BJP BRITISH JOURNAL OF PHARMACOLOGY
BPS BRITISH PHARMACOLOGICAL SOCIETY

BJP British Journal of Pharmacology

DOI:10.1111/j.1476-5381.2010.00978.x
www.brjpharmacol.org

RESEARCH PAPER

Utility of organotypic raphe slice cultures to investigate the effects of sustained exposure to selective 5-HT reuptake inhibitors on 5-HT release

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Keywords
antidepressant; SSRI; 5-HT; 5-hydroxytryptaminergic neuron; raphe slice culture; augmentation; 5-HT$_1$ autoreceptors; glutamatergic system; AMPA/kainate receptors

Received
5 February 2010
Revised
11 June 2010
Accepted
12 July 2010

BACKGROUND AND PURPOSE
Selective 5-hydroxytryptamine (5-HT, serotonin) reuptake inhibitors (SSRIs) are widely used antidepressants and their therapeutic effect requires several weeks of drug administration. The delayed onset of SSRI efficacy is due to the slow neuroadaptive changes of the 5-hydroxytryptaminergic (5-HTergic) system. In this study, we examined the acute and chronic effects of SSRIs on the 5-HTergic system using rat raphe slice cultures.

EXPERIMENTAL APPROACH
For organotypic raphe slice cultures, mesencephalic coronal sections containing dorsal and median raphe nuclei were prepared from neonatal Wistar rats and cultured for 14–16 days.

KEY RESULTS
Acute treatment with citalopram, paroxetine or fluoxetine (0.1–10 μM) in the slice cultures slightly increased extracellular 5-HT levels, while sustained exposure for 4 days augmented the elevation of 5-HT level in a time-dependent manner. Sustained exposure to citalopram had no effect on tissue contents of 5-HT and its metabolite, expression of tryptophan hydroxylase or the membrane expression of 5-HT transporters. The augmented 5-HT release was attenuated by Ca^{2+}-free incubation medium or treatment with tetrodotoxin. Experiments with 5-HT$_{1A/B}$ receptor agonists and antagonists revealed that desensitization of 5-HT$_1$ autoreceptors was not involved in the augmentation of 5-HT release. Finally, co-treatment with an α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)/kainate, but not an *N*-methyl-D-aspartate, receptor antagonist, suppressed this augmentation.

CONCLUSION AND IMPLICATIONS
These results suggest that sustained exposure to SSRIs induces the augmentation of exocytotic 5-HT release, which is caused, at least in part, by the activation of AMPA/kainate receptors in the raphe slice cultures.

Abbreviations
5-HIAA, 5-hydroxyindolacetic acid; 8-OH-DPAT, 8-hydroxy-2-(di-n-propylamino)tetralin; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; DMSO, dimethyl sulfoxide; GTPγS, guanosine 5′-[γ-thio]triphosphate; KRH, Krebs–Ringer–Henseleit; MDMA, 3,4-methylenedioxymethamphetamine; NMDA, *N*-methyl-D-aspartate; PBS, phosphate-buffered saline; SERT, 5-HT (serotonin) transporter; SSRI, selective 5-HT (serotonin) reuptake inhibitor; TPH, tryptophan hydroxylase; TTX, tetrodotoxin

Introduction

Depression is a major health problem around the world, and the lifetime prevalence of major depressive disorder is 10 to 20% (Kessler *et al.*, 2003). Because the sites of action of early types of antidepressant drugs, such as the tricyclic antidepressants and monoamine oxidase inhibitors, lie mostly in the monoaminergic system, major depressive disorder has been associated with hypofunction of the central monoaminergic system, in particular the 5-hydroxytryptaminergic (5-HTergic) system (Owens and Nemeroff, 1994; Belmaker and Agam, 2008). Selective 5-hydroxytryptamine (5-HT, serotonin) reuptake inhibitors (SSRIs) are now one of the more commonly used antidepressant drugs in clinical use. SSRIs are largely devoid of the adverse effects found with use of the tricyclic antidepressants. Their therapeutic actions are thought to be due to inhibition of the 5-HT transporter (SERT), which transports 5-HT from the synaptic cleft to the presynaptic compartment. Acute administration of SSRIs as well as other antidepressants such as the tricyclic antidepressants immediately increase extracellular 5-HT levels, although this increase depends on the type of antidepressants, the brain area and the route of administration (Bel and Artigas, 1992; Fuller, 1994; Beyer and Cremers, 2008). However, a therapeutic response usually requires 2–6 weeks of drug administration (Schechter *et al.*, 2005). This delayed onset of effect suggests that sustained treatment with SSRIs induces slow neuroadaptive changes in the monoaminergic system, especially the 5-HTergic system.

A number of behavioural, neurochemical and electrophysiological studies have emphasized the importance of the 5-HTergic system in the pathophysiology of depression and the therapeutic actions of antidepressants including SSRIs (Vandermaelen and Aghajanian, 1983; Chaput *et al.*, 1986; Mann, 1999; Van der Stelt *et al.*, 2005). Furthermore, *in vitro* studies have revealed the acute and chronic effects of antidepressants on cell lines, dissociated neurons, astrocytes and other systems (Pákáski *et al.*, 2005; Iceta *et al.*, 2007; Ohno *et al.*, 2007). However, the long-term effects of antidepressants on the 5-HTergic system (i.e. the effects on neural circuits and extracellular 5-HT levels), and the interaction between 5-HTergic and other neurons such as glutamatergic neurons, are still not well characterized. Furthermore, no reports of *in vitro* studies thus far have successfully analysed the alterations in the 5-HTergic system as a result of long-term exposure to SSRIs. In the mammalian brain, the principal source of the 5-HTergic innervation of the forebrain is the dorsal and medial raphe nuclei located in the midbrain (Jacobs and Azmitia, 1992). Recently, we have established rat organotypic raphe slice cultures containing functional 5-HTergic neurons, and have reported that sustained exposure to 3,4-methylenedioxymethamphetamine (MDMA) and methamphetamine causes augmentation of 5-HT release (Higuchi *et al.*, 2008; Nagayasu *et al.*, 2010). Thus, using raphe slice cultures, we can assess the effects of the acute and sustained exposure to SSRIs on the 5-HTergic system *in vitro*.

