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The effect of dopamine on adult hippocampal neurogenesis

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Abbreviated Running Title: Effect of DA on adult hippocampal neurogenesis

Key Words: D1-like receptor; D2-like receptor; progenitor cell; stem cell; antidepressant drug

Abbreviations: ADP; adult dentate gyrus-derived neural precursor cells, bFGF; basic fibroblast growth factor, BrdU; 5-bromo-2-deoxyuridine, CNTF; ciliary neurotrophic factor, FBS; fetal bovine serum, GAPDH; Glyceraldehyde 3-phosphate dehydrogenase, GCL; granule cell layer, GFAP; glial fibrillary acidic protein, HBSS; hanks balanced salt solution, NPC; neural precursor cells, PBS; phosphate buffered saline, PS; penicillin-streptomycin, SGZ; subgranular zone, SVZ; subventricular zone, SSRI; selective serotonin reuptake inhibitor, Tuj-1; Neuron-specific class III β–tubulin

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1. Introduction

In the adult mammalian brain, neurogenesis mainly occurs in two regions, subventricular zone (SVZ) situated through the lateral wall of the lateral ventricles and subgranular zone (SGZ) in the dentate gyrus (DG) of hippocampus (Kempermann, 2006). Neural precursor cells (NPCs) reside in the SGZ proliferate, survive, and differentiate into neuron and glia. This differential step of neurogenesis is affected by many factors, including environment, stress, hormone and drugs (Dranovsky and Hen, 2006, Duman et al., 2001). Stress of various kinds, are considered to be a risk factor of depression, decrease the proliferation of NPCs (Gould et al., 1997; Pham et al., 2003). As clinical therapeutic effect, chronic treatment with different classes of antidepressant drugs such as selective serotonin reuptake inhibitors (SSRIs), tricyclic antidepressants (TCAs), and noradrenalin reuptake inhibitors increase the proliferation (Airan et al., 2007, Malberg et al., 2000, Santarelli et al., 2003). It is also reported that suppression of hippocampal neurogenesis by the irradiation prevents anti-depressive effects induced by fluoxetine or imipramine treatment (Airan et al., 2007, Santarelli et al., 2003). These results suggest neurogenesis might be involved in the action mechanism of antidepressant drugs and/or the pathophysiology of depression. However, it remains unknown in detail how antidepressant drugs regulate adult hippocampal neurogenesis

and how the modified neurogenesis shows anti-depressive effects.

Dopamine (DA) is involved in the regulation of motivation, volition, interest/pleasure, and attention/concentration, all of which are likely to be impaired in depressed patients. Several previous reports suggest that depression may often be accompanied by a relative hypo-dopaminergic state (Papakostas, 2006). Roy et al. (1985) reported lower cerebrospinal fluid of homovanilic acid, a chief metabolite of DA. Several neuroimaging studies showed increased D2/D3-receptor and decreased DA transporter bindings in some brain regions of depressive patients (D'Haenen H and Bossuyt, 1994, Meyer et al., 2001, Shah et al., 1997). Therefore, the dopaminergic system may be a target for the pharmacological treatment of depression. In fact, it has been reported that DA receptor agonists such as bromocriptine, pramipexole or the combination therapy of them with antidepressants may be beneficial effects in the treatment for refractory and bipolar depression (Aiken, 2007, Inoue et al., 2010, Inoue et al., 1996).

Several studies suggested that DA controls the differential steps of adult hippocampal neurogenesis. SGZ receives dopaminergic projection from ventral tegmental area (Baker et al., 2004, Gasbarri et al., 1997, Hoglinger et al., 2004). Experimental depletion of DA in rodents decreased the cell proliferation and survival of NPCs in SGZ

(Hoglinger et al., 2004, Khaindrava et al., 2011). Furthermore, postmortem study showed that number of NPCs was reduced in SGZ of patients suffered from Parkinson disease (Borta and Hoglinger, 2007). However, dopaminergic modulation of adult hippocampal neurogenesis has not been investigated well.

NPCs in adult hippocampus are classified into stem/progenitor cells of four types based on stages of neural development (Kempermann, 2006). We have recently established the culture system of adult rat dentate gyrus-derived neural precursor cells (ADPs), which correspond to type 2a early progenitor cells in the adult hippocampus (Boku et al., 2009, Masuda et al., 2012). Encinas (2006) showed that fluoxetine increased the early progenitor cell classified as type 2 *in vivo* study. In the present study, to clarify the direct effect of DA on NPCs, we examined the effect of DA on the proliferation of ADPs. In addition, we examined the effect of DA receptor agonists on adult rat hippocampal neurogenesis *in vivo*. These results showed that DA promoted the increase of ADPs via D1-like receptor and D1-like receptor agonist promoted the survival of newborn cells in the adult hippocampus. On the other hand, D2-like receptor agonist did not affect both proliferation and survival.

2. Material and methods

2.1. Animals

Male Sprague-Dawley (SD) rats (7 weeks old) were obtained from Japan SLC, Inc. (Shizuoka, Japan). The rats were housed in groups of three or four in a temperature-controlled environment (22 ± 1 °C) with free access to food and water. The subjects were maintained on a 12 h light/ dark cycle (light phase; 06:00-18:00). The experiments began after 1 week for an acclimatization period.

All experiments were approved by the Hokkaido University School of Medicine Animal Care and Use Committee and were in compliance with the Guide for the Care and Use of Laboratory Animals.

2.2. *Drugs*

Dopamine hydrate was purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). (±)-SKF38393, (±)-SKF-81297, quinpirole, pramipexole dihydrochloride and R-SCH23390 were purchased from Sigma-Aldrich Corp. (St. Louis, MO). Bromocriptine mesilate was donated by Nihon Ciba-Geigy K.K (Tokyo, Japan). Sulpiride was donated by Fujisawa Pharmaceutical Co. Ltd (Osaka, Japan). Each binding affinities of compounds for D1-like and D2-like receptor of rat striatum was described at Table 1 (Andersen, 1988, Chen et al., 2011, Mottola et al., 1992, Neumeyer et al., 2003).

