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Involvement of serotonin neurotransmission in hippocampal neurogenesis and behavioral responses in a rat model of post-stroke depression

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ABSTRACT

Ischemia-stimulated dentate gyrus (DG) neurogenesis is hypothesized to be an etiological factor of post-stroke depression (PSD) and a potential target of selective serotonin (5-hydroxytryptamine; 5-HT) reuptake inhibitors (SSRIs) in PSD. Clinical investigations have explored the strategy of augmenting SSRIs action by combination with a 5-HT1A receptor antagonist. We investigated the relative importance of the effects on ischemia-stimulated neurogenesis and depressive-like behavior of WAY-100635 versus citalopram at different dose levels in PSD animals. Adult rats were exposed to a chronic mild stress paradigm after ischemic surgery. Decreased sucrose consumption was indicative of the core depressive syndrome anhedonia. Proliferating cells and their fate were monitored by bromodeoxyuridine labeling protocols up to 28 days after ischemia. Expression of the 5-HT1A receptor in DG was also examined. The current findings confirmed the ability of WAY-100635 to augment SSRIs pharmacological efficacy and SSRIs-induced elevation of post-stroke DG neurogenesis. Specifically, WAY-100635 and citalopram in different dose combinations display their relative importance in ischemia-stimulated neurogenesis probably through reinforcing serotonergic neurotransmission and/or density of 5-HT1A receptor in DG. The present data extend our understanding that increase of ischemia-induced DG neurogenesis can be interpreted as a valid index, to an extent, or even a prerequisite for an efficient co-treatment strategy.

1. Introduction

Post-stroke depression (PSD) is a common mood disturbance that occurs in stroke patients and that potentially affects their recovery (Hackett et al., 2005). The development of selective serotonin (5-hydroxytryptamine; 5-HT) reuptake inhibitors (SSRIs) as antidepressant agents has demonstrated that increasing the extracellular concentration of 5-HT in brain is an efficacious treatment for PSD (Wiart et al., 2000; Rampello et al., 2004). Certainly, these findings suggest that clinical PSD patients display deficits in serotonergic neurotransmission that can be reversed by SSRIs, such as fluoxetine and the most selective SSRI citalopram.

On the other hand, whereas SSRIs immediately block reuptake, clinical efficacy is only seen after prolonged treatment and it can be several weeks before significant antidepressant action is observed. At the clinical level, the β-adrenoceptor/5-HT1A receptor antagonist pindolol accelerated the lag therapeutic effects of SSRIs in open-label and placebo-controlled trials from 1994 (Artigas et al., 1994; Blier and Bergeron, 1995). There is a significant accumulation of data for SSRIs co-treatment with either pindolol (Hjorth, 1996; Hjorth and Auerbach, 1996; Dawson and Nguyen, 2000) or more selective 5-HT1A antagonists such as WAY-100635 (Romero et al., 1996a,b; Hjorth et al., 1997; Dawson, 1998) to enhance extracellular concentrations of 5-HT. These clinical and preclinical trials have fueled efforts on developing therapeutic protocols to optimize the treatment of depression.

Additionally, an intriguing property of the adult hippocampus is its capability to generate new neurons throughout life, called neurogenesis (Abrous et al., 2005). Stress and antidepressants have been shown to influence hippocampal neurogenesis, which is hypothesized to be an etiological factor of depression (Dranovsky and Hen, 2006; Jacobs et al., 2000; Kempermann and Kronenberg, 2003). Thus, neurogenesis provides another explanation for the "therapeutic lag" observed for SSRIs. Jacobs (2002) has suggested that this lag is due to the time it takes for newly formed cells to migrate and form neurons that are fully and functionally integrated into the existing brain circuitry. Our prior study indicated that depressive behaviors in ischemic rats were accompanied by reduced ischemia-stimulated hippocampal neurogenesis, including proliferating cells and their survival and neurogenic fate, and that these effects were reversed by citalopram administration; this observation provides vital information for the etiology of PSD, and also a potential therapeutic target for SSRIs resulting from their enhancement of serotonergic synaptic transmission in PSD (Wang et al., 2008). This also grew out of a longstanding interest in serotonin and its 1A receptor.

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As a kind of neurotransmitters, it has long been reported that 5-HT exert an important mitogenic action in the central neural system (CNS) during development (Lauder et al., 1981; Whitaker-Azmitia, 1991). In the adult CNS, serotonin plays an important role in neuronal and synaptic plasticity, and its action on the serotonin 5-HT1A receptor is particularly significant in this regard (Azmitia and Whitaker-Azmitia, 1997). It was also found that the powerful mitogenic effect of fenfluramine (a releaser of serotonin throughout the CNS) on the granule cell layer of the adult rat dentate gyrus (DG) could be completely blocked by pretreatment with a specific 5-HT1A antagonist (Jacobs, 2002). Our previous study indicated that PSD animals have significantly decreased expression of 5-HT1A receptors on the protein and mRNA levels, and all these effects were reversed by citalopram. The dysfunction of 5-HT1A receptors in the DG may play an important role in the hippocampal neurogenesis of PSD and become a potential target for therapeutic intervention in rat.

We hypothesize that augmentation of citalopram treatment by combined pharmacotherapy with the 5-HT1A receptor antagonist WAY-100635 and ischemia-stimulated neurogenesis may be causally connected. However, information on the relative importance of 5-HT1A receptor blockers versus 5-HT reuptake inhibitors is lacking. Thus the current studies were carried out in an attempt to monitor the effects of WAY-100635 and citalopram co-administered in different dose combinations on ischemia-stimulated neurogenesis, and the density of the 5-HT1A receptor and its encoding mRNA in the DG. This will also help to explain the reasons behind the variability in the response to SSRIs among PSD patients.

2. Materials and methods

2.1. Animals and experimental protocol

Male Sprague–Dawley rats (210–250 g) were housed two per cage (40 cm long×25 cm wide×15 cm high) maintained at 21±2 °C in a 12-h light:12-h dark cycle (lights "on" at 7:00 a.m.), food and tap water were available adlibitum. The animals were acclimatized to the environment over 2 weeks, while being trained to consume 1% (w/v) sucrose solution for taste adaptation. All experiments were conducted in compliance with the National Institute of Health Guidelines for the Care and Use of Laboratory Animals (NIH Publications No. 80-23), revised in 1996.

