effects on hippocampal neurogenesis (Malberg et al., 2000; Wakade et al., 2002; Wang et al., 2004; Halm et al., 2004), which is in line with our result. This result seems valid because haloperidol is a potent dopamine D2 antagonist and basically exerts its pharmacological action by blocking D2 receptors. This result is also replicated by a recent study that another D2 receptor antagonist sulpiride had no effect on the cell number of adult rat DG-derived neural precursor cells (Takamura et al. 2014). Keilhoff et al. exceptionally reported that very small amount of haloperidol increased BrdU-positive cells in DG and normalized vitamin D-deficient-induced reduction of cell proliferation (Keilhoff et al., 2010). The difference between these results may be interpreted as follows; administration of haloperidol does not affect or would rather decrease neurogenesis or cell proliferation at a normal therapeutic dose, whereas, subclinical dose of this drug may act in a quite different way. Although the effect of different doses of haloperidol on neurogenesis has not been investigated yet, this notion has been supported by the studies of ketamine, a glutamate NMDA (N-methyl-D-aspartate) receptor antagonist. At a normal dose, ketamine interferes with cell proliferation and differentiation of neural stem cells (Huang et al., 2015), on the contrary, subanesthetic doses of ketamine applied subchronically enhance neurogenesis in SGZ (Keilhoff et al., 2004). In any case, it is unlikely that haloperidol produces its therapeutic actions through stimulation of neurogenesis under the normal dose for treatment.

As mentioned above, it is controversial whether atypical antipsychotics increase neurogenesis in the hippocampus, thus we should closely discuss our findings. Xu et al. demonstrated that chronic administration of 5 or 10 mg/kg of quetiapine increase number of BrdU-labeled cells in DG at a dose-dependent manner (Xu et al., 2006). It is also demonstrated that 2 mg/kg of olanzapine, but not 0.5 mg/kg, administration increased the number of neurons both in the DG and prelimbic cortex as the same extend as fluoxetine, a selective serotonin reuptake inhibitor (SSRI) (Kodama et al., 2004). Interestingly, they also demonstrated that most new

born cells differentiated into neurons in the DG, in contrast to the prelimbic cortex, where approximately 20% of new born cells differentiated into endothelial cells but not neurons, and concluded that their results represent antidepressant effect through reverse of the loss of glia in depressed patients. Compared to conventional antipsychotics including haloperidol, the action mechanism of atypical antipsychotics is more complex and thus still remains unclear. Although 'atypical' was originally referred as effectiveness against the positive symptoms in schizophrenia with little extrapyramidal symptoms (EPS), it has been shown now that this class of drugs is also effective on improvement of cognition and/or mood disorder. These pharmacological effects of atypical antipsychotics reflect more than dopamine D2 antagonism and suggest morphological changes of the several parts of brain including hippocampal formation. Increased neurogenesis seen in this area may contribute to such advantages of atypical antipsychotics that this class of drugs improves not only positive symptoms but also negative symptoms or mood disorder.

On the other hand, Maeda et al. reported that clozapine, administered orally, protects phencyclidine-induced decrease in the number of BrdU-labeled cells but had little influence on cell proliferation by its self (Maeda et al., 2007). This result suggests another possibility of the relation between atypical antipsychotics and neurogenesis; they reinstate altered neurogenesis for some reason because similar effect is also confirmed in restraint stress (Luo et al., 2005) and ischemia (Bi et al., 2009). Another fascinating study is that chronic administration of 0.5 mg/kg of clozapine increased BrdU-positive cells in DG, but 20 mg/kg of this drug had no effect (Halim et al., 2004). It is interesting that Wakade et al. reported that risperidone and olanzapine increase neurogenesis in SVZ, but this effect is not observed in the hippocampus (Wakade et al., 2002). Similarly, Green et al demonstrated that olanzapine significantly increased cell proliferation in the SVZ and PFC but not the DG (Green et al., 2006). We did not examine the neurogenesis of these regions in this study, because neurogenesis in this area has not been detected in human. Furthermore, it is shown that all new cells are non-neural when the differentiation of the newly born cells has been followed (Madsen et al., 2005). For these reasons, we took a negative view to the significance of neurogenesis in SVZ and PFC even though neurogenesis increases in these regions when antipsychotics are administered. In either case, these discrepancies between the studies might be explained by the methodological differences, mainly due to the route of drug administration; difference between osmotic pumps used in our experiment and intraperitoneal or subcutaneous injection or oral administration. It is known that osmotic pumps serve some preferable aspect such as to reduce stress to the animals, minimize unwanted experimental variables, and hold the drug concentration constant (Kapur et al., 2003; Remington et al., 2011). Any factor listed here can alter neurogenesis and cell proliferations and it is likely that our method test drug potency by minimizing such extra undesirable factors.