In this study, we examined the effects of acute treatment and sustained exposure to SSRIs, in particular citalopram, paroxetine and fluoxetine, on extracellular 5-HT levels in raphe slice cultures. Here, we show that sustained exposure of the slice cultures to SSRIs induced augmentation of exocytotic 5-HT release. Furthermore, we investigated the mechanisms underlying the neuroadaptive changes of 5-HTergic system induced by sustained exposure to SSRIs.

Methods

Preparation of rat organotypic raphe slice cultures

All animal care and experimental procedures were in accordance with the ethical guidelines of the Kyoto University Animal Research Committee. Organotypic slice cultures were prepared as previously described (Higuchi *et al.*, 2008) with slight modifications. Briefly, Wistar rats at post-natal days 2–3 (Nihon SLC, Shizuoka, Japan) were anaesthetized with hypothermia, decapitated, and the brain was isolated. Coronal sections (350 μm thickness) containing dorsal raphe nuclei and median raphe nuclei were identified by visual inspection with the aid of the Atlas of the Developing Rat Brain (Paxinos *et al.*, 1991), and prepared using a tissue chopper (Narishige, Tokyo, Japan). Four slices were placed on each 30 mm Millicell-CM insert (pore size 0.4 μm; Millipore, Billerica, MA, USA) and the inserts were transferred to a six-well culture plate. Culture medium, consisting of 50% minimal essential medium/HEPES (containing 10 $mg \cdot L^{-1}$ tryptophan), 25% Hank's balanced salt solution and 25% heat-inactivated horse serum (containing approximately 0.5 $mg \cdot L^{-1}$ tryptophan; Gibco, Invitrogen Japan, Tokyo, Japan) supplemented with 6.5 $mg \cdot mL^{-1}$ glucose and 2 mM L-glutamine, 100 $U \cdot mL^{-1}$ penicillin G potassium and 100 $\mu g \cdot mL^{-1}$ streptomycin sulfate (Gibco), was supplied at a volume of 0.7 mL per well. Slice cultures were maintained at the liquid/air interface for 14–16 days in an incubator at 37°C in an atmosphere of 5% CO_2, and subsequently used in experiments. The culture

medium was exchanged for fresh medium on the day following culture preparation, and on every second day thereafter.

Measurement of extracellular 5-HT level and tissue content

Measurements of extracellular 5-HT levels and tissue content were performed as previously described (Higuchi *et al.*, 2008). Briefly, for measurement of extracellular 5-HT levels, culture inserts were transferred and washed in 0.7 mL Krebs–Ringer–Henseleit (KRH) buffer (146 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$, 1.2 mM $CaCl_2$, 10 mM D-glucose, 15 mM HEPES, 5 mM HEPES-Na, 0.2 mM ascorbic acid; pH 7.4) three times. After the washes, the inserts were pre-incubated in KRH buffer for 15 min, then transferred to 0.7 mL KRH buffer containing drugs and incubated for 30 min. After incubation, KRH buffer was collected, and 5-HT (in 20 µL samples) was immediately analysed by high-performance liquid chromatography (HPLC) with an electrochemical detection system (Eicom, Kyoto, Japan). For measurement of 5-HT and its metabolite, 5-hydroxyindolacetic acid (5-HIAA) in tissues after drug treatment, slices were collected, homogenized, and sonicated in 500 µL ice-cold 0.1 M $HClO_4$ containing 10 mM $Na_2S_2O_5$ and 1 mM EDTA, and placed on ice for 15 min. Protein concentrations were measured using the Bradford protein assay (Bio-Rad, Hercules, CA, USA). Homogenates were centrifuged at 18 000× *g* for 15 min at 4°C, and supernatants (20 µL) were analysed with HPLC with an electrochemical detection system. 5-HT and 5-HIAA were quantified by reference to a linear calibration curve ranging from 1 to 100 nM. The detection limits for both 5-HT and 5-HIAA were estimated to be around 0.5–0.6 fmol per 50 µL sample.

Sustained drug exposure protocol

Slice cultures were incubated with culture medium containing drugs. Immediately prior to drug exposure (Day 0), 1 day (Day 1), 2 days (Day 2) and 4 days (Day 4) after the onset of drug exposure, extracellular 5-HT levels were measured by incubating cultures with KRH containing drugs for 30 min following the pre-incubation for 15 min. For sustained exposure, slices were maintained with culture medium containing drugs for 4 days, whereupon slice cultures were washed in KRH buffer three times, treated with the drugs for 30 min, and then extracellular 5-HT levels were measured. In some experiments, we used Ca^{2+}-free KRH with 2 mM EGTA and 50 µM 1,2-bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid tetrakis (acetoxymethyl ester) (Dojindo Laboratories, Kumamoto, Japan), a cell-permeable cytosolic Ca^{2+} chelator.

Western blot

For the detection of SERT in the membrane fraction, slice cultures from three culture inserts were harvested in 1 mL isotonic buffer, homogenized with a Teflon–glass homogenizer and centrifuged at 1000× *g*. Supernatants were centrifuged at 18,000× *g* for 30 min, and the resulting pellets were resuspended in water and stored at –20°C. Thawed samples were centrifuged at 40 000× *g* for 20 min, and pellets were resuspended in 20 mM Tris–HCl buffer containing 1% Triton-X (Nacalai tesque, Kyoto, Japan), 1% protease inhibitor cocktail (Merck, Darmstadt, Germany) and 1% phosphatase inhibitor cocktail (Sigma-Aldrich, Saint-Louis, MO, USA); this suspension was used as a synaptosomal membrane fraction. For the detection of tryptophan hydroxylase (TPH), slices were homogenized with 100 µL 20 mM Tris–HCl buffer containing 1% Triton-X, sonicated and centrifuged at 1000× *g*, and the supernatants analysed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Resolved proteins were transferred to a polyvinylidene fluoride membrane and probed with anti-TPH antibody (1:3000 dilution) or goat anti-actin antibody (1:10 000), anti-SERT antibody (1:1000) or anti-Na^+/K^+-ATPase antibody (1:5000). Proteins were visualized by Immobilon Western HRP substrate (Millipore) and detected by Chemidoc XRS (Bio-Rad).