Based on the baseline sucrose preference test and the open field test (OFT) scores during the adaptation period, left middle cerebral artery occlusion (MCAO) was carried out by introducing an intraluminal thread via the left common carotid artery (Koizumi et al., 1986), and the survivors with neurological score (Longa et al., 1989) ≥1 but <4 then were randomly divided into PSD + (5 mg/kg) citalopram group (PSD + C), PSD + (5 mg/kg) citalopram + (0.3 mg/kg) WAY100,635 group (PSD + C5 + W0.3), PSD + (10 mg/kg) citalopram + (0.3 mg/kg) WAY100,635 (PSD + C10 + W0.3) group and PSD + (10 mg/kg)citalopram + (0.1 mg/kg) WAY100,635 (PSD + C10 + W0.1) group. A detailed description of the PSD animal model methodology used has been reported with minor modifications (Wang et al., 2009a). In brief, 14-day chronic mild stress (CMS) protocol begins just after neurological score. The citalopram-treated group received daily citalopram in the dose of 5 mg/kg for further 14 days, PSD + C + W also for further 14 days. Citalopram (Lunbeck A/S, Copenhagen, Denmark) and WAY100,635 (N-{2 [4-(2-methoxyphenyl)-1-piperazinyl] ethyl}-N-(2-pyridinyl) cyclohexanecarboxamide) (Sigma) were dissolved in 0.9% saline and administered intraperitoneally (i.p.), in a volume of 1 ml/kg.

All groups were randomly divided into matched subgroups firstly for experiment on proliferation by 14 days after ischemia). To label proliferating cells, BrdU (Sigma), 50 mg/kg in saline i.p., was given twice daily, at 8-h intervals, on consecutive days (days 12 and 13 after ischemia). Rats were killed the day after the last dose. The protocol of BrdU labeling is provided in Fig. 1. Secondly, all groups were randomly divided into matched subgroups for experiment on survival and differentiation by each time-point (21 and 28 days after ischemia). To label proliferating cells for long-term studies on survival and differentiation, animals were injected with BrdU (50 mg/kg, i.p.) three times a day at 4-h intervals on 4 consecutive days (8–11 days after ischemia) and sacrificed at 21 and 28 days after ischemia (see Fig. 2. for schematic illustration of administration paradigm). There are five to seven rats per group.

2.2. PSD animal model and behavioral tests

PSD animal model was established according to MCAO plus CMS (MCAO + CMS) treatment procedure with minor modifications. A description of methodology and behavioral tests used are depicted in our former study (Wang et al., 2009a). Briefly, permanent MCAO was carried out by introducing an intraluminal thread via the left common carotid artery (Koizumi et al., 1986). The CMS regimen included totally 7 different stressors, which were arranged day and night in random order for 14 consecutive days as follows: food and water deprivation (20 h, followed by sucrose preference test), water deprivation (18 h, followed by a 1-h exposure to empty bottles), 45° cage tilt (17 h), overnight illumination (lights on for a total of 36 h), soiled cage (200 ml water in 100 g sawdust bedding, 21 h), swimming in 4°C water (5 min), paired caging (2 h). With the exception of general handling, CON group animals were left undisturbed in their home cage (two per cage) and had no contact with the stressed animals.

During the adaptation period, the baseline sucrose preference test and OFT scores were employed, sucrose preference test was also employed after 14-day CMS. In OFT, the open field was consisted of a wooden box (75 cm square chamber, 40 cm high walls) with painted black for the wall and white for the floor, which was divided by 1 cm wide black lines into 25 (5×5) equal squares. The animals were placed at the centre of the open field arena and tested in a quiet room. The numbers of locomotor activity (at least three paws in a quadrant), and rearing behavior (animal standing upright on its hind legs) were recorded for 5 min. In sucrose preference test, the animals were allowed to consume water and 1% sucrose solution for 1 h after 20-h food and water deprivation. The position of the 2 bottles (left/right sides of the cages) was varied randomly from trial to trial. For each trial, the position of the 2 bottles was counterbalanced across the rats in each group. During the test, both bottles were removed after 30 min and weighed. They were replaced by another pair of preweighed bottles (the position of the 2 bottles was then reversed).

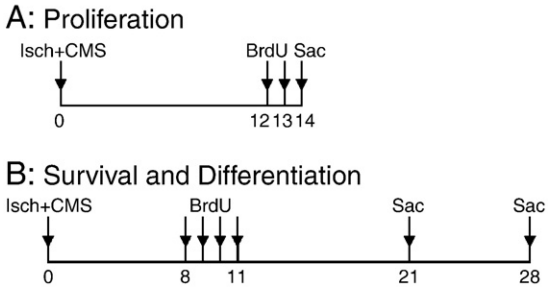

Fig. 1. Experimental designs and group assignments. Animals were subjected to CMS procedure following ischemic occlusion and citalopram-treated alone or citalopram-co-treated with WAY-100635 respectively. (A) Proliferation studies were conducted in rats injected with bromodeoxyuridine (BrdU) twice daily (50 mg/kg) at 8-h intervals on 2 consecutive days (days 12 and 13 after ischemia and CMS). Rats were sacrificed the day after the last dose. (B) Survival and differentiation study was conducted in rats injected with BrdU (50 mg/kg) three times a day at 4-h intervals on 4 consecutive days (days 8–11 after ischemia) and sacrificed at days 14, 21 and 28 after ischemia and CMS. Isch, ischemic injury; CMS, chronic unpredicted mild stress; Sac, sacrifice.