2.3. Isolation and culture of ADP

ADPs were isolated from DG of adult male SD rats (8 weeks) as described in a previous report (Boku et al., 2009). Briefly, rats were deeply anesthetized with sodium pentobarbital (Dainippon Sumitomo Pharma Co. Ltd., Osaka, Japan) and were then decapitated. The brains were removed from 8 rats immediately and were placed in cold Hank's balanced salt solution. The coronal sections with a thickness of 1mm were cut using brain slicer (Muromachi Kikai Co. Ltd., Tokyo, Japan). The DG region of hippocampus was dissected carefully under a dissecting microscope to exclude all regions containing subependymal tissues. The dissected DG tissue was minced with a scalpel blade and digested using a mixture of 2.5 U/ml papain (Worthington Biochemical Corp., Lakewood, NJ), 1 U/ml dispase II (F. Hoffman La Roche Ltd., Indianapolis, IN) and 250 U/ml Deoxyribonuclease I (Worthington) in a water bath at 37 °C for 30 min. The cell mixture was passed through a 70 μm cell strainer. The fraction containing ADP was separated using Percoll gradient centrifugation (GE Healthcare, Uppsala, Sweden). After separation, the cells were washed and suspended with DMEM/F12 medium (Sigma-Aldrich) containing 10% fetal bovine serum (FBS; Invitrogen Corp., Carlsbad, CA) and 1 % penicillin/streptomycin (Invitrogen). The

suspension cells were plated onto a non-coated plate. After overnight culture at 37°C, 5% CO_2, the medium was exchanged for proliferation medium consisting of Neurobasal (Invitrogen), B27 supplement minus vitamin A (Invitrogen), 2 mM L-Glutamine (Invitrogen), 1% penicillin/streptomycin, and 20 ng/ml basic fibroblast growth factor (Invitrogen). Subsequently, half of the medium was change new proliferation medium every two or three days. At the 80-90% confluency, cells were passaged by trypsinization onto poly L-ornithine-laminin-coated dishes. The cell density for plating was approximately 1×10^4 cells /cm^2.

2.4. Cell Counting of ADP

Viable cells were quantitated using an Alamar Blue assay, which is a rapid and simple non-radioactive assay. $1x10^4$ cells were seeded on laminin- ornithine coated 96-well plates in 100 μl/well of proliferation medium. After overnight incubation, to examine the effect of DA receptor ligands on ADP proliferation, cells were treated with each drug at each concentration for 3 days. To examine the effect of DA receptor antagonists on DA-promoted ADP proliferation, antagonists were applied to culture medium 1h before 30 μM of DA was added and the cells were cultured for 3 day. Three days after the treatment, 10 μl/well of Alamar Blue solution (Invitrogen) was added to

medium, and cells were incubated at 37°C for 3 h. Then the absorbance was measured using a Varioskan Flash (Thermo Fisher Scientific Inc., Waltham, MA) microplate reader at 570 and 600 nm, and cell number based on percentage reduction of Alamar Blue was calculated as described in the manufacture's protocol. Although the DA concentration in the extracellular fluid of the rat hippocampus is under normal conditions are reported as 0.1 nM (Simpson et al., 2001), micromolar concentration range of DA was used in *in vitro* study evaluating the effect of DA on proliferation of the cell derived from SVZ (Hoglinger et al., 2004, O'Keeffe et al., 2009b). Therefore, we used DA at broad concentrations of 1-100 μM. The rages of other compounds used in this study were determined by preliminary experiments.

2.5. RNA extraction and reverse transcription –polymerase chain reaction (RT-PCR)

Total RNA was extracted from cells with RNeasy Extraction kit (Qiagen Inc., Hilden, Germany). Total RNA was converted to cDNA with Quantitect Reverse Transcription kit (Qiagen). Polymerase chain reaction (PCR) was performed with AmpliTaq Gold 360 master mix (Applied biosystems, Foster City, CA) in the GeneAmp PCR system 9700 (Applied biosystems). The conditions of PCR were: 95°C for 10 min, followed by 40 cycle of 95°C for 30 s, 60°C for 30 s and 72°C for 1 min, and finally 72°C for 7 min.

The sequences of forward and reverse primers and product size are presented in Table 2. PCR products were analyzed in a 3% agarose gel in Tris-boric acid EDTA buffer containing ethidium bromide. Images of gels were obtained using image analysis software (Kodak 1D; Eastman Kodak Company, Rochester, NY).

2.6. In vivo experimental design and sample collection

For the experiment to evaluate the drug effect on the proliferation, rats (8 weeks old) were either administrated (±)-SKF38393 (10 or 30 mg/kg), pramipexole (0.3 or 1 mg/kg) or saline daily by intraperitoneal (i.p.) injection for 21 days. 24 h after the last drug administration, rats were once received injection of 5-bromo-3′-deoxyuridine (BrdU, 75 mg/kg, i.p.).

For the experiment to evaluate the drug effect on the cell survival, rats were received injection of BrdU (75 mg/kg, i.p.) once. From next day, rats were administrated either (±)-SKF38393 (10 or 30mg/kg, i.p.), pramipexole (0.3 or 1 mg/kg) or saline once a day for 21 days. The doses of drugs used in this study were based on previous reports (Cooper et al., 1990, D'Aquila et al., 2000, Gambarana et al., 1995, Winner et al., 2009). 24 h after the last injection, rats were anaesthetized with sodium pentobarbital and transcardinally perfused with 0.1 M cold phosphate-buffered saline (PBS), followed by

4% cold paraformaldehyde. The brains were removed and post-fixed overnight in 4% paraformaldehyde and cryoprotected in 30% sucrose.

2.7. Immunohistochemistry

A freezing microtome was used to collect serial coronal 30 μm sections through the entire hippocampus. Every twelfth section was slide-mounted for peroxidase BrdU immunolabeling. The sections were incubated 0.01 M citric acid at 90 °C, digested in trypsin (0.1%) in Tris buffer containing 0.1% $CaCl_2$ for 10 min, denatured in 2N HCl for 30 min, blocked in 3% normal horse serum for 20 min, and incubated overnight at 4 °C with mouse monoclonal antibody against BrdU (1:50; Becton Dickinson, CA) in PBS containing 3% normal horse serum and 0.2% Tween 20. On the next day, the sections were incubated in biotinylated secondary antisera (IgG, 1:200; Vector Laboratories) for 60 min, incubated in avidin-biotin-horseradish peroxidase (1:50; Vector Laboratories) for 60 min and reacted in the solution of 3,3'-diaminnobenzidine (Vector Laboratories). The sections were counterstained with Cresyl Violet.

2.8. Quantitation of BrdU-labelled cells

The numbers of BrdU-positive cells were counted in right side of section with a coded one-in-twelve series section. Cells were counted as being in the SGZ if they were

touching or in the SGZ. Cells that were located more than two cells away from the SGZ were counted as in the hilus. All BrdU-positive cells were counted regardless of the size or shape through a 100 × objective with Olympus BX-60 (Olympus Optical, Tokyo, Japan) throughout the rostrocaudal extent of granule cell layer (GCL). The total numbers of BrdU positive cells were multiplied by twelve and reported as total number of cells per right side region.