There are certainly some limitations in our experiment. First, the doses we adopted in this study are only valid for the tested species (young C57BL/6J male mice) because pharmacokinetics of drugs including antipsychotics differs among the species and strain of the animal employed in each study (for review, Riviere et al., 1997). Second, as discussed above, the routes of administration of drugs, e.g.

subcutaneously, intraperitoneally, orally, or via osmotic minipump, are crucial because altered first-pass effect and other metabolic considerations could influence drug concentration and eventually neurogenesis in the brain. Third, we killed the animals and counted BrdU-labeled cells only one time point from the BrdU administration. There are some discussions about duration between BrdU injections and killing animals. Furthermore, as review by Taupin, BrdU is not a marker of the S-phase of cell cycles but a thymidine analogue, therefore, studying neurogenesis with BrdU requires distinguishing cell proliferation and neurogenesis from other events involving DNA synthesis including DNA repair (for review, Taupin, 2007). Further studies using other reliable cell proliferation markers such as proliferating nuclear antigen (PCNA) or Ki-67 are needed to eliminate ambiguity over the regimen with BrdU. Despite of these limitations, we think our study is remarkable because this is the first study that examines six of the major antipsychotics simultaneously in contrast to previous studies that approach this problem cover only a few drugs. Furthermore, it should be emphasized that we have quantified BrdU-labeled cells by means of stereology. As is well described elsewhere, direct, two-dimensional counting is prone to a number of artifacts that can lead to an erroneous conclusion in the cell counts and the technique of quantitative stereology allows precise and unbiased measurement of three-dimensional parameters (Howell et al., 2002). Few previous studies adopted this method, which can contribute to an increased risk of an erroneous conclusion.

Finally, the mechanism by which atypical antipsychotics, but not haloperidol, increase BrdU-positive cells in the hippocampus is not clear. Haloperidol is a potent dopamine D2 antagonist and essentially exerts its pharmacological action by blocking D2 receptors. Whereas, it is well known that atypical antipsychotics, by and large, act as 5-HT receptor inverse agonists, but also act on many other receptors. Although it remains unclear whether antipsychotics, especially atypical antipsychotics, exerts its therapeutic effect(s) through the increase of hippocampal neurons and further studies are necessary to clarify this question, our data suggest that differences of neurogenesis among these drugs may, at least in part, account for their pharmacological profiles and their therapeutic potency.

4. Experimental procedures

4.1 Animals

Male C57BL/6J mice (Clea Japan, Tokyo, Japan), 10 weeks of age at the start of the experiments, were used. They were housed four to five per cage with ad libitum access to food and water. All animals were maintained in an air-conditioned, pathogen-free room with a time-controlled lighting system. The handling and killing of animals was carried out in accordance with nationally prescribed guidelines. Ethical approval for the study was granted by the Animal Care and Use Committee of Kyushu University, Fukuoka, Japan and the Institutional Animal Care and Use Committee of Kurume University School of Medicine, Fukuoka, Japan.

4.2 Drugs

Haloperidol, quetiapine and clozapine were purchased from Sigma-Aldrich (St. Louis, MO, USA). Other atypical antipsychotics were generously provided by each manufacturer; aripiprazole from Otsuka Pharmaceutical Co., Ltd. (Tokyo, Japan), risperidone from Janssen Pharmaceutica NV (Beerse, Belgium), and olanzapine from Eli Lilly and Co. (Indianapolis, IN, USA). For immunohistochemistry, mouse monoclonal antibody against BrdU (1170376, 1:800) was obtained from Roche Diagnostics Japan (Tokyo, Japan). All drugs were dissolved in distilled water with a drop of lactic acid, and then the pH was adjusted to 5 to 7 with 1 N sodium hydroxide.