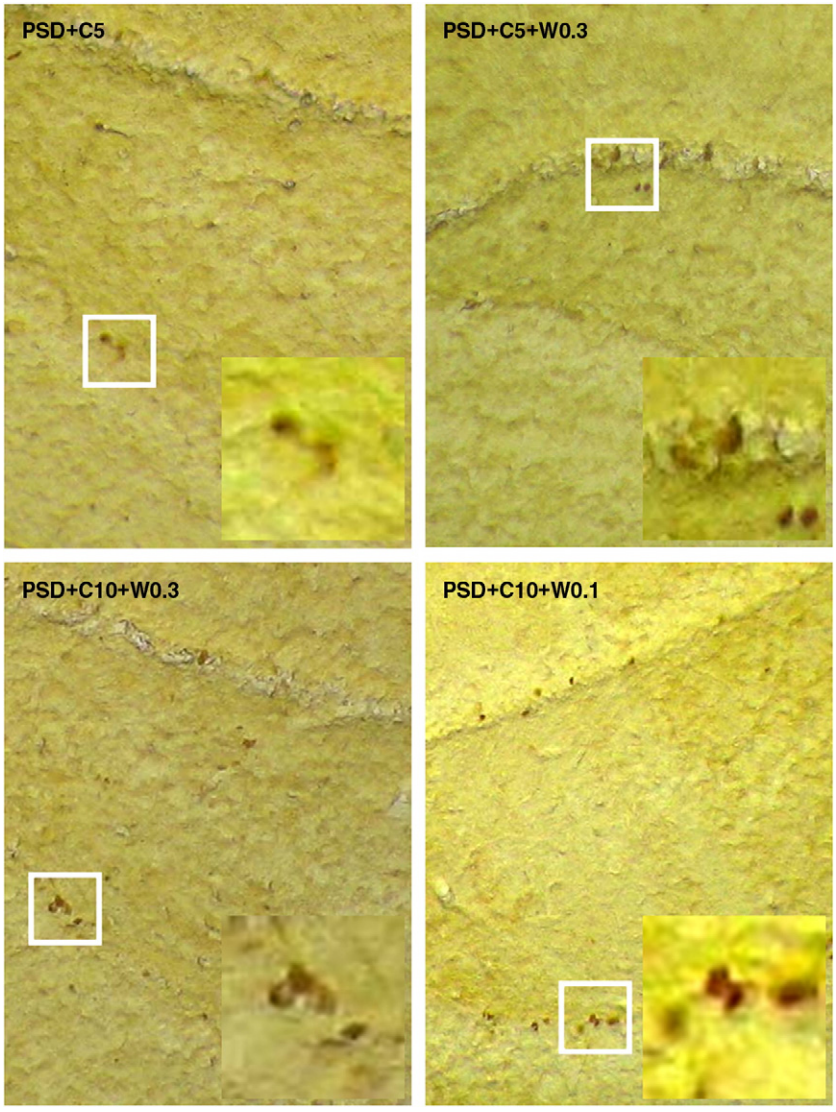

Fig. 2. Representative images illustrating bromodeoxyuridine (BrdU, 50 mg/kg, i.p; twice daily, at 8-h intervals) immunostaining in the hippocampal dentate gyrus (DG) of post-stroke depression (PSD) citalopram-treated (PSD + C) and citalopram-co-treated (PSD + C + W) groups. Proliferating (Labeled) cells are found as clusters in the subgranular zone (SGZ). The experiment was repeated three times with similar results (original magnification, 20×; inset, 40×).

A baseline preference test was performed before the CMS procedure. The sucrose preference (SP) was calculated according to the following ratio: SP = sucrose intake (g) / sucrose intake (g) + water intake (g).

2.3. Immunohistochemistry and image data analysis

Immunohistochemistry staining for brain tissue was performed on coronal sections (40 μm). For detection of BrdU-labeled nuclei, tissue samples were pretreated with 0.1 M citric acid, pH6.0 at 75°C for 30 min, followed by 2 M HCl for 30 min at 37°C, and rinsed in 0.1 M boric acid for 10 min. Sections were incubated in 3% H_2O_2 for 15 min, and in blocking solution (3% normal goat serum/1% BSA/0.3% Triton X-100 in PBS) for 2 h at room temperature, and with mouse mono-clonal anti-BrdU antibody (Chemicon, 1:100) at 4°C overnight. After washing step, sections were incubated with biotinylated goat anti-mouse secondary antibody (Sigma, 1:100) and placed in avidin-peroxidase complex solution for 1 h, respectively. The horseradish peroxidase reaction was detected with 0.05% diaminobenzidine (DAB) and 0.03% H_2O_2. To determine the phenotype of progenitor cells, double immunofluorescence was conducted to detect the colocalization of BrdU with either NeuN or GFAP. Serial sections were incubated with 2 M HCl for 30 min at 37°C. After washing, sections were incubated in blocking solution, then with mouse mono-clonal anti-BrdU antibody (Chemicon, 1:150 dilution) either with rabbit mono-clonal anti-NeuN (Chemicon, 1:50) or with rabbit mono-clonal anti-GFAP (Neomarker, 1:100) overnight at 4°C. After washing, sections were incubated for 1 h with rhodamine-conjugated goat anti-mouse lgG (Vector, 1:100) and FITC-conjugated goat anti-rabbit lgG (Vector, 1:100).

The number of BrdU-labeled cells revealed by DAB histochemistry were quantified with a bright-field microscope at 50× magnification, by an investigator blind to treatment history, on 6 coronal sections of 40 μm thickness spaced 240 μm apart within the hippocampus (bregma −2.12 mm to bregma −6.30 mm) (Paxinos and Watson, 1997). BrdU-labeled cells were counted in the GCL and the SGZ, defined as a two-cell body width along the base of the GCL and considered part of the GCL. BrdU-labeled cells that were located more than two cells away from the GCL were classified as being located in the corresponding hilar region. BrdU-immunoreactive cells in the uppermost focal plane were excluded. The numbers of labeled cells were expressed as the mean number of BrdU-positive cells per section. The numbers of BrdU/NeuN and BrdU/GFAP double-labeled cells in the left GCL and hilar, was quantified by using Zeiss LSM510 laser scanning microscope (Carl Zeiss) equipped with an 40× objective at excitation/emission wavelengths of 535 nm/565 nm(rhodamine, red), 470 nm/505 nm(FITC, green).

The BrdU-positive cells were first randomly selected in DG and analyzed in their entire *z*-axis with a 0.5 μm step to exclude false double-labeling. A minimum of 50 BrdU-labeled cells were analyzed from each rat, and numbers of double-labeled cells were expressed as percentages of the total number of BrdU-labeled cells.

2.4. Real-time PCR

After routine tissue preparation, the total RNA was isolated using Trizol reagent kit (Invitrogen) according to the manufacturer's protocol, and then a total of 5 mg RNA was used as the template to obtain first strand cDNA by random priming using the Promega RT System. The following primer pair was designed for the 5-HTR1A receptor gene using Primer Premier 5.0: forward, 5′-CCA GGG CAA CAA CAC CAC A-3′ and reverse, 5′-GAT CAG TAA CCG CCA AGG AG-3′. Template (1 ml) was mixed with the Master Mix (including 10× SYBR Green PCR buffer, 25 mM $MgCl_2$, 2.5 mM dNTP, 10,000× Sybergreen, and Taq DNA polymerase) and with 20 μM of each primer. The reaction mixture was made up to a final volume of 25 μl using RNase-free deionized water. The amplification conditions were as follows: 2 min at 30 °C and 10 min at 95 °C (Taq DNA polymerase activation) followed by 40 cycles of 20 s at 94 °C (denaturation), 20 s at 55 °C (annealing) and 30 s at 72 °C (extension). Real-time RT-PCR was performed by monitoring the increase in the fluorescence of the SYBR Green dye using the Rotor-Gene 3000 Real-time PCR apparatus (Corbett Research) according to the manufacturer's instructions. Each sample was tested in triplicate and samples obtained from three independent experiments were used for analysis of relative gene expression normalized by β-actin control bands using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). CT was defined as the threshold cycle of PCR at which amplified product was first detected. $2^{-\Delta\Delta CT}$ can be calculated with the formula: ΔΔCT = (CT, 5-HTR1A-CT, β-actin) control − (CT, 5-HTR1A-CT, β-actin) sample.