2.9. Data analysis

Results were expressed as the mean and standard error of the means (SEM). Statistical analyses were performed using one-way ANOVA with a Dunnett's post hoc test in dose-response experiments, two-way ANOVA with Dunnett's post hoc test in the experiments for effect of DA receptor antagonists on DA-promoted ADP proliferation and Student's t-test in the experiments for effect of SKF38393 on proliferation of NPC in *vivo*. In the case that the data were not normally distributed or homogenous for variance, a Kruskal-Wallis analysis followed by a Steel test for non-parametric analyses was performed to detect any significant effects of treatments.

3. Results

3.1. Effect of DA on ADP proliferation

We firstly investigated the effect of DA on ADP proliferation. One-way ANOVA showed significant difference related to treatment with dopamine on the cell number of ADPs ($F(5, 30) = 4.55$; $p = 0.0033$). *Post-hoc* analyses revealed that the cell number of ADPs was increased significantly by treatment with 30 and 100 μM DA compared to the control (114 ± 2 % ($p<0.01$), 114 ± 4 % ($p<0.05$); Dunnett's test, respectively) (Fig. 1). This result indicated DA directly promoted the proliferation of ADPs.

3.2. mRNA expression pattern of DA receptor on ADP

DA receptors are classified into two major types, D1-like receptors (D1 and D5) and D2-like receptors (D2, D3 and D4) according to structural homologies and shared messenger cascades. To evaluate the expression pattern of DA receptors on ADPs, we performed RT-PCR analysis. The results showed that all subtypes of DA receptor mRNAs were detected (Fig. 2).

3.3. Effects of D1- and D2-like receptor antagonists on DA-promoted ADP proliferation

We next examined the effects of DA receptor antagonists (Fig. 3A, C) and the

co-administration of DA receptor antagonists with DA (Fig. 3B, D) on the cell number of ADPs. One-way ANOVA showed no difference related to treatment with neither D1-like receptor antagonist R-SCH23390 ($F(5, 18) = 0.39$; $p = 0.85$) nor D2-like receptor antagonist sulpiride ($F(5, 12) = 0.62$; $p = 0.69$) on the cell number of ADPs (Fig. 3A, C). On the other hand, two-way ANOVA showed significant difference related to treatment with R-SCH23390 on DA-increased number of ADPs ($F(4, 18) = 9.34$; $p = 0.0003$). *Post-hoc* analyses revealed that R-SCH23390 significantly attenuated DA-induced increase of ADPs at the dose of 10, 30 and 100μM ($p<0.05$, $p<0.01$ and $p<0.01$; Dunnett's test, respectively) (Fig. 3B). On the contrary, sulpiride did not ($F(4, 12) = 0.85$; $p = 0.52$, Two-way ANOVA) (Fig. 3D).

3.4. Effects of D1- and D2-like receptor agonists on ADP proliferation

We next examined the effect of D1- and D2- like receptor agonists on the cell number of ADPs. One-way ANOVA showed significant difference related to treatment with D1-like receptor agonist (±)-SKF38393 and (±)-SKF82197 on the cell number of ADPs ($F(5, 18) = 21.7$; $p<0.0001$, $F(5, 12) = 77.7$; $p<0.0001$, respectively). *Post-hoc* analyses revealed that (±)-SKF38393 at doses of 100 μM significantly increased the cell number of ADPs (135 ± 3%, $p<0.01$; Dunnett's test) and that (±)-SKF81297 at doses of

30 μM significantly increased (122 ± 2%, p<0.01; Dunnett's test) (Fig. 4A and B). On the other hand, D2-like receptor agonist quinpirole did not (F (5, 18) =1.19; *p*=0.35, one-way ANOVA) (Fig. 4C). Moreover, D2-like receptor agonists including pramipexole (F (5, 12) =1.08; *p*=0.42, one-way ANOVA) and bromocriptine (F (5, 12) =8.47; *p*=0.0012, one-way ANOVA, p<0.01; post hoc Dunnett's test) did not increase the cell number of ADPs either (Fig. 4D and E).

3.5 Effects of DA receptor agonists on proliferation of NPCs in adult rat hippocampus

DA increased the cell number of ADPs via D1-like but not D2-like receptor *in vitro*. To investigate the effects of DA receptor agonists on the proliferation of NPCs in adult hippocampus *in vivo*, rats were given (±)-SKF38393, a D1-like receptor agonist, or pramipexole, a D2-like receptor agonist, daily for 21 days, followed by the BrdU administration to label dividing cells. BrdU-labelled cells were seen more in SGZ than hilus region (Fig. 5A, B, and C). Most of them formed cluster. Student's t-test showed that treatment of (±)-SKF38393 at the dose of 10 mg/kg/day had no effect on the number of BrdU-positive cells in SGZ (4209 ± 281) (t=1.34; *p*=0.20) and hilus (1176 ± 98) (t=1.43; *p*=0.17) of DG compared to the control (3681 ± 276 in SGZ, 993 ± 82 in hilus) (Fig. 5D, E). Furthermore, treatment of (±)-SKF38393 at the dose of 30

mg/kg/day also had no effect on the number of BrdU-positive cells in SGZ (2125 ± 288) (t=1.11; p=0.28) and hilus (682± 107) (t=1.47; p=0.16) of DG compared to the control (1816 ± 144 in SGZ, 474 ± 76 in hilus) (Fig. 5F, G). On the other hand, treatment of pramipexole at the dose of 0.3 and 1 mg/kg/day did not affect the number of BrdU-positive cells in SGZ (3280 ± 401, 2510 ± 356, respectively) (F (2, 15) =1.66; p=0.92) and hilus (790 ± 180, 772 ± 128, respectively) (F (2, 15) =1.10; p=0.36) of DG compared to the control (2698 ± 392 in SGZ, 810 ± 160 in hilus) neither (Fig. 5H, I).

3.6. Effects of DA receptor agonists on survival of newborn cells in adult rat hippocampus

We next investigated the effect of DA receptor agonists on the survival of newborn cells in adult rat hippocampus. BrdU-labeled neuclei were almost round and seen separately in the GCL (Fig. 6A, B, C). Kruskal-Wallis test showed significant difference related to treatment with of (±)-SKF38393 on the number of BrdU-positive cells in GCL (H=9.02; p=0.013) and one-way ANOVA showed significant difference related to treatment with of (±)-SKF38393 on the number of BrdU-positive cells in hilus (F (2, 24) =3.82; p=0.036, one-way ANOVA). Post-hoc analyses revealed that treatment of (±)-SKF38393 at the dose of 10 and 30 mg/kg/day increased significantly the number of

BrdU-positive cells both in GCL (4653± 444 ($p<0.01$), 3994 ± 396 ($p<0.05$); Steel test, respectively) and hilus (1572 ± 244 ($p<0.05$), 1657 ± 164, ($p<0.05$); Dunnett's test, respectively) of DG compared to the control (3033 ± 138 in SGZ, 1011 ± 103 in hilus) (Fig. 6D, E). On the other hand, one-way ANOVA showed that treatment of pramipexole at the dose of 0.3 and 1 mg/kg/day did not affect the number of BrdU-positive cells in SGZ (6384 ± 409, 5766 ± 488, respectively) ($F(2, 15) = 0.91$; $p=0.42$) and hilus (2044 ± 208, 1786 ± 142, respectively) ($F(2, 15) = 0.57$; $p=0.58$) of DG compared to the control (5542 ± 471 in SGZ, 1788 ± 230 in hilus) (Fig. 6F, G).