[^{35}S]guanosine 5′-[γ-thio]triphosphate binding assay

[^{35}S]guanosine 5′-[γ-thio]triphosphate (GTPγS) binding assay was performed according to the method of Alper and Nelson (1998), with some modifications. Briefly, following sustained exposure to drugs, slices were collected in 1 mL of 50 mM Tris–Cl buffer (pH 7.4) and homogenized using a Teflon–glass homogenizer. Homogenates were centrifuged at 39 800× *g* for 10 min at 4°C, and the resulting pellets were resuspended in 1 mL of Tris buffer. Suspensions were incubated for 10 min at 37°C in a shaking water bath. After incubation, suspensions were centrifuged at 39 800× *g* for 10 min, and the pellets were resuspended in 1 mL Tris buffer. Suspensions were briefly homogenized with a Teflon–glass homogenizer and centrifuged at 39 800× *g* for 10 min. Resulting pellets were resuspended in Tris buffer and stored at –80°C until the day of assay. On the day of the assay, membrane suspensions were thawed and centrifuged at 39 800× *g* for 10 min. Resulting pellets were resuspended in 200 µL of assay buffer (160 mM NaCl, 67 mM Tris base, 4 mM $MgCl_2$, 0.267 mM EGTA; pH 7.4, 0.136 mM dithiothreitol was added immediately before use), and protein concentrations were measured. Membrane suspensions were diluted with

assay buffer to a concentration of 40 μg protein/100 μL. Membrane suspensions (40 μg·sample^{-1}), ligands (dissolved in water), guanosine 5′-diphosphate (0.3 mM final concentration, dissolved in assay buffer) and the [^{35}S]GTPγS (0.1 nM final concentration, dissolved in assay buffer) were mixed on ice, and incubated for 20 min in a shaking water bath at 37°C. Reactions were terminated by rapid filtration through glass-fiber filters (GF/C, Whatman, Kent, UK), followed by three washes with ice-cold water. Filters were collected into vials and Clear-sol II (Nacalai tesque) was added. Radioactivity was measured using liquid scintillation spectrometry. Non-specific binding was determined by the amount of [^{35}S]GTPγS bound in the presence of 300 μM GTP and subtracted from all samples. Basal [^{35}S]GTPγS binding was defined as the binding when no ligand was added to the assay tube.

Statistical analysis

Data are presented as means ± SEM. Differences between two groups were compared using Student's *t*-test. Data with more than two groups were compared by one-way analysis of variance (ANOVA). The time-course data were analysed by one-way ANOVA for repeated measures. *Post hoc* comparisons were made by Bonferroni correction. Differences of $P < 0.05$ were considered statistically significant.

Materials

All SSRIs were purchased from Sigma-Aldrich. Citalopram and fluoxetine were dissolved in phosphate-buffered saline (PBS), paroxetine was dissolved in dimethyl sulfoxide (DMSO), and stock solutions were stored at –20°C until use. On the day of treatment, stock solutions were thawed and diluted in PBS or DMSO to the concentration of 100 times the final concentration. The 100X solutions were further diluted in 0.7 mL of buffer or culture medium. Final concentration of DMSO in vehicle for paroxetine was 1%, which had no effect on the 5-HT release and tissues in the slice cultures. Tetrodotoxin (TTX) was purchased from Wako Pure Chemical Industries (Osaka, Japan). [^{35}S]GTPγS (1250 Ci/mmol) was purchased from PerkinElmer (Waltham, MA, USA). Antibodies against SERT and actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies against TPH, and Na^+/K^+-ATPase were purchased from Millipore, Sigma-Aldrich and Abcam (Cambridge, MA, USA), respectively. All other chemicals, unless otherwise noted, were purchased from Sigma-Aldrich. Receptor and drug nomenclature follows Alexander *et al.* (2009).

Results

Effects of acute treatment and sustained exposure to SSRIs on extracellular 5-HT levels

SSRIs are known to induce acute increases of extracellular 5-HT level *in vivo* (Beyer and Cremers, 2008). First, we examined the effect of acute treatment with SSRIs on extracellular 5-HT levels in raphe slice cultures. Treatment with citalopram, paroxetine or fluoxetine (0.1, 1, 10 μM) caused a slight increase in extracellular 5-HT levels (Figure 1A–C). Paroxetine and fluoxetine had a tendency to increase 5-HT levels at concentrations greater than 1 μM. One-way ANOVA showed that the effects of acute treatment with paroxetine and fluoxetine were significant ($F_{3,21} = 3.567$, $P < 0.05$ and $F_{3,22} = 3.135$, $P < 0.05$, respectively). Citalopram also had a tendency to increase 5-HT level, although this effect was not significant ($F_{3,21} = 2.483$, $P = 0.089$).

Next, we examined the effects of sustained exposure to SSRIs on extracellular 5-HT level (Figure 1D–F). Slice cultures were exposed to SSRIs for 4 days in culture medium (sustained exposure), and then the slices were treated with SSRIs in KRH for 30 min on Day 4 (treatment on Day 4). The cultures exposed to vehicle (PBS for citalopram and fluoxetine; 1% DMSO for paroxetine) displayed low extracellular 5-HT levels after treatment for 30 min on Day 4 with citalopram, paroxetine or fluoxetine (10 μM), similar to those observed by acute treatment with respective SSRIs (see Figure 1A–C). In the cultures which had sustained exposure to citalopram, paroxetine or fluoxetine (0.1, 1, 10 μM) for 4 days, extracellular 5-HT levels were further increased after treatment with the respective SSRIs (10 μM) for 30 min in comparison to the levels measured in cultures exposed to vehicle. One-way ANOVA showed that the increases in extracellular 5-HT levels induced by sustained exposure to SSRIs were statistically significant (citalopram: $F_{3,19} = 8.773$, $P < 0.001$; paroxetine: $F_{3,20} = 9.213$, $P < 0.001$; fluoxetine: $F_{3,13} = 6.135$, $P < 0.01$). Citalopram and paroxetine displayed a bell-shaped curve of concentration dependence, with subsequent increases following sustained exposure to citalopram and paroxetine at a concentration of 10 μM being lower than those induced at a concentration of 1 μM. Compared with vehicle-exposed cultures, cultures following sustained exposure to citalopram (1 μM), paroxetine (1 μM) or fluoxetine (10 μM) showed significant increases in 5-HT levels. However, the increase in extracellular 5-HT levels following sustained fluoxetine exposure was lower than that induced by citalopram and paroxetine.