4.3 Drug administration

After mice were habituated to the new environment for 2-weeks, haloperidol (1 mg/kg/day, n=10), quetiapine (20 mg/kg/day, n=8), aripiprazole (3 mg/kg/day, n=9), clozapine (20 mg/kg/day, n=9), olanzapine (2 mg/kg/day, n=9), risperidone (0.5 mg/kg/day, n=11), or vehicle (n=9) were administered via intraperitoneally implanted micro-osmotic pumps (Alzet, Model 1004, Durect Corp. Cupertino, CA) for 21 days. Although the dose of an antipsychotic chosen in animal studies presents crucial importance, the literature reveals no standard rationale across studies (Kapur et al., 2000). Actually, it is often arbitrarily chosen, as is typically seen in haloperidol being administered between the lowest dose (0.05mg/kg) and the highest dose (5mg/kg) (Morais et al., 2017; Halim et al., 2004). Therefore, in this study we decided the dose regimen according to the previous studies shown to be effective both in behavior and biochemistry (Centonze et al., 2004; Bi et al., 2009; Li et al., 2004; Halim et al., 2004; Kodama et al., 2004; Wakade et al., 2002). Then, all mice were injected intraperitoneally with 50 mg/kg BrdU once a day on the 18th, 19th and 20th days. There is a debate with regard to the dose of BrdU to administer to label S-phase cells of adult animals. Burns and Kuan reported that 50-100 mg/kg of BrdU is sufficient for labeling the S-phase proliferative cells in mice (Burns and Kuan, 2005). On the other hand, Cameron and McKay showed that in the adult rat, systemic injections of 50 mg/kg of BrdU label at most 45% of S-phase cells in the DG and a single higher dose of BrdU saturated the S phase population without causing overt damage to the labeled cells (Cameron and McKay, 2001). However, at least to our knowledge, such foundation is lacking for the mouse. For these reasons, in this study we use multiple injections of 50 mg/kg of BrdU method according to the previous study by Kempermann et al. (Kempermann et al., 1998).

4.4 Tissue processing and immunohistochemistry

24 hours after the final BrdU injection, the animals were deeply anesthetized with pentobarbital (100 mg/kg, i.p.) and perfused intracardially with saline, followed by cold 4% paraformaldehyde in PBS. The brains were removed, immersed for 12 h in the same 4% paraformaldehyde fixative at 4° C, and cryoprotected in 20 and 30% sucrose in PBS for 48 h at 4° C. Brains were then embedded in Tissue-Tek OCT (optimum cutting temperature) compound and stored at -80° C until use. Serial coronal sections (40 μm) were cut on a cryostat, collected as free-floating sections in PBS and then processed immediately for immunohistochemistry (IHC), according to previously described methods (Kajitani et al., 2006). Briefly, free-

floating sections were incubated in 50% formamide in 2 × SSC for 2h at 65° C. The sections were washed in PBS-T (0.3% Triton X-100 in PBS) and immersed in H2O for 10 min. Next, the sections were treated with 2N HCl at room temperature for 30 min, to denature nuclear DNA, and then treated with Tris-HCl (pH 7.5) for 10 min. After washing in PBS, sections were incubated with an anti-BrdU antibody over night at 4° C. Sections were then incubated for 1 h with secondary antibody (biotinylated horse anti-mouse; Vector laboratories, Burlingame, CA, USA), followed by amplification with the Vectastain ABC KIT (Vector laboratories, Burlingame, CA, USA), and bound antibodies were visualized with the VEC SK-4100 DAB substrate kit (Vector Laboratories). Digital images were acquired using an Axioskop2 Plus microscope, equipped with an AxioCam CCD camera (Carl Zeiss Microimaging Japan, Tokyo, Japan).

4.5 Counting BrdU-positive cells using unbiased stereological counting techniques
BrdU-positive cells were quantified by using unbiased stereological techniques, according to previous described methods (Yutsudo et al., 2013). In summary, the number of BrdU-positive cells in the GCL of DG was determined in eight to nine coronal sections, 240 μm apart (bregma -1.35 to -3.51 mm), using a semiautomatic stereology system (Stereo Investigator, MBF Bioscience, Williston, VT, USA). Cells that met the counting criteria through a 60-125-μm axial distance were counted according to the optical dissector principle (Kempermann et al., 1998). Corresponding volumes of both regions were also determined. The GCL or hilus reference volume was determined by summing the traced area for each section multiplied by the distance between sections sampled.