2.5. Western blotting

Tissue was dissected on ice, flash frozen in liquid nitrogen and washed three times with cold PBS. Each sample was then homogenized in ice-cold lysis buffer (20 mmol/l Tris, pH 7.5; 150 mmol/l NaCl; 1 mmol/l EDTA; 1 mmol/l EGTA; 1% Triton X-100; 2.5 mmol/l Na-pyrophosphate; 1 mmol/l b-glycerophosphate; 1 mmol/l Na3VO4; 1.l g/ml leupeptin and 1 mmol/l PMSF) and centrifuged at 14,000 rpm for 20 min. The protein concentration of the supernatant was determined spectrophotometrically (Thermo). Loading buffer was added to the samples, and they were boiled for 3 min. Then 50 μg protein was loaded onto a 10% Bis–Tris gel (Invitrogen) for electrophoresis. The resulting bands were transferred to a PVDF membrane, which was then blocked with Tris-buffered saline (TBS) containing 1% bovine serum albumin and 5% non-fat milk powder (w/v). The membrane was incubated overnight at 4 °C with primary antibodies, and immunoreactivity was detected using Horseradish Peroxidase-bound secondary antibody and visualized with enhanced chemiluminescence using 1:1000 5-HT1A receptors antibody (Santa Cruz Biotechnology). The intensity of the signal of each target gene was normalized to that of a β-actin internal control. The expression levels of the target gene and β-actin protein were quantified from the blots by densitometry (TotalLab, version 1.1, UK), and the density ratio of the bands was calculated. These data represented the relative expression of the target gene protein. All data were expressed relative to the control.

2.6. Statistical analysis

Values are presented as means ± S.E.M. Sucrose test (sucrose preference) was analyzed using a repeated measurement ANOVA with treatment (PSD + C, PSD + C5 + W0.3, PSD + C10 + W0.3, PSD + C10 + W0.1) and day (baseline, days 14, 21, and 28) as two factors. Quantity of immunofluorescent signal data, gene expression and protein level was performed by using one-way ANOVA. (One-way or two-way) ANOVAs were supported by the Bonferroni post hoc tests for multiple, comparisons, with $p<0.05$ considered significant.

3. Results

3.1. Behavioral tests

Results from two-way ANOVA indicated that chronic administration of a combination of an SSRI agent with a 5-HT1A receptor antagonist enhanced sucrose preference [$F(3, 80)=51.1$, all $p<0.0001$]. Subsequent Bonferroni's post hoc tests indicated potentiation of increases at 2 doses of citalopram co-treatment with WAY-100635 on days 14 and 21 (all $p<0.001$). Simultaneous administration of 0.3 mg/kg WAY-100635 enhanced citalopram (5 mg/kg)-induced sucrose preference; the effect increased when 0.3 mg/kg WAY-100635 was concurrently administered with a higher dose of citalopram (10 mg/kg). WAY-100635 at 0.1 mg/kg induced higher relative sucrose intake than 0.3 mg/kg WAY-100635 co-administered with 10 mg/kg citalopram. On day 28, among the co-treatment groups only 0.1 mg/kg WAY-100635 produced increased sucrose preference compared with other groups including co-treatment and citalopram-only groups (all $p<0.001$). The data are shown in Table 1.

3.2. Hippocampal neurogenesis

BrdU-labeled cells were located exclusively near the SGZ and frequently formed clusters after injection (Fig. 2). The phenotype of BrdU-positive cells was determined using markers for NeuN and GFAP (Fig. 3). In the previous study (Wang et al., 2009b by Behavioural pharmacology), we found one dose of citalopram (5 mg/kg) co-treatment with WAY-100635 (0.3 mg/kg) enhanced proliferation on day 14 and neuronal differentiation in DG on day 28. Here, two doses of citalopram co-treatment with WAY-100635 enhanced the numbers of proliferating cells on day 14 and their differentiation into neurons in DG on day 28 (Fig. 4). Administration of 0.3 mg/kg WAY-100635 enhanced citalopram (5 mg/kg)-induced proliferation ($p<0.01$) and neurogenic differentiation ($p<0.001$); the effects increased when 0.3 mg/kg WAY-100635 was combined with a higher dose of 10 mg/kg citalopram ($p<0.05$ and $p<0.001$ respectively). WAY-100635 at 0.1 mg/kg induced elevated proliferation and neurogenic differentiation compared with 0.3 mg/kg WAY-100635 co-administered with 10 mg/kg citalopram (all $p<0.001$). Simultaneously, the GFAP signal was mainly detected in the hilus. In comparison with the PSD + C animals, PSD + C + W groups produced a lower proportion of double-labeled cells (all $p<0.001$). There was no difference among different doses of PSD + C + W groups. The number of BrdU-immunoreactive cells and their phenotypes in the DG and hilus on day 28 are shown in Fig. 5.

Table 1
Relative sucrose all baseline and at 14, 21,days after MCAO.

Group(n)	Baseline	Day 14	Day 21	Day 28
PSD + C5(6)	70.2±1.2	51.3±1.1	56.8±1.0	69.2±1.4
PSD + C5 + W0.3(6)	69.8±1.3[a]	61.0±0.9[a]	64.5±1.1[a]	71.8±1.3
PSD + C10 + W0.3(6)	70.3±1.1[ab]	68.8±0.7[ab]	73.0±0.7[ab]	73.3±1.3
PSD + C10 + W0.1(6)	69.7±1.0[ac]	76.3±1.4[ac]	81.0±1.3[ac]	84.0±1.6[ac]

Relative sucrose intake (%) in all groups at baseline and at 14, 21,days after MCAO is expressed as the mean ± S.E.M. The number in parentheses is the number (n) of animals in each group. PSD, post-stroke depression; C5 or 10, citalopram (5 mg/kg or 10 mg/kg). W0.3 or 0.1, WAY-100635 (0.3 mg/kg or 0.1 mg/kg). [a]$p<0.001$, vs. PSD + C5 value. A statistically significant difference was found compared with PSD + C5 group ([a], $p<0.001$), and between PSD + C5 + W0.3 and PSD + C10 + W0.3 groups ([b], $p<0.001$), between PSD + C10 + W0.3 and PSD + C10 + W0.1 groups ([c], $p<0.001$).