Next, we examined the time course of the increase induced by sustained exposure to

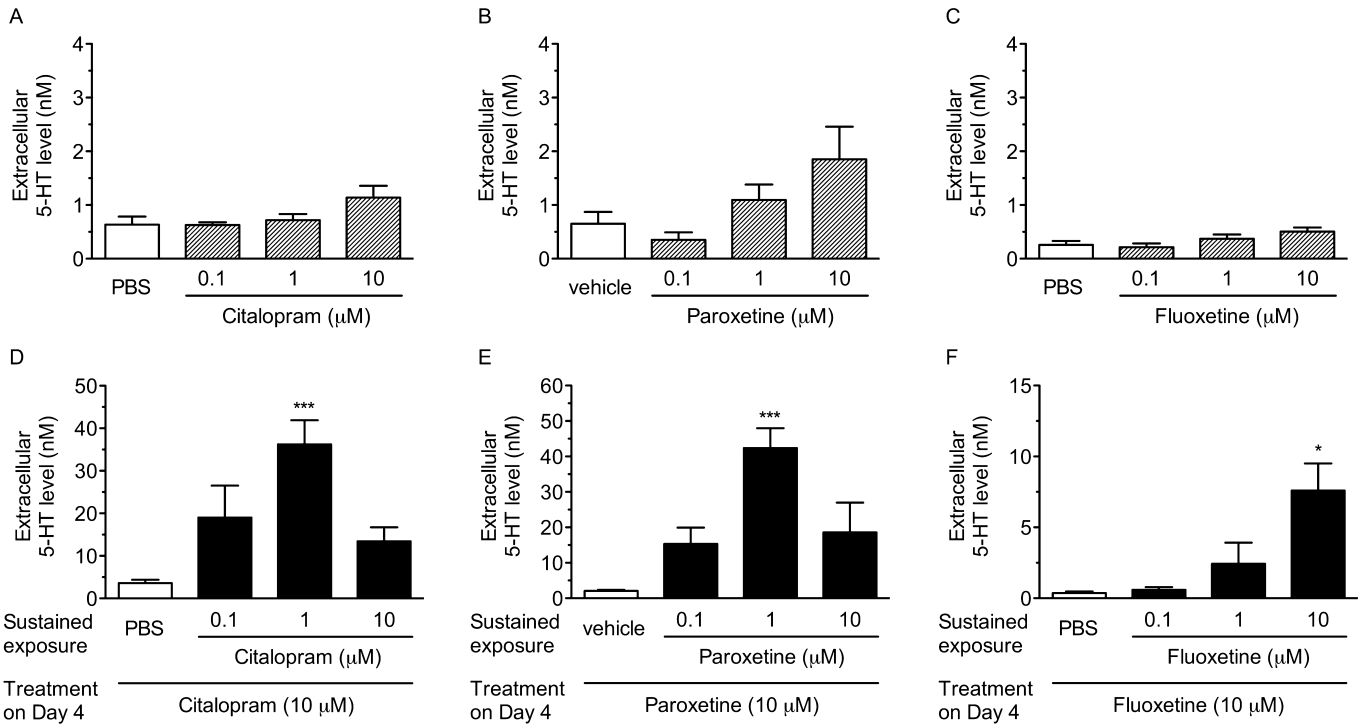

Figure 1

Effects of acute treatment or sustained exposure to citalopram (A, D), paroxetine (B, E) and fluoxetine (C, F) on the extracellular 5-HT levels in raphe slice cultures. (A–C) At Day 0, slice cultures were treated with citalopram, paroxetine, fluoxetine (0.1, 1, 10 μM) or vehicle [phosphate-buffered saline (PBS) for citalopram and fluoxetine; 1% dimethyl sulfoxide for paroxetine] in Krebs–Ringer–Henseleit for 30 min, and extracellular 5-HT levels were then determined. (D–F) Following sustained exposure to selective 5-HT (serotonin) reuptake inhibitor (SSRIs) in culture medium for 4 days, slice cultures were treated with 10 μM of the corresponding SSRI for 30 min on Day 4, and extracellular 5-HT levels determined. Values represent the means of the 5-HT concentration ± SEM. *$P < 0.05$, ***$P < 0.001$ versus vehicle-exposed cultures. $n = 3$–8.

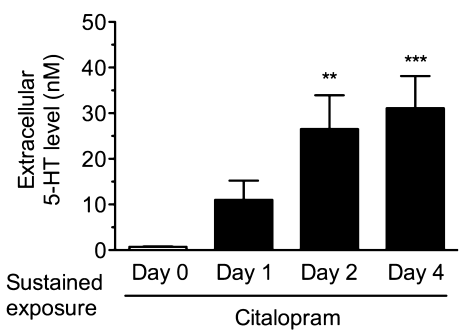

Figure 2

Time course of the increase in extracellular 5-HT levels following sustained exposure to citalopram. Slice cultures were exposed to citalopram (1 μM) for 4 days. Prior to sustained exposure (Day 0), 1 (Day 1), 2 (Day 2) and 4 (Day 4) days after the initiation of sustained exposure, slice cultures were treated with citalopram (1 μM) for 30 min, and then the extracellular 5-HT levels were determined. Values represent the means of the 5-HT concentration ± SEM. **$P < 0.01$, ***$P < 0.001$ versus Day 0. $n = 7$.

citalopram, one of the most selective SSRIs (Figure 2). Consistent with the results of acute treatment, immediately prior to drug exposure (Day 0), treatment with citalopram (1 μM) for 30 min did not increase extracellular 5-HT levels. Subsequently, sustained exposure to citalopram for 1, 2 or 4 days steadily and significantly increased extracellular 5-HT levels after treatment with citalopram (1 μM) for 30 min (one-way repeated measures ANOVA: $F_{3,18} = 10.59$, $P < 0.001$). The significant increases of extracellular 5-HT level were observed on Days 2 and 4, compared with Day 0.