4.6 Statistical analyses
The data are expressed as means ± SEM. Stereological counts were compared by one-way analysis of variance (ANOVA), with p-values of <0.05 regarded as statistically significant. Then, an all pairwise multiple comparisons post hoc procedure by Dunnett's test was used for further analysis between each treatment group. The densities of BrdU-positive cells in haloperidol-treated and control groups were compared by one-tailed t-test. All statistical analyses were carried out using JMP 13.1.0 software (SAS Institute, Cary, NC, USA).

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Conflicts of Interest
The authors declare no conflicts of interest associated with this manuscript.

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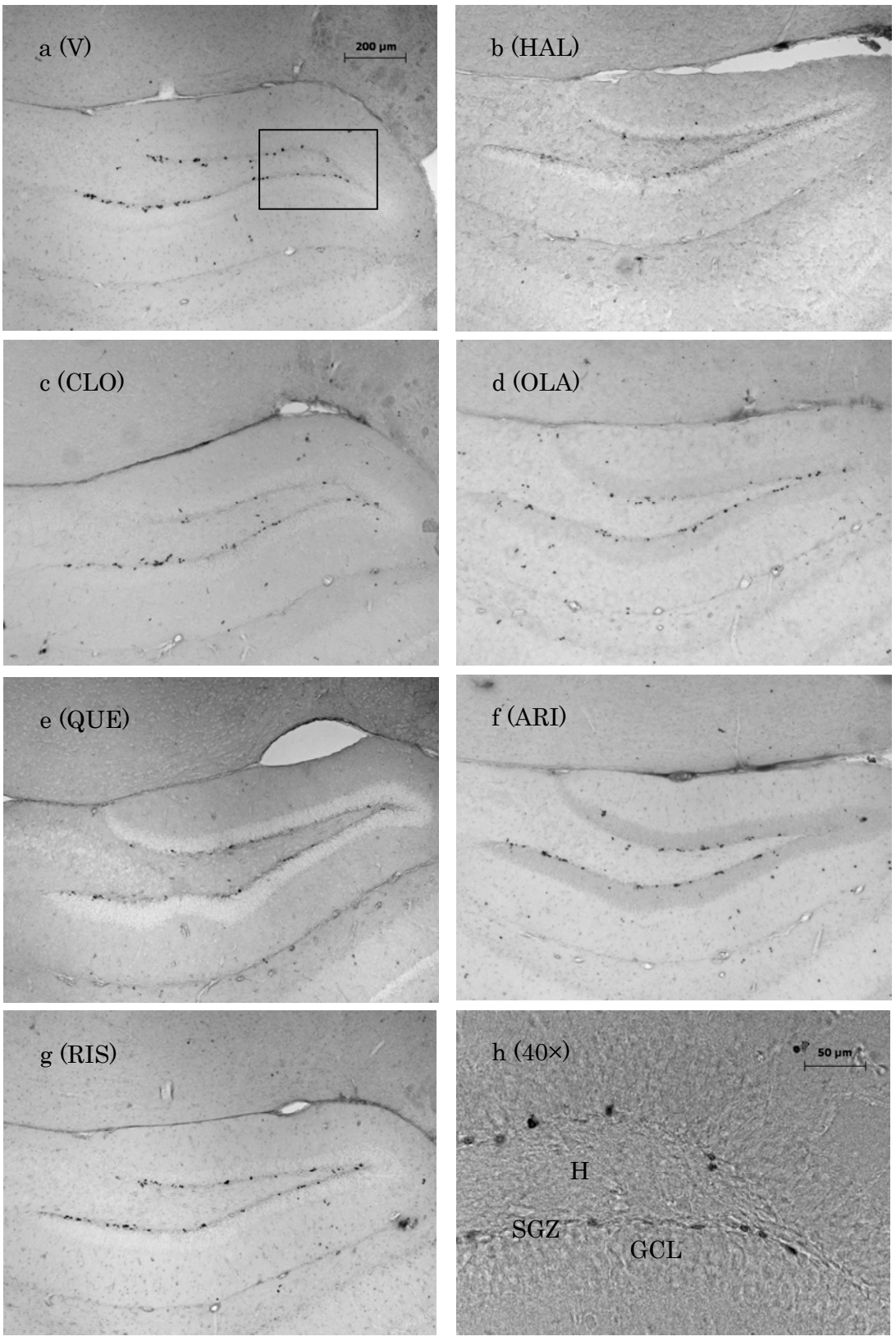
a (V)
200 µm
b (HAL)
c (CLO)
d (OLA)
e (QUE)
f (ARI)
g (RIS)
h (40×)
50 µm
H
SGZ
GCL