4. **Discussion**

The aim of present study is to investigate the effect of DA on adult hippocampal neurogenesis. First, we showed that DA directly increased the cell number of ADPs. This DA-promoting effect for proliferation is consistent with *in vivo* study showing that drug-induced depletion of DA decreases NPC proliferation in SGZ of adult hippocampus (Hoglinger et al., 2004, Winner et al., 2009). ADPs expressed all subtypes of DA receptor (D1-D5) mRNAs as progenitor cells *in vivo* (Mu et al., 2011).

Next, we investigated which subtype of receptor mediated the DA-promoted ADP proliferation using pharmacological methods. D2-like receptor agonists such as bromocriptine, pramipexole or the combination therapy of them with antidepressants are effective in the treatment for refractory and bipolar depression (Aiken, 2007, Inoue et al., 2010, Inoue et al., 1996), and several preclinical studies have shown that D2-like receptor agonist promoted the proliferation of neurospheres derived from SVZ (O'Keeffe et al., 2009a, Winner et al., 2009). From these findings, we expected that DA increased an ADP proliferation thorough D2-like receptor. However, the results indicated not D2-like but D1-like receptor. Several studies showed 0.3-30 μM of DA promoted the proliferation of neurospheres derived SVZ mediated by D2-like receptor (Hoglinger et al., 2004, O'Keeffe et al., 2009b). Compared with these studies, relative high concentration of DA was required to promote the proliferation of ADPs in this

study. This reason might be explained by the different affinities of DA for DA receptors (Table. 1). DA shows a lower affinity for D1-like receptors than D2-like receptors (Missale et al., 1998). Thus, higher concentration DA might be required to promote the proliferation of ADPs.

We investigated the effect of DA receptor agonists on the proliferation of NPCs in the adult rat hippocampus. While we evaluated the effect of (±)-SKF38393 and (±)-SKF81297 *in vitro* assay, we used only (±)-SKF38393 as a D1-like agonist *in vivo* assay. The reason that we use (±)-SKF38393 *in vivo* experiments was that (±)-SKF38393 is used generally used as a D1-like receptor agonist *in vitro* and *in vivo* assay. While we evaluated the effect for ADP proliferation with three D2-like receptor agonists, pramipexole, bromocriptine and quinpirole *in vitro* experiments, we used pramipexole for *in vivo* experiments. Among three D2-like receptor agonists we used in this study, only bromocriptine decreased the proliferation of ADP suggesting an extra D2-like receptor mechanism on the proliferation. Pramipexole is used for refractory depression in clinical (Aiken, 2007) and promoted the proliferation of NPC in SVZ of DA-depletion model (Winner et al., 2009). From these reasons, we selected pramipexole for *in vivo* experiments. Although D1-like receptor agonists promoted the proliferation of ADPs, chronic administration of (±)-SKF38393 had no effect on the proliferation of

NPCs *in vivo.* Hoglinger et al. (2004) showed that dopamine depletion of DA induced the decrease of NPC in DG and it was recovered in parallel with dopaminergic re-innervation. Thus, DA may play an important role in proliferation homeostasis in normal animals. The maintenance medium did not contain DA in the case of culture with ADPs. So, we might detect the stimulative effect of D1-like receptor agonist on proliferation *in vivo* assay. On the other hand, in our *vivo* experiments, physiological DA existed in hippocampus of normal rats might put the effect of (±)-SKF38393 in the shade. Chronic treatment with pramipexole also did not affect the proliferation on NPC in adult hippocampus of rats. This result was consistent with *in vitro* result. As like the effect of (±)-SKF38393 for proliferation *in vivo* results, there is the possibility that physiological DA might put the effect of pramipexole in the shade. However, a previous study showed that chronic treatment with pramipexole increases the proliferation of NPC in SVZ but not in SGZ of drug-induced DA depletion model rats (Winner et al., 2009). From these results, pramipexole might not affect the proliferation of NPC in DG regardless of the presence of DA. Yang et al. (2008) demonstrated that treatment of D2-like receptor agonist quinpirole increased the proliferation of NPCs mediated by ciliary neurotrophic factor (CNTF) in glial cells of mouse hippocampus. There are some differences in experimental conditions between them and us. One difference is spices

used in experiments, we used rats and Yang used mice. Recently, it is reported that serotonin 1A receptor agonist increased the adult hippocampal neurogenesis in rats but not mice (Arnold and Hagg, 2012). Thus, this difference is possible reason. Other difference is the period of drug administration, which they used intermittent injections for only 3 days. It is possible that chronic treatments induce adaptive changes in D2-like receptors. Some studies evaluated the effect of haloperidol, an antipsychotic with D2-like receptor antagonistic activities. Acute and chronic treatment of haloperidol showed that haloperidol did not affect the proliferation of NPC in DG, while one study showed an increase (Dawirs et al., 1998, Kippin et al., 2005, Malberg et al., 2000). These discrepancies results in these studies might be due to difference in experiments conditions including experimental design, drug dosage and species, because haloperidol is not selective D2-like receptor antagonist (Bymaster et al., 1996). Thus, it is difficult to determine the effect of D2-like receptor on proliferation from these studies. Another study showed S33138, a selective D3 receptor antagonist, or D3 receptor knockout (KO) increased the number of NPC in DG (Egeland et al., 2012). They also showed interesting results that S33138 decreased the proliferation of NPC in DG of D3-KO mouse. Although S33138 is selective D3 receptor antagonist, it has weak D2 receptor antagonistic activity (Millan et al., 2008). Thus, D2 antagonistic activity of S33138 is

relatively enhanced in D3 KO mouse. This results suggested that blockade of D2 receptor deceased proliferation in DG. So, DA can act through multiple receptor subtype to exert NPC. Future studies are required to evaluate the effect of each DA receptor subtype for the proliferation using more selective DA receptor agonists or antagonists.