Effect of sustained exposure to citalopram for 4 days on the tissue content of 5-HT and 5-HIAA and TPH expression

Following sustained exposure to 1 μM citalopram for 4 days, the tissue contents of 5-HT and 5-HIAA in slice cultures were measured and found to be unaffected by sustained exposure to citalopram (Figure 3). Furthermore, Western blots revealed that sustained exposure to citalopram for 4 days had no effect on the expression level of TPH, the rate-limiting enzyme of 5-HT synthesis (Figure 3).

Effect of sustained exposure to citalopram on membrane expression of SERT

Because extracellular 5-HT levels are regulated by the activity of SERT on the plasma membrane, we

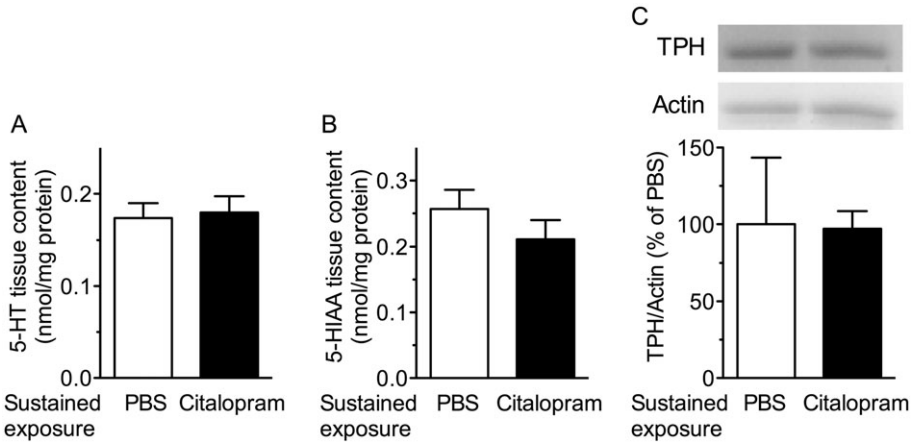

Figure 3

Effects of sustained exposure to citalopram on 5-HT (A) and 5-hydroxyindolacetic acid (5-HIAA) (B) content of slices, and on the expression of tryptophan hydroxylase (TPH) (C). Slice cultures were exposed to citalopram (1 μM) for 4 days. Slices were collected on Day 4 with no subsequent treatment, and 5-HT (A) and its metabolite 5-HIAA (B) contents were measured. Values represent means of 5-HT or 5-HIAA levels (nmol·mg^{-1}·protein) ± SEM. n = 12-13. (C) The expression of TPH protein was determined by Western blot analysis. Actin was used as a loading control. Upper panel shows representative blots of TPH and actin. Lower graph shows densitometric analysis of TPH expression. TPH levels were normalized against actin. The values of citalopram-exposed slices are expressed relative to those of phosphate-buffered saline (PBS)-exposed slices. n = 4–5.

investigated whether sustained citalopram exposure affected the membrane expression of SERT by Western blot. Immunoreactivity for SERT was detected at 70 kDa and results are shown in Figure 4. Sustained exposure to citalopram for 4 days had no effect on the expression of SERT in membrane fractions. However, as a positive control, treatment with a selective 5-hydroxytryptaminergic neurotoxin, 5,7-dihydroxytryptamine (200 μM), for 2 days significantly decreased the expression of SERT in membrane fractions (Supporting Information Figure S1).

Sustained exposure to citalopram induced augmentation of Ca^{2+}- and action potential-dependent exocytotic 5-HT release

We investigated whether Ca^{2+}- and action potential-dependent release of 5-HT was involved in the increased extracellular 5-HT levels induced by sustained citalopram exposure (Figure 5). Following sustained exposure to citalopram (1 μM) for 4 days, slices were treated with citalopram (1 μM) under Ca^{2+}-free conditions or in the presence of 1 μM TTX. In PBS-exposed cultures, the Ca^{2+}-free conditions had no effect on the extracellular 5-HT level. In the citalopram-exposed cultures, Ca^{2+}-free conditions significantly attenuated the increase of extracellular 5-HT levels (Figure 5A). In PBS-exposed cultures, TTX alone tended to inhibit the extracellular 5-HT level, although the difference was not significant. In the citalopram-exposed cultures, co-treatment with TTX significantly attenuated the increase of extracellular 5-HT levels (Figure 5B).

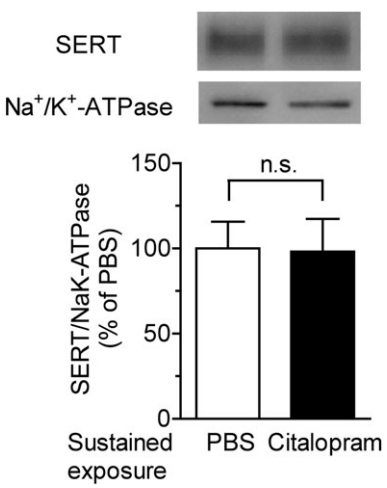

Figure 4

Effects of sustained exposure to citalopram on the membrane expression of 5-HT (serotonin) transporter (SERT). Slice cultures were exposed to citalopram (1 μM) for 4 days. Slices were collected without treatment on Day 4, and homogenized. Membrane fractions of homogenates were prepared as described in Materials and Methods. The expression of SERT in the membrane fraction was determined by Western blot analysis. Na^+/K^+-ATPase was used as a loading control. A representative blot is shown above the graph. SERT levels were normalized against the Na^+/K^+-ATPase signal. The values of citalopram-exposed slices are expressed relative to those of phosphate-buffered saline (PBS)-exposed slices. n.s., not significant; n = 7.