Figure 1

BrdU immunohistochemistry in the hippocampus after chronic antipsychotic treatment. Mice were treated with antipsychotics or vehicle for 21 days and received injections of BrdU. (a-g) Representative photomicrographs (10× magnification) are shown from vehicle (a; V), haloperidol (b; HAL), clozapine (c; CLO), olanzapine (d; OLA), quetiapine (e; QUE), aripiprazole (f; ARI), or risperidone (g; RIS). (h) The majority of the BrdU-labeled cells are located in the subgranular zone (SGZ) of the hippocampus, the narrow region between the granule cell layer (GCL) and hilus (H), which is shown in the high magnification (40×).

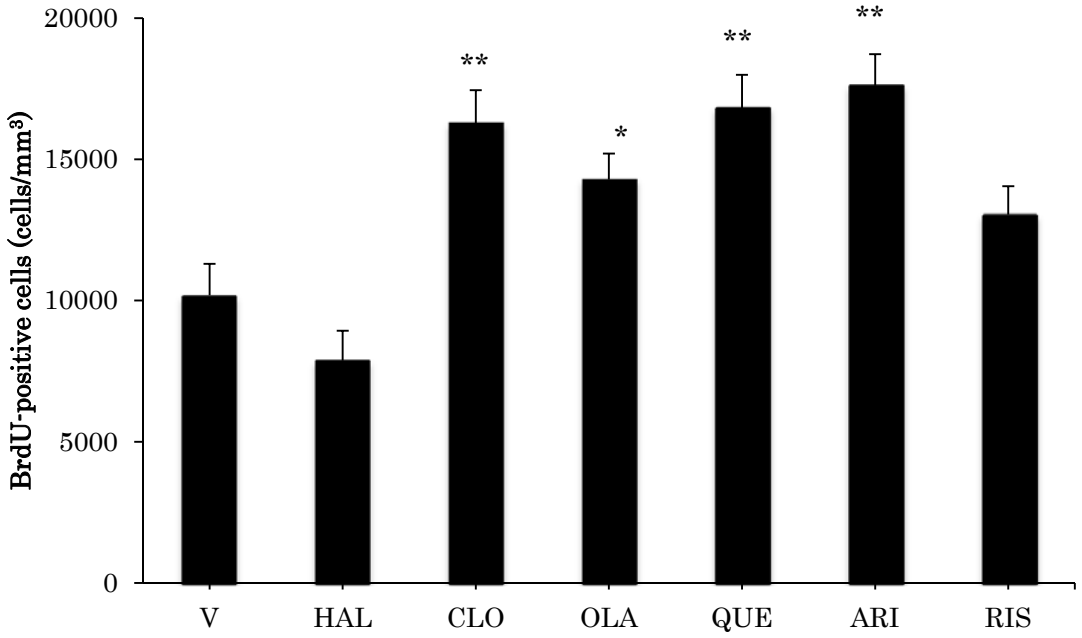

Figure 2

The density of BrdU-positive cells in the hippocampus of adult mouse is increased after chronic atypical antipsychotic administration. Mice were treated with haloperidol (HAL; n=10), olanzapine (OLA; n=10), clozapine (CLO; n=8), quetiapine (QUE; n=8), arpiprazole (ARI; n=9), risperidone (RIS; n=11), or vehicle (V; n=9)) for 21 days, and received BrdU injection once a day on the 18th, 19th, and 20th days. Then, mice were killed 24 hours after the last BrdU injection. Values are mean ± SEM. $^{**}p < 0.01$, $^{*}p < 0.05$ significantly different from vehicle control ($F_{6,58}$=10.5, $p < 0.0001$)