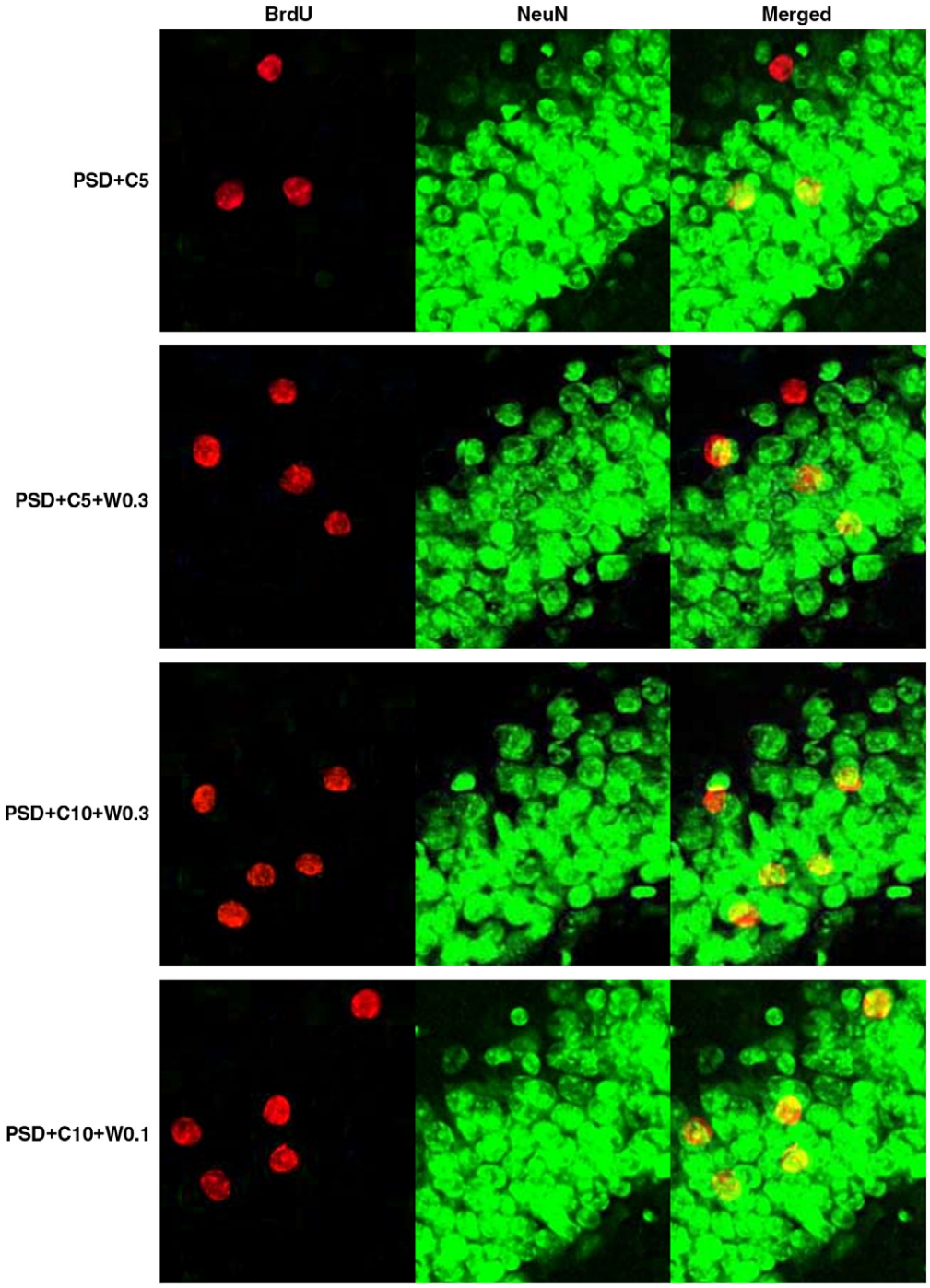

Fig. 3. Confocal microscopy of a representative double-stained hippocampal section showing cells labeled with bromodeoxyuridine (BrdU) (Left, red), neuron specific nuclear protein (NeuN) (Centre, green), and glial fibrillary acidic protein (Centre, green); BrdU and NeuN or GFAP labeling are shown in both isolated and overlapping cases (Right). The experiment was repeated thrice, and similar results were obtained each time (original magnification, 20×; inset, 40×).

The numbers of BrdU-labeled cells did not differ among the 4 groups on days 21 and 28, indicating that WAY-100635 administered in combination with citalopram did not increase the survival of the newborn cells in PSD animals, compared with citalopram treatment alone (Fig. 6).

3.3. Expression of 5-HT1A receptors in the dentate gyrus

Western immunoblotting and real-time RT-PCR were then used to investigate the protein level and gene expression of 5-HT1A receptors in the dentate gyrus on days 14 and 28. For each group, the fluctuations in protein synthesis of 5-HT1A receptors were consistent with the alterations of mRNA expression.

There were no differences in gene expression or protein level of 5-HT1A receptors when the animals were administered the same dose (5 mg/kg or 10 mg/kg) of citalopram, whether or not they were co-treated with any concentration of WAY-100,635. When co-treated with 0.3 mg/kg WAY-100,635, a high concentration of citalopram (10 mg/kg) produced elevated expression of 5-HT1A receptors at two time points compared with a low dose (5 mg/kg) (all $p<0.001$). The data are shown in Figs. 7 and 8.

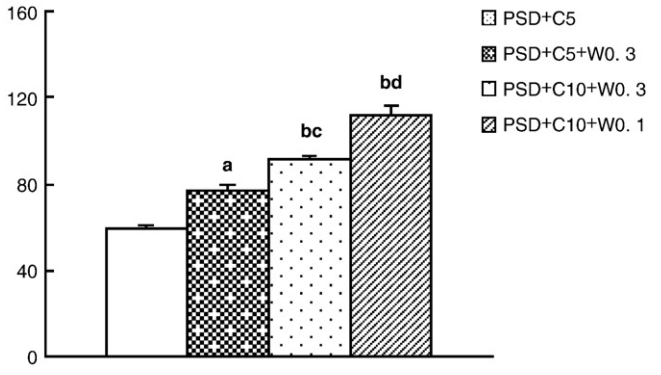

Fig. 4. Cell proliferation in the dentate gyrus of the hippocampus on days 14 after ischemia. The number of bromodeoxyuridine-labeled cells per section of the dentate gyrus in each group is expressed as the mean ± S.E.M. PSD, post-stroke depression; C5 or 10, citalopram (5 mg/kg or 10 mg/kg). W0.3 or 0.1, WAY-100635 (0.3 mg/kg or 0.1 mg/kg). A statistically significant difference was found compared with PSD + C5 group ($^{a}p<0.01$; $^{b}p<0.001$), and between PSD + C5 + W0.3 and PSD + C10 + W0.3 groups ($^{c}p<0.05$), between PSD + C10 + W0.3 and PSD + C10 + W0.1 groups ($^{d}p<0.001$).