A previous study showed that DA depletion decreased cell survival in DG (Khaindrava et al., 2011). To examine the effect of DA receptor agonists on survival of newborn cells, BrdU was injected once followed by the administration of (±)-SKF38393 or pramipexole for 21 days. The result showed significant increase of cell number for BrdU-labeled cells in chronic D1-like receptor stimulation rat compared with control. The survival-promoting activity of astrocytes induced by apomorphine, a potent D1/D2 agonist, is partially mediated by D1-like receptor (Li et al., 2006). Using the primary culture of rat striatal neurons, Amano et al. (1994) showed that DA-induced neuroprotection against kainic acid by the increase of intercellular cAMP mediated D1-like receptor activation. Thus, our results seem to be consistent with previous reports.

Our *in vitro* and *in vivo* findings together suggest that the clinical effect of D2-like receptor agonists for depression might not be related to hippocampal neurogenesis. It is reported that the efficacy of fluoxetine is blocked in some, but not all, behavioral

paradigms in corticosterone-treated mice where hippocampal neurogenesis is abolished by X-irradiation (David et al., 2009). It suggests antidepressant action is mediated by both hippocampal neurogenesis-dependent and -independent mechanism. Unlike SSRI and TCA, DA receptor agonists may exhibit rapid antidepressant action (Willner, 1997). Furthermore, a previous study reported that the anti-depressive action of pramipexole might be mediated through nucleus accumbens (Kitagawa et al., 2009). Thus, the therapeutic action of pramipexole may rely on the neurogenesis-independent mechanism.

At the present time, it remains unclear how activation of D1-like receptor promotes some aspects of adult hippocampal neurogenesis. The major signaling cascade underlying D1-like receptor is cAMP cascade. Our previous report showed that noradrenaline directly increased the proliferation of ADPs via β2 receptor (Masuda et al., 2012). As with D1-like receptor, the stimulation of β2 receptor activates Gs protein coupled to adenylyl cyclase. Thus, the increase of intracellular cAMP might be one of the possible mechanisms in promoting the proliferation. Inhibition of phosphodiesterase-4, an enzyme that catalyzes the hydrolysis of cAMP, stimulate cAMP-CREB cascade and increase the survival of newborn cells (Li et al., 2009, Nakagawa et al., 2002, Sasaki et al., 2007). Therefore, cAMP cascade might be also

involved in the survival of newborn cells in the adult hippocampus thorough the activation of D1-like receptor.

5. Conclusion

Here we have shown that DA promoted the increase of ADPs via D1-like receptor and D1-like receptor agonist promoted the survival of newborn cells in the adult rat hippocampus. These results suggested that D1-like receptor might play, at least in part, a role in adult hippocampal neurogenesis and the activation of D1-like receptor might have a therapeutic potential for depression. Hippocampal neurogenesis is as a process mainly composed by proliferation, survival and differentiation on newborn cells. In this paper, we evaluated the effect of dopamine on proliferation and survival but differentiation. In further study, it is needed to evaluate the effect of dopamine on differentiation.

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Conflicts of Interest

Dr. S. Nakagawa has received honoraria from GlaxoSmithKline, Eisai, Pfeizer, Daiichi-Sankyo, Meiji Seika Pharma, Ono Pharmaceutical and Eli Lilly, and has recived research/grant support from Dainippon Sumitomo Pharma, Pfeizer, Eli Lilly, Eisai and Ono Pharmaceutical. Dr. Inoue has received honoraria from GlaxoSmithKline, Pfeizer, Astellas, Eli Lilly, Mitsubishi Tanabe Pharma, Mochida Pharmaceutical Otsuka Pharamaceutical, Meiji Seika Pharma, Asahi Kasei Pharma, Sionogi, Janssen Pharmaceutical, Takeda Pharamaceutical and Yoshitomi Pharmaceuticccal, has received research/grant support from Otsuka Pharmaceutical, and is a member of the advisory boards of GlalxoSmithKline, Eli Lilly, Mochida Pharmaceutical and Mistubishi Tanabe Pharma. Other authors have no potential conflicts of interests.

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Table Legends

Table.1 The affinities of drugs for D1-like and D2-like receptor of rat striatum.

Table. 2 The PCR primer sequence and product size

Figure Legends

Fig. 1. Effect of dopamine on ADP proliferation. ADPs were incubated in the proliferation medium with dopamine for 3 days. The cell number of ADPs was determined using Alamar Blue assay. Data are presented as percentages of control. Data are expressed as mean ± SEM from six independent experiments. $^{**}p < 0.01$, $^{*}p < 0.05$ compared with control. ADP, adult rat dentate gyrus-derived neural precursor cell. Cont, control.

Fig. 2. mRNA expression pattern of GAPDH as an internal control and dopamine receptors on ADPs. The RT-PCR products were analyzed in 3 % agarose gels stained with ethidium bromide with a standard DNA marker.

Fig. 3. Effects of dopamine receptor antagonists on ADP proliferation. (A, C) The effect of D1-like receptor antagonist R-SCH23390 or D2-like receptor antagonist sulpiride on ADP proliferation. The cells were incubated with them for 3 days. (B, D) The effect of R-SCH23390 or sulpiride on dopamine-promoted ADP proliferation. The ADPs were incubated with R-SCH23390 or sulpiride for 1 h prior to adding 30 μM dopamine,

followed by their incubation for 3days. Data are expressed as mean ± SEM from three or four independent experiments. ***p* <0.01 compared with control, ##*p* <0.01, #*p* <0.05 compared with 30 μM dopamine alone. Cont, control.

Fig. 4. Effects of dopamine receptor agonists on ADP proliferation. (A,B) The effect of D1-like receptor agonist on ADP proliferation. The ADPs were incubated with (±)--SKF38393 or (±)-SKF81297 for 3 days. (C-E) The effect of D2-like receptor agonists on ADP proliferation. The ADPs were incubated either with quipirole, pramipexole or bromocriptine for 3 days. Data are expressed as mean ± SEM from three or four independent experiments. ***p* <0.01 compared with control. Cont, control.

Fig. 5. Effects of dopamine receptor agonists on the proliferation of newborn cell in adult rat hippocampus. Rats were received single injection of BrdU after (±)-SKF39383 or pramipexole treatment for 21 days, and were killed 24 h after BrdU injection. Shown are representative photomicrographs of BrdU positive cells (arrows) in hippocampus from vehicle (A), 10 mg/kg (±)-SKF38393 (B) and 1 mg/kg pramipexole (C). The histogram shows the quantification of BrdU-positive cells in the SGZ (D, F and H) and hilus (E, G and I). The data are presented as the mean ± SEM number of BrdU-positive

cells in the subgranular zone (SGZ) or hilus of the hippocampus (n=6-9). Scale bars: 100 μm.