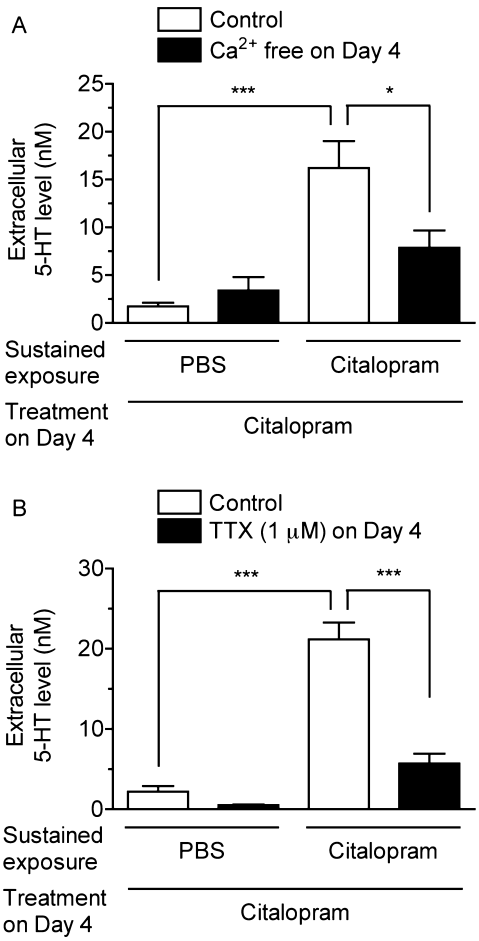

Figure 5

Effects of Ca^{2+}-free external solution and tetrodotoxin (TTX) treatment on the increase in extracellular 5-HT levels. Following sustained exposure to citalopram (1 μM) in culture medium for 4 days, slices were treated with citalopram (1 μM) in Ca^{2+}-free Krebs–Ringer–Henseleit (KRH) (A) or in the presence of TTX (1 μM) in KRH (B) for 30 min on Day 4, and the extracellular 5-HT levels were determined. Values represent the means of 5-HT concentration ± SEM. $^*P < 0.05$, $^{***}P < 0.001$. $n = 7$–11 (A), $n = 4$ (B).

Desensitization of 5-HT$_{1A}$ and 5-HT$_{1B}$ receptors following sustained exposure to citalopram

Several reports have indicated that desensitization of 5-HT autoreceptors (i.e. the 5-HT$_{1A,1B}$ receptors) occurs after long-term administration of SSRIs *in vivo* (Dremencov *et al.*, 2000; Le Poul *et al.*, 2000; Hensler, 2003). In order to examine whether 5-HT$_{1A}$ and 5-HT$_{1B}$ receptors were desensitized in our raphe cultures after sustained citalopram exposure, we performed a [^{35}S]GTPγS binding assay using membrane suspensions obtained from slices following sustained drug exposure. In PBS-exposed cultures, treatment with 8-hydroxy-2-(di-n-propylamino)tetralin (8-OH-DPAT) (10 μM), a 5-HT$_{1A}$ receptor agonist, or CGS12066A (10 μM), a 5-HT$_{1B}$ receptor agonist, was sufficient to induce specific [^{35}S]GTPγS binding. There were no significant differences in specific [^{35}S]GTPγS binding stimulated by 8-OH-DPAT (10 μM) between the sustained PBS- and citalopram-exposed cultures, although there was a tendency for specific binding to decrease in citalopram-exposed cultures (Figure 6A). When the slice cultures were exposed to 8-OH-DPAT (10 μM) for 4 days, 8-OH-DPAT-stimulated specific [^{35}S]GTPγS binding was significantly decreased compared with that in PBS-exposed cultures (Supporting Information Figure S2). However, CGS12066A (10 μM)-stimulated specific [^{35}S]GTPγS binding to the membrane in sustained citalopram cultures was significantly lower than that from the sustained PBS-exposed cultures, which was at a similar level to basal [^{35}S]GTPγS binding (Figure 6B).

The effects of 5-HT$_1$ receptor agonists on the augmented 5-HT release following sustained citalopram exposure were also examined. On Day 4 of sustained citalopram exposure, cultures were treated with citalopram and 8-OH-DPAT (10 μM) for 30 min and showed significant suppression of the augmented 5-HT release (Figure 6C), while the corresponding treatment with CGS12066A (1 μM) had no effect (Figure 6D).

Next, we investigated whether 5-HT$_{1A}$ and 5-HT$_{1B}$ receptors contributed to the development of the effects of sustained exposure to citalopram. For this, we added antagonists of 5-HT$_{1A}$ (WAY100635, 1 μM) or of 5-HT$_{1B}$ receptors (SB224289, 10 μM) to the culture medium along with citalopram for 4 days. The antagonists were also present during the 30 min drug treatment on Day 4. By themselves, the 5-HT$_{1A}$ antagonist or the 5-HT$_{1B}$ antagonist each did not affect 5-HT release in sustained PBS-exposed cultures. Similarly, sustained co-treatment and treatment on Day 4 with either receptor antagonist had no significant effect on the augmented 5-HT release (Figure 7). As shown in Supporting Information Figure S3, the decrease in CGS12066A (10 μM)-stimulated specific [^{35}S]GTPγS binding to the membranes prepared from sustained citalopram-exposed cultures was reversed by co-treatment with 10 μM SB224289. Thus, there was no significant difference in [^{35}S]GTPγS binding between sustained PBS-exposed and citalopram and SB224289-co-treated cultures.