4. Discussion

This animal study complements previous studies on the ability of 5-HT1A receptor blocking agents to achieve more efficacious

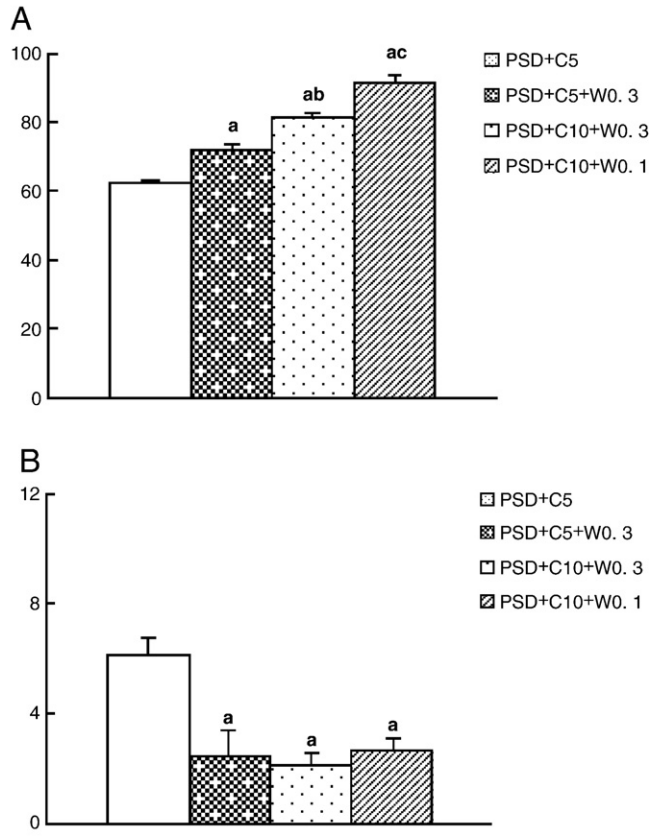

Fig. 5. Cell differentiation in the dentate gyrus of the hippocampus on days 28 after ischemia. The number of BrdU-immunoreactive cells and their neuronal (A) or glial (B) phenotypes in the hippocampal dentate gyrus is expressed as the mean ± S.E.M. PSD, post-stroke depression; C5 or 10, citalopram (5 mg/kg or 10 mg/kg). W0.3 or 0.1, WAY-100635 (0.3 mg/kg or 0.1 mg/kg). NeuN, a neuronal marker in the nucleus of mature mammalian neurons; GFAP, glial fibrillary acidic protein. A statistically significant difference was found compared with PSD + C5 group ($^{a}p<0.001$), and between PSD + C5 + W0.3 and PSD + C10 + W0.3 groups ($^{b}p<0.001$), between PSD + C10 + W0.3 and PSD + C10 + W0.1 groups ($^{c}p<0.001$).

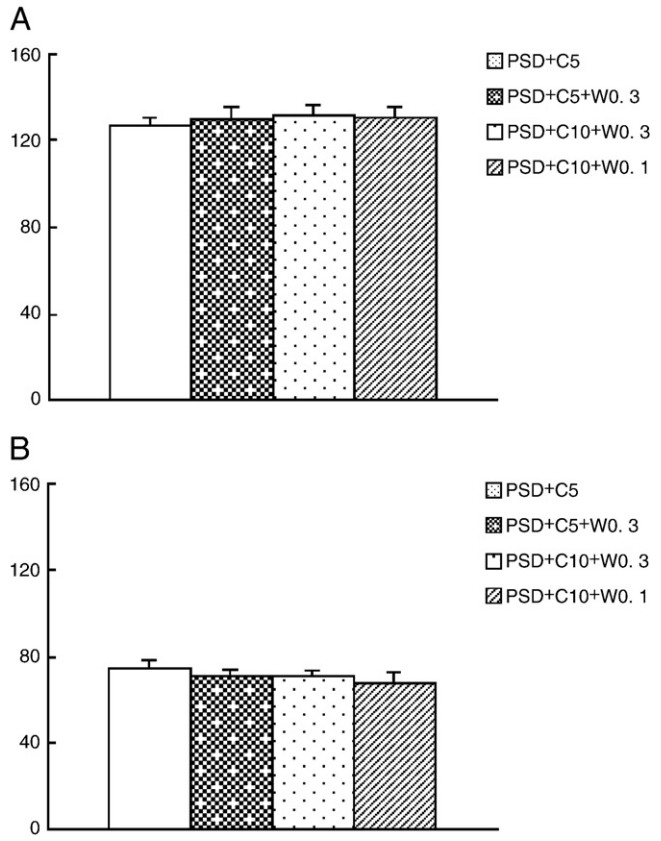

Fig. 6. Cell survival in the dentate gyrus of the hippocampus on days 14 (A) and 28 (B) after ischemia. The number of bromodeoxyuridine-labeled cells per section of the dentate gyrus is expressed as the mean ± S.E.M. PSD, post-stroke depression; C5 or 10, citalopram (5 mg/kg or 10 mg/kg). W0.3 or 0.1, WAY-100635 (0.3 mg/kg or 0.1 mg/kg).

treatment and augment 5-HT reuptake inhibitor-induced elevation of post-stroke DG neurogenesis in PSD. Specifically, the present data demonstrate that an increase of ischemia-induced DG neurogenesis can be interpreted as a valid index, to an extent, or even a prerequisite for an efficient co-treatment strategy. WAY-100635 and citalopram when co-administrated in different dose combinations displayed their relative importance in influencing this interaction on ischemia-stimulated neurogenesis, probably through reinforcing serotonergic neurotransmission and/or expression of the 5-HT1A receptor in the DG.