Fig. 6. Effects of dopamine receptor agonists on the survival of newborn cells in the adult rat hippocampus. Rats were received chronic administration of (±)-SKF38393 or pramipexole for 21 days after single BrdU injection (75 mg/kg). Rats were killed 24 h after the last administration. Shown are representative photomicrographs of BrdU positive cells (arrows) in the hippocampus from vehicle (A), 10 mg/kg (±)-SKF38393 (B) and 1 mg/kg pramipexole (C). The Histogram shows the quantification of BrdU positive cells in the GCL (D and F) and hilus (E and G). The data are presented as the mean ± SEM number of BrdU positive cells. (D) ††p <0.01, †p <0.05 compared with vehicle (Steel test). (E) **p <0.01 compared with vehicle (n=6-9). Scale bars: 20 μm.

Figure1

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Figure.1

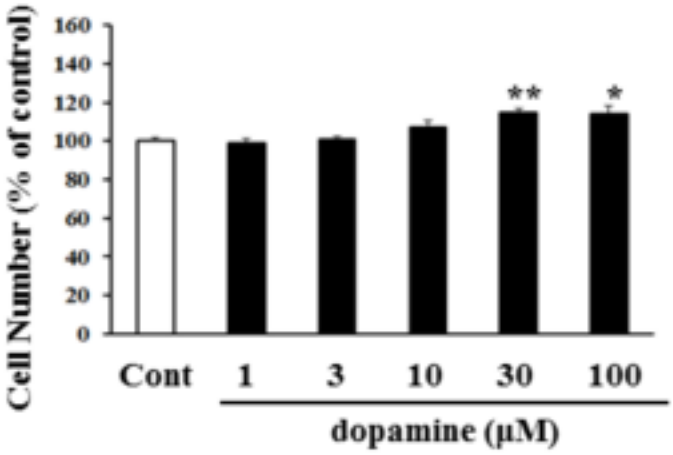


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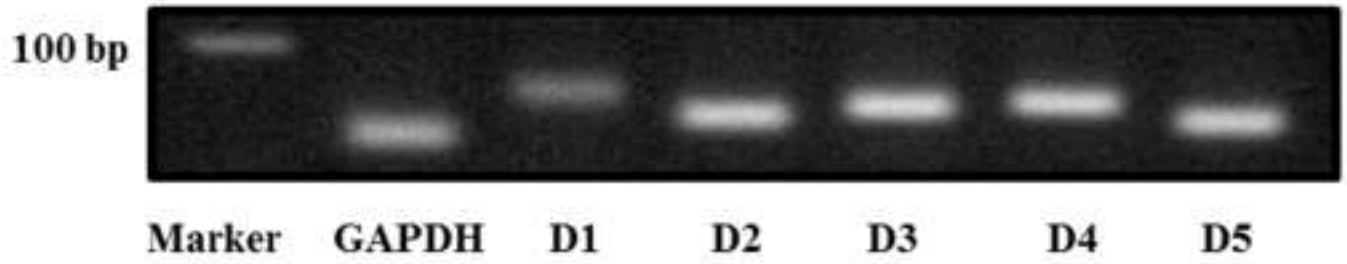


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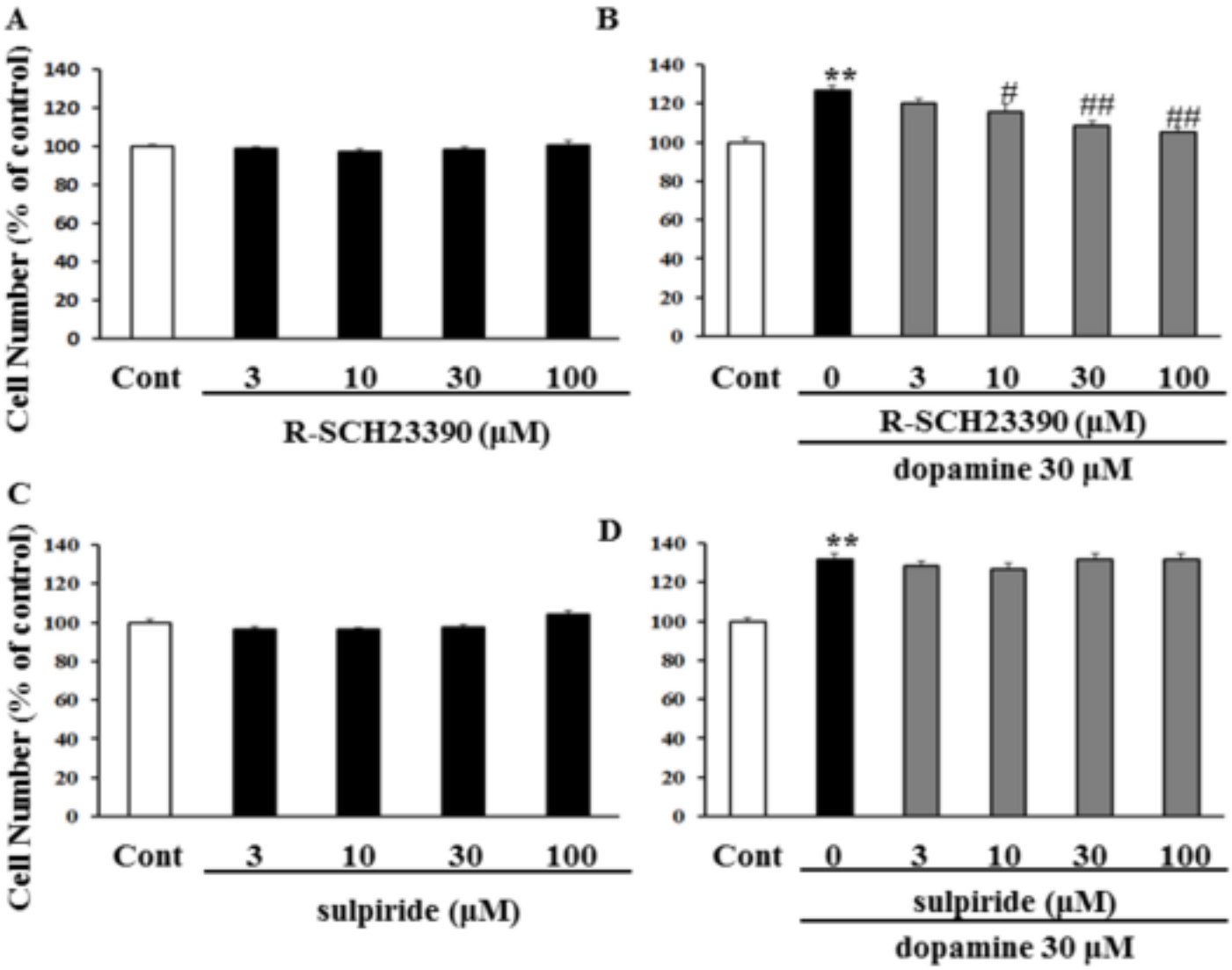