Involvement of glutamate receptors in the augmentation of 5-HT release

We examined the involvement of glutamate receptors in the augmentation of 5-HT release following sustained exposure to citalopram, because there is evidence that the glutamatergic system is involved in the pathophysiology of depression and is responsible for the therapeutic effects of antidepressants

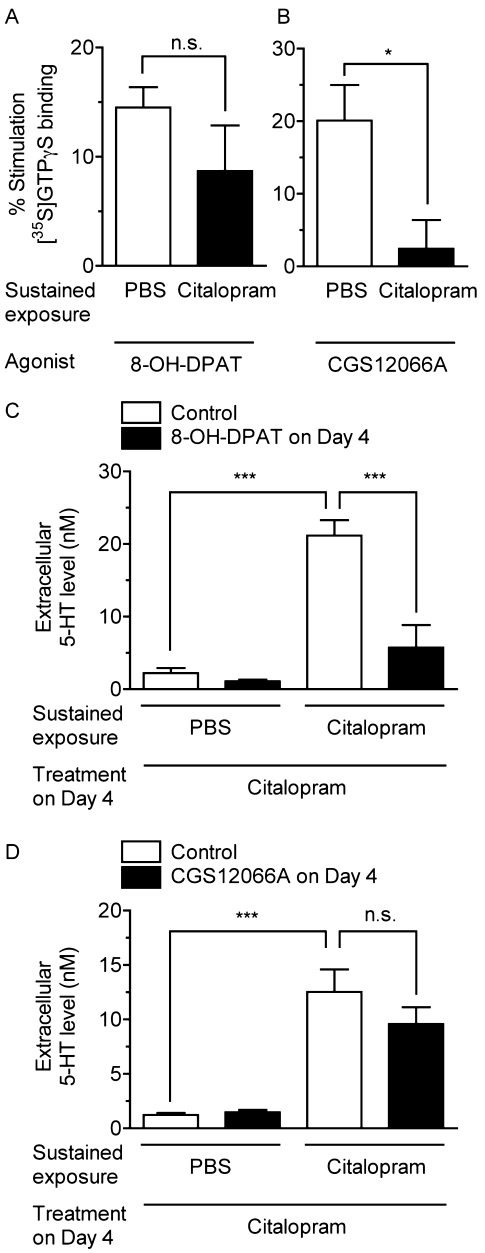

Figure 6

Effects of the 5-HT$_{1A}$ or 5-HT$_{1B}$ receptor agonists on [^{35}S]GTPγS binding (A, B) and on the augmentation of 5-HT release (C, D) following sustained exposure of slice cultures to citalopram. (A, B) Following sustained exposure to citalopram (1 μM) in culture medium for 4 days, slices were collected without citalopram treatment on Day 4 and membrane suspensions were prepared as described in Methods. 8-OH-DPAT-stimulated (A) and CGS12066A-stimulated (B) [^{35}S]GTPγS binding was measured. Values represent the means of percent stimulation of [^{35}S]GTPγS binding normalized to basal binding. n.s., not significant, *P < 0.05. n = 4–5. (C, D) Following sustained exposure to citalopram (1 μM) in culture medium for 4 days, slices were treated with citalopram (1 μM) in the presence of 8-OH-DPAT (10 μM) or CGS12066A (1 μM) in Krebs-Ringer-Henseleit for 30 min on Day 4, and the extracellular 5-HT levels were determined. Values represent the means of the 5-HT concentration ± SEM. n.s., not significant, ***P < 0.001. n = 4–6 (C), n = 5–6 (D).

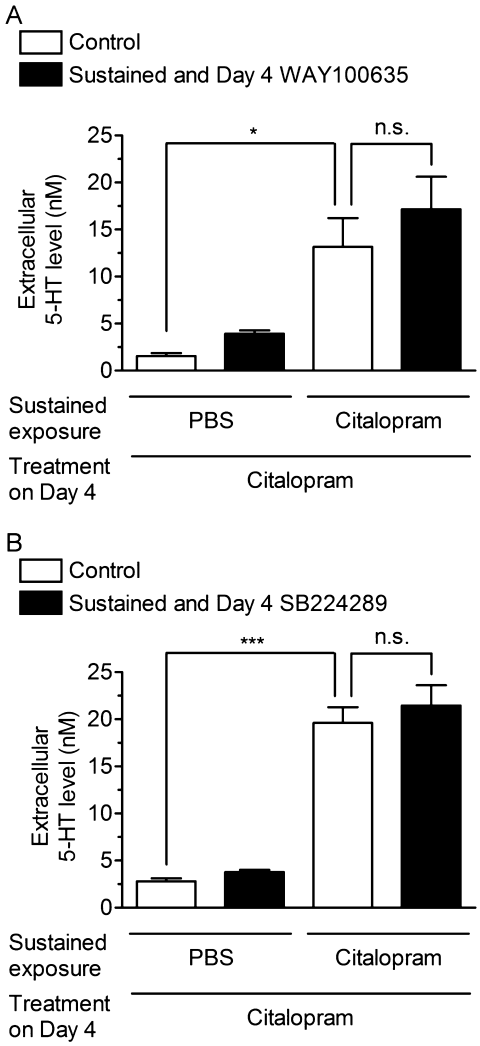

Figure 7

Effects of sustained co-treatment with the 5-HT$_{1A}$ or 5-HT$_{1B}$ antagonist and citalopram on the augmentation of 5-HT release. Following sustained exposure to citalopram (1 μM) with or without WAY100635 (1 μM; A) or SB224289 (10 μM; B) in culture medium for 4 days, slices were treated with citalopram (1 μM) in the presence or absence of WAY100635 (1 μM; A) or SB224289 (10 μM; B) in Krebs-Ringer-Henseleit for 30 min on Day 4, and the extracellular 5-HT levels were determined. Values represent the means of the 5-HT concentration ± SEM. n.s., not significant, *P < 0.05, ***P < 0.001. n = 3.