In agreement with our former finding (Wang et al., 2009a), the current MCAO + CMS protocol induced anhedonia (Willner et al., 1992) that were consistent with behavioral correlates of depressive-like symptoms in post-stroke humans. PSD is generally considered to be associated with low serotonergic transmission. 5-HT reuptake-inhibiting agents have been shown to increase extracellular 5-HT in both terminal and cell body areas. The increase in extracellular 5-HT in the cell body region activates 5-HT1A autoreceptors, thereby decreasing 5-HT neuronal firing (Chaput et al., 1986). The latter process counteracts the primary effect of the increase in synaptic 5-HT in the nerve terminal region expected from the inhibition of reuptake and contributes to a pronounced “therapeutic lag”. Hence, there are indications that blockade of 5-HT1A autoreceptors potentiates SSRI-induced elevation of extracellular 5-HT in the rat in vivo, and the behavioral effect of SSRIs (Gartside et al., 1995; Artigas et al., 1996; Hashimoto et al., 1997; Mitchell and Carroll, 1997; Grignaschi et al., 1998; Trillat et al., 1998). In the present study, the high potency of administration of either 0.3 mg/kg or 0.1 mg/kg the selective 5-HT1A receptor blocker WAY-100635 to enhance the therapeutic efficacy of citalopram (5 mg/kg or 10 mg/kg) possibly resulted from WAY-

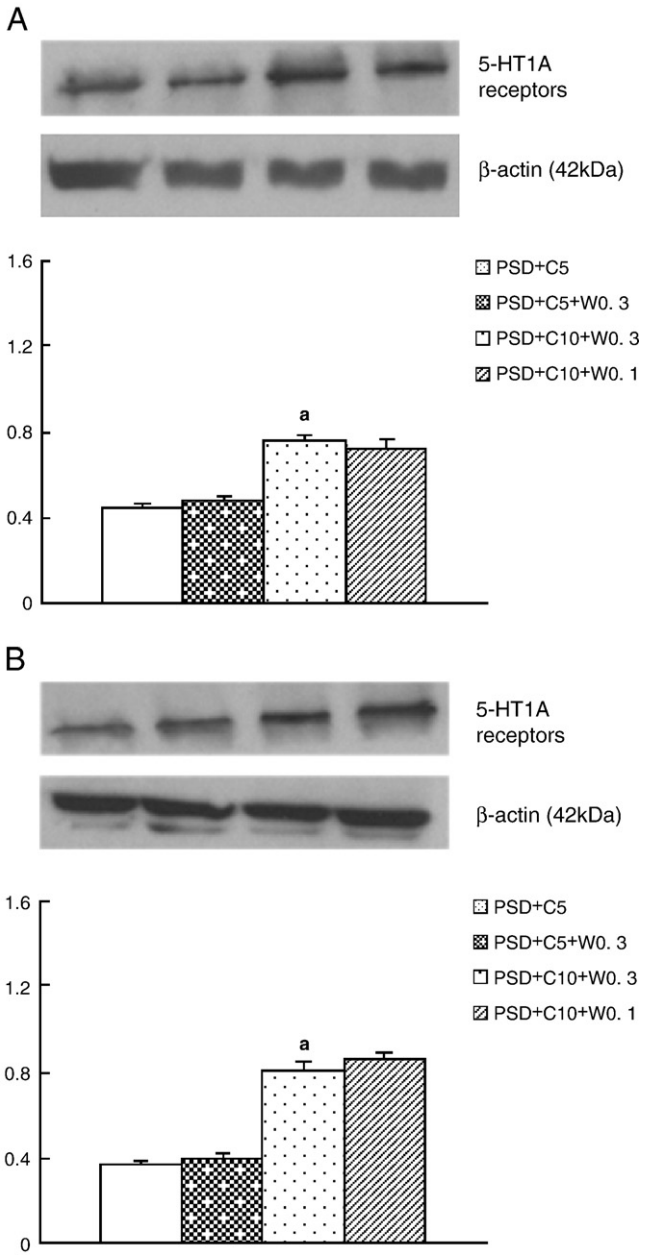

Fig. 7. Relative protein expression of 5-HT1A receptors in all groups by Western blotting analysis on the days 14 (A) and 28 (B) after ischemia and CMS. A representative blot is shown (upper panel), and data from densitometric scanning of the blots and calculation of the density ratio of the bands are shown (lower panel). Values are means ± S.E.M. PSD, post-stroke depression; C5 or 10, citalopram (5 mg/kg or 10 mg/kg); W0.3 or 0.1, WAY-100635 (0.3 mg/kg or 0.1 mg/kg). A statistically significant difference was found between PSD + C5 + W0.3 and PSD + C10 + W0.3 groups ($^a p < 0.001$).

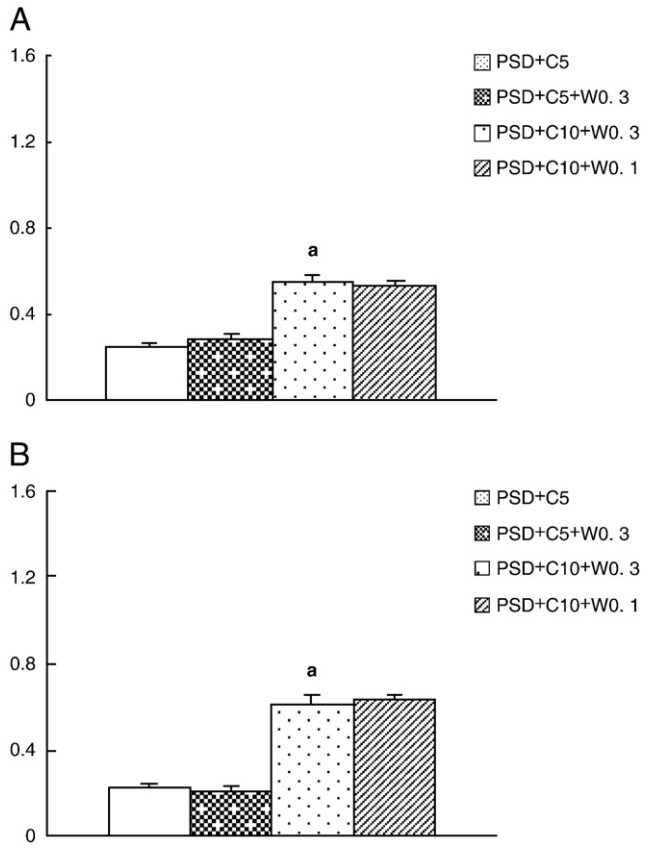

Fig. 8. Relative gene expression of 5-HT1A receptors in all groups by Real-time PCR on the days 14 (A) and 28 (B) after ischemia and CMS. Values are means ± S.E.M. PSD, post-stroke depression; C5 or 10, citalopram (5 mg/kg or 10 mg/kg); W0.3 or 0.1, WAY-100635 (0.3 mg/kg or 0.1 mg/kg). A statistically significant difference was found between PSD + C5 + W0.3 and PSD + C10 + W0.3 groups ($^a p < 0.001$).

100635 promoting citalopram-induced cell proliferation. The numbers of proliferating cells labeled with BrdU on day 14 after ischemia and their neuronal differentiation on day 28 in DG were both elevated. It has long been known that 5-HT exert an important mitogenic action in the CNS during development and plays an important role in neuronal and synaptic plasticity in the adult CNS. 5-HT was also found to be involved in regulating SGZ neurogenesis (Brezun and Daszuta, 1999, 2000), they reported that either depletion of brain 5-HT or neurotoxic destruction of brain serotonergic neurons resulted in a significant reduction in granule cell neurogenesis in the adult rat DG. So blockade of 5-HT1A autoreceptors potentiating SSRI-induced elevation of extracellular 5-HT seems to be responsible the increased post-stroke DG neurogenesis, which was implicated in the biological factor and an important index for the augmented pharmacological efficacy in relation to the "clinical" or behavioral effects.