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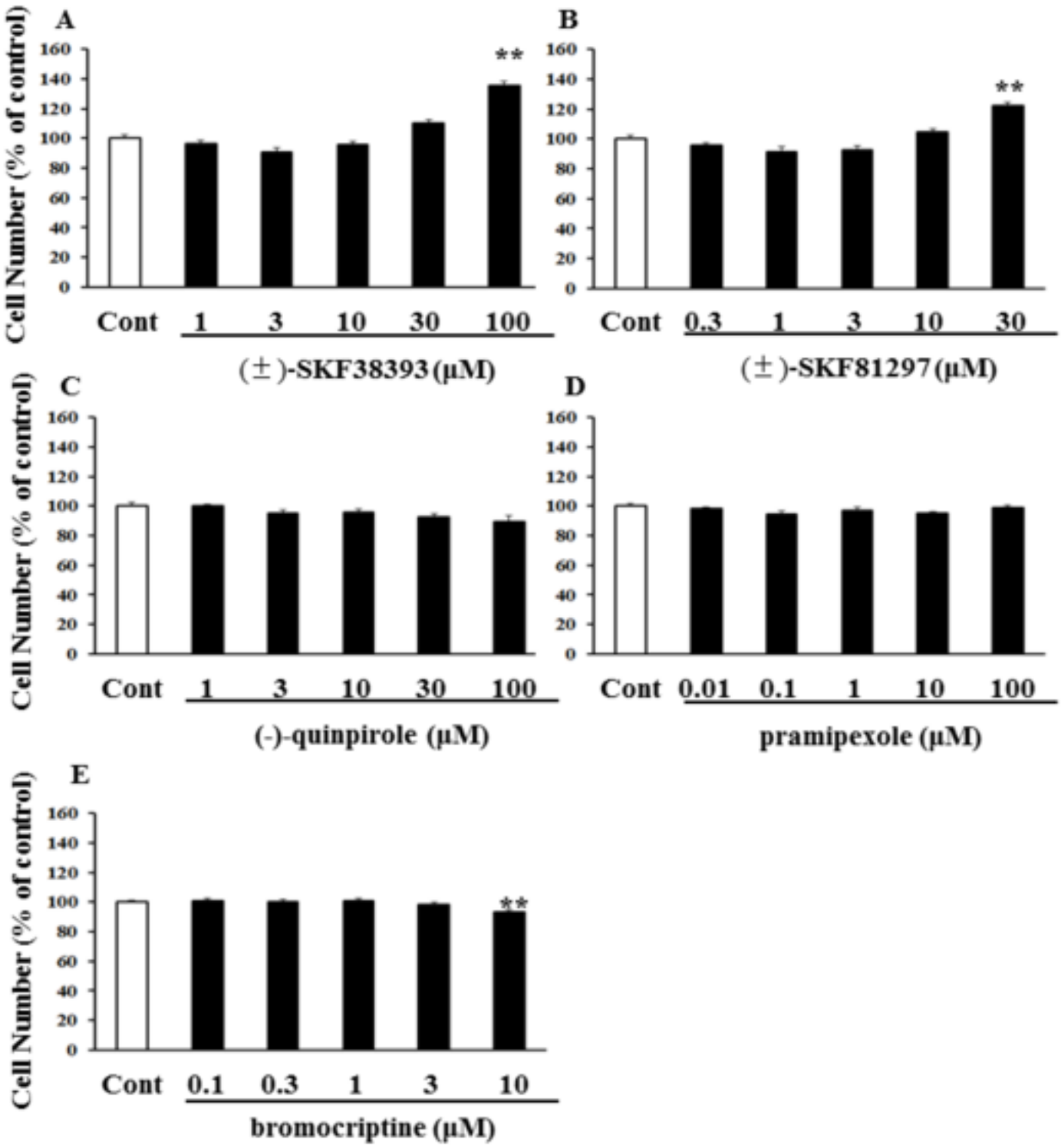


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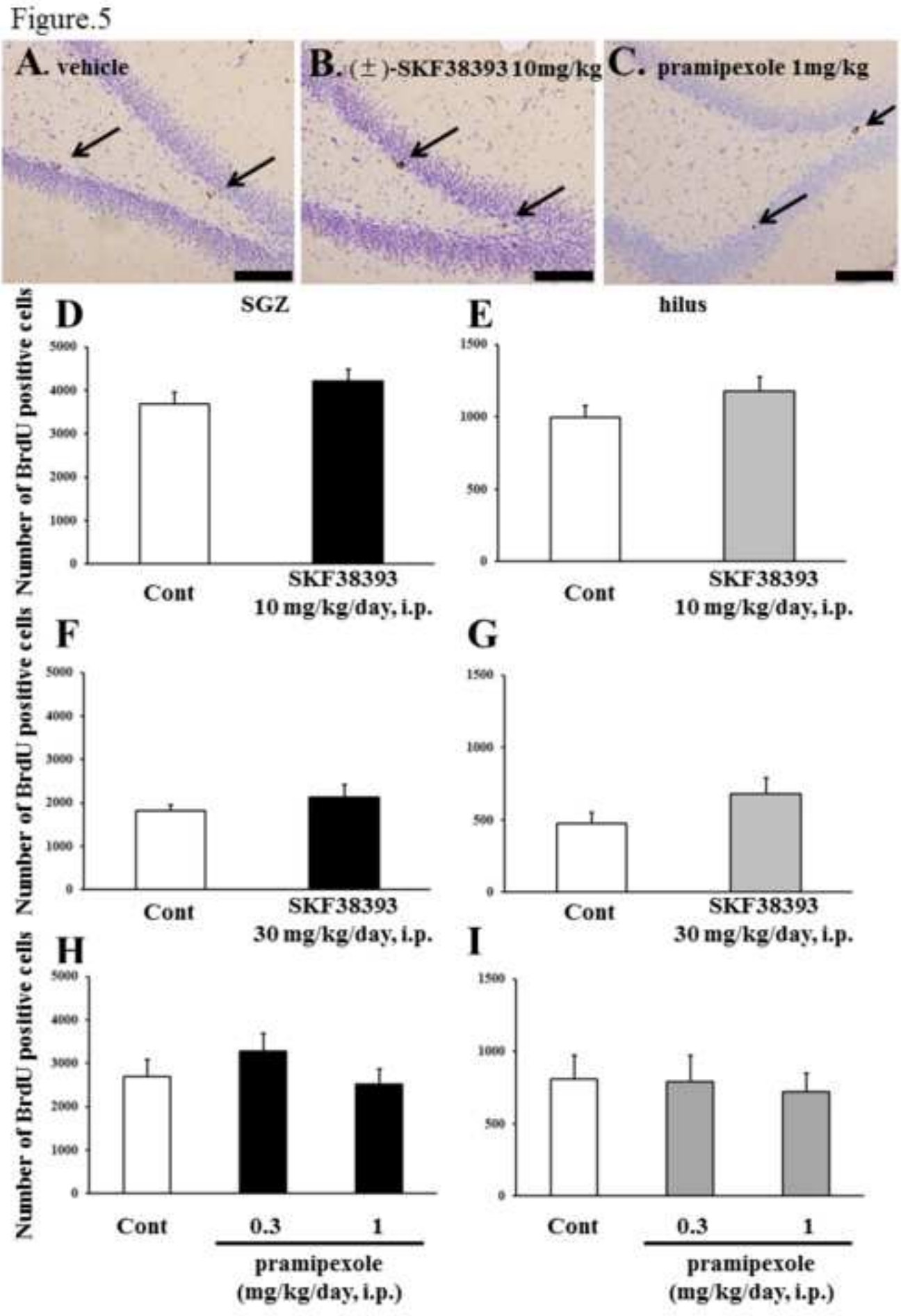


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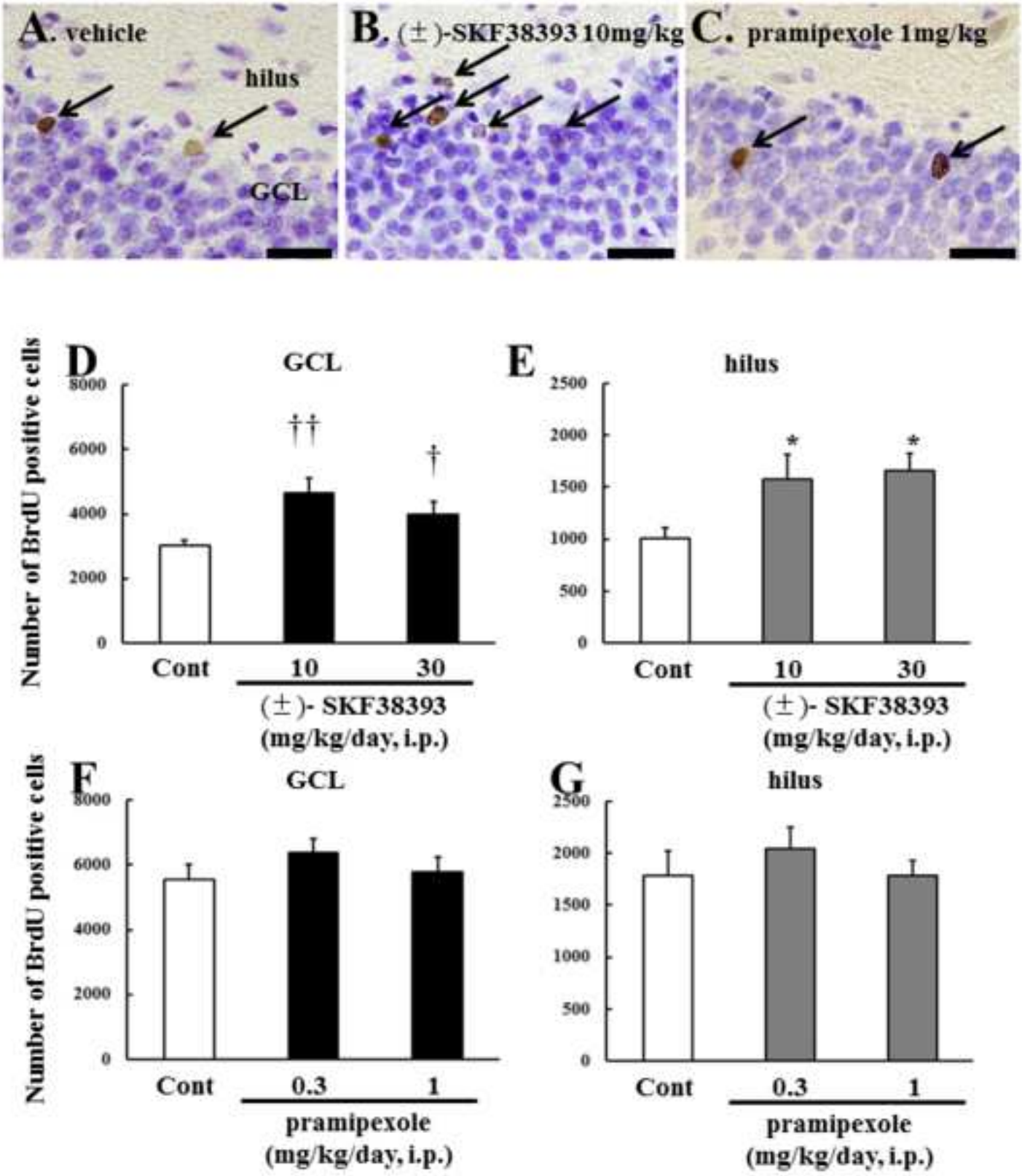


Table1
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Table 1

DA receptor ligand	Ki (nM)	
	D1-like receptor	D2-like receptor
DA	500	70
(±)-SKF38393	18	9300
(±)-SKF81297	1.9	1270
Quinpirole	>5000	720
Pramipexole	>10000	39
Bromocriptine	>10000	8
SCH23390	0.14	895
Sulpiride	>10000	70

Table2
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Table 1:

Primers		Sequences	Product size (bp)
GAPDH	Forward	5'-AGCTGGTCATCAATGGGAAA -3'	63
	Reverse	5'-ATTTGATGTTAGCGGGATCG -3'	
D1	Forward	5'-TCCAGAAAGGAAGGGTAGGAG-3'	80
	Reverse	5'- CCTTTCCAATCTGAAAACTGCT -3'	
D2	Forward	5'-TGAACAGGCGGAGAATGG-3'	70
	Reverse	5'-CTGGTGCTTGACAGCATCTC-3'	
D3	Forward	5'-GATGGTGGTCATTGTGCTTG-3'	74
	Reverse	5'-GGGTATTAAGAACGTGAGTCAGGA-3'	
D4	Forward	5'-CCTCCATCTTCAACCTGTGC-3'	76
	Reverse	5'-TGCTGGTTGTAGCGCAGT-3'	
D5	Forward	5'-CATGTCTCAAATCTCTCCAACG-3'	69
	Reverse	5'-CAGTCAAGCTCCCAGACAGA-3'	