An inconsistent result from our analysis was that elevated cells proliferation in the combination treatment group did not produce changes in their survival. Nevertheless, more neurons generated from them. It is the most important for newborn cells to neuronal differentiation, for new born neuron can extend axons along the mossy fiber pathway to the CA3 pyramidal cell layer and exhibit long-term potentiation (van Praag et al., 1999), and finally integrate into the existing hippocampal circuitry. Some potential factors, for example, post-stroke apoptosis could mediate the loss of progenitors after proliferation during the phase of maturation in the SGZ (Bingham et al., 2005).

In the current study, a 0.3 mg/kg dose of WAY-100635 augmented the high-dose citalopram (10 mg/kg)-induced increase of relative sucrose intake more than that of low-dose citalopram (5 mg/kg). Furthermore, the effect on DG neurogenesis of high-dose citalopram (10 mg/kg) was significantly potentiated by WAY-100635 (0.3 mg/kg). Systemic administration of citalopram produced a roughly dose-dependent increase of ischemia-stimulated DG neurogenesis. Additionally, it should be noted that elevated gene expression and protein levels of 5-HT1A receptors seems to be also critically dependent upon the dose of citalopram administered. An increased extracellular level of 5-HT was not measured in the present study, but it has been reported that (0.5 mg/kg or 5.0 mg/kg s.c.) citalopram dose-dependently produced high dialysate 5-HT in the rat ventral hippocampus in vivo.

The finding that the potentiation effect is dependent on the dose of citalopram eliciting significant post-stroke neurogenesis in DG possibly results from two facts: first, the increase in synaptic 5-HT

in the terminal region expected from citalopram inhibition of reuptake and its reinforcement of the 5-HT neuronal firing and release of the transmitter attenuated by WAY-100,635. On the other hand, enhanced expression of 5-HT1A receptors should also be taken into account.

The potential role of 5-HT1A receptors in the function and structure (e.g. DG neurogenesis) of hippocampus-related depressive disorders has been reported earlier (Gould, 1999; Radley and Jacobs, 2002), and the 5-HT1A receptor is present at a particularly high concentration in the hippocampus, especially in the DG. Animal studies suggest that increases in neurotransmission at postsynaptic 5-HT1A receptors may mediate the therapeutic effects of some antidepressant drugs (Welner et al., 1989), and many studies suggest that increases in adult neurogenesis after SSRI administration require the activation of 5-HT1A receptors (Santarelli et al., 2003), which is consistent with the results that 5-HT1A receptor antagonists or knockouts decrease or lack, respectively, cell proliferation in the dentate gyrus (Radley and Jacobs, 2002; Santarelli et al., 2003). In our previous study, we identified a decrease in 5-HT1A mRNA levels in hippocampus in chronically stressed post-stroke rats. This reduction of 5-HT1A mRNA was significantly ameliorated in rats administered citalopram. These results were paralleled by citalopram-induced increase in cell proliferation in the DG of PSD rats. All these results contribute to a better understanding of the potential role of the 5-HT1A receptor in mediating the neuroprotective effects of citalopram on cell proliferation and subsequent DG neurogenesis response to post-stroke stress.

It is also interesting to note that when citalopram was administered at 10 mg/kg the potentiation effects observed in this study seem to be related to the concurrent WAY-100635 dose. In addition to the dependence of augmentation of SSRIs plasma level, Hjorth et al. also showed that the augmentation effect is critically dependent on the dose of 5-HT 1A antagonist (Hjorth et al., 1997). It was reported that WAY-100635 (0.01–0.3 mg/kg) dose-dependently potentiated the citalopram-induced increase of extracellular 5-HT in the rat ventral hippocampus. However, systemic administration of 0.1 mg/kg of WAY-100635 is unexpectedly more effective than 0.3 mg/kg for elevating citalopram-induced robust neurogenesis and increasing sucrose preference. It can be speculated that the effect of 0.3 mg/kg WAY-100635 on decreased DG neurogenesis resulted from its tonic inhibition of 5-HT1A receptors, which mediate the effects of 5-HT on DG neurogenesis. In turn, net augmentation of DG neurogenesis elicited by 0.1 mg/kg WAY-100635 likely reflects the effects of decreased extracellular 5-HT in the rat ventral hippocampus, secondary to weak inhibition of 5-HT1A receptors in DG by WAY-100,635. Additionally, by comparison, low or high concentrations of WAY-100635 concurrent with 10 mg/kg citalopram did not produce different densities of the DG 5-HT1A receptor or its encoding mRNA. All these again support the findings that gene expression and protein level of 5-HT1A receptors in DG seem to be exclusively dependent upon the dose of citalopram administrated. The relative importance that the dose of WAY-100635 displays in the interaction is a result of its antagonism of DG 5-HT1A receptors.

The interpretation and analysis of the data presented in this study have certain limitations. First, since plasma half-lives of drugs are generally much shorter in rodents than in humans, the doses established in the current study probably are not equivalent to human plasma levels, contributing to the diverse results. Second, BrdU is the preferred technique for labeling newborn cells, but WAY-100635 cannot be excluded as a putative factor involved in altering the number of BrdU-labeled cells.

In summary, the current findings are specific to PSD, showing that a two-week WAY-100635 treatment markedly augments the antidepressant effects of citalopram in PSD animals. The behavioral change seems to occur with a parallel modification of ischemia-stimulated DG neurogenesis, however, PSD appears instead to be multifactorial in origin and consistent with the biopsychosocial model of mental illness, rather than "pure" psychosocial mechanism. In our previous study, we identified a decrease in 5-HT1A mRNA levels in hippocampus in chronically stressed post-stroke rats indicating biological mechanism, according to which ischemic insults directly affect neural circuits including serotonin system involved in mood regulation after stroke. So we cannot exclude these biological factors involving in post-stroke DG neurogenesis, which can be interpreted as a prerequisite for an effective combined pharmacotherapy strategy. The relative importance of the dose of WAY-100635 versus that of citalopram in ischemia-stimulated neurogenesis probably depends on prevention of 5-HT1A autoreceptor-based negative feedback and/or modification of the 5-HT1A receptor protein or mRNA in the DG. The relative importance of their doses in the interaction will be helpful for the design of therapeutic strategies based on SSRIs + 5-HT1A antagonist combinations for PSD.

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