(Paul and Skolnick, 2003). Sustained PBS-exposed cultures were co-treated with the NMDA receptor antagonist MK-801 (10 μM) or with the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)/kainate receptor antagonist 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) (10 μM) over the course of sustained citalopram exposure and during the 30 min drug treatment on Day 4. The levels of extracellular 5-HT were not significantly altered with these drug treatments. Sustained co-treatment and treatment on Day 4 with MK-801 (10 μM) had no effect on the augmentation of 5-HT

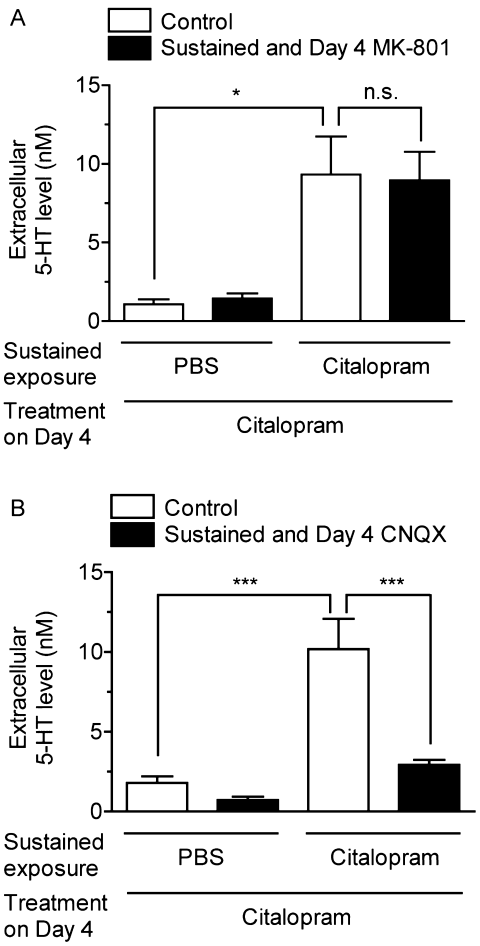

Figure 8

Effects of sustained exposure to glutamate receptor antagonists on the augmentation of 5-HT release. Following sustained exposure to citalopram (1 μM) with or without MK-801 (10 μM) (A) or 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) (10 μM) (B) in culture medium for 4 days, slices were treated with citalopram (1 μM) in the presence or absence of MK-801 (10 μM) or CNQX (10 μM), respectively, in Krebs-Ringer-Henseleit for 30 min on Day 4, and extracellular 5-HT levels were determined. Values represent the means of the 5-HT concentration ± SEM. n.s., not significant, $*P < 0.05$, $***P < 0.001$. $n = 4–5$ (A), $n = 6$ (B).

release following sustained citalopram exposure (Figure 8A). However, sustained co-treatment and treatment on Day 4 with CNQX (10 μM) significantly suppressed the augmentation of 5-HT release, the levels of which were comparable to the levels following sustained PBS exposure (Figure 8B).

Discussion and conclusions

The effects of antidepressant drugs are often assayed *in vivo* using the forced swimming test, the tail suspension test and other tests in which the effect of antidepressants can be observed, even with acute drug administration. Because few conventional *in vivo* methods allow assessment of the time dependency of antidepressants, an assay system in which the delayed onset of antidepressant effects can be observed would be advantageous. The organotypic slice cultures retain neural and synaptic functions which enable analysis over a relatively long time and may offer advantages in analysis of the neural and molecular mechanisms underlying antidepressant efficacy of chronic treatment with SSRIs. As previously described (Higuchi *et al.*, 2008; Nagayasu *et al.*, 2010), we have established an organotypic slice culture, using tissue from the raphe nuclei. These raphe slice cultures exhibit 5-HT biosynthesis and contain 5-HT and its metabolite 5-HIAA, at approximately the same or slightly higher levels than those previously reported in the raphe nucleus of adult rats (Cransac *et al.*, 1996). When acute raphe slice preparations were perfused with standard artificial cerebrospinal fluid, 5-HT synthesis was immediately decreased and 5-HT_{1A} autoreceptor-mediated regulation of 5-HTergic neuronal firing rate was diminished; both these defects were corrected by adding the 5-HT precursor, tryptophan, to the slices (Liu *et al.*, 2005; Evans *et al.*, 2008). However, because our raphe slice cultures were maintained for 14–16 days in culture medium containing tryptophan ($10\ mg{\cdot}mL^{-1}$), the tissue contents of 5-HT and 5-HIAA were maintained. Furthermore, we have shown that the slice cultures have functional 5-HTergic neurons with the ability to release 5-HT in response to stimulation, and are able to reuptake 5-HT through SERT (Higuchi *et al.*, 2008; Nagayasu *et al.*, 2010). Taken together, these findings suggest that the raphe slice cultures are suitable for the analysis of the mechanisms underlying 5-HTergic neural plasticity following sustained exposure to SSRIs *in vitro*. However, we should mention that the raphe slice cultures were prepared from neonatal rats at postnatal days 2–3, in which 5-hydroxytryptaminergic neurons are still immature and developing (Moll *et al.*, 2000; Petrunich *et al.*, 2008).

We previously reported that acute treatment with MDMA and methamphetamine induced a large increase in 5-HT release in the same slice culture system (Higuchi *et al.*, 2008; Nagayasu *et al.*, 2010). Unlike SSRIs, MDMA and methamphetamine are substrate-type releasers, which are transported into the nerve terminals and cause rapid 5-HT release via a reverse transport-mediated mechanism (Howell and Kimmel, 2008). In this study, acute treatment with the SSRIs induced a slight increase in extracellular 5-HT levels, although the effect of citalopram was not significant, as we previously reported (Higuchi *et al.*, 2008). The small increase in

5-HT levels induced by acute SSRI treatment is likely to be due to the inhibition of 5-HT reuptake by the blockade of SERT. Consistent with these results, many *in vivo* microdialysis studies have revealed that acute treatment with SSRIs elicited an increase in extracellular 5-HT levels in the raphe nuclei (Bel and Artigas, 1992; Invernizzi *et al.*, 1992; Fuller, 1994). These findings may also indicate weak spontaneous activity of raphe 5-HTergic neurons in slice cultures under normal conditions. The weak spontaneous activity may be due to the lack of tonic noradrenergic input from the locus coeruleus that is essential for normal firing rate of raphe 5-hydroxytryptaminergic neurons observed *in vivo* (Vandermaelen and Aghajanian, 1983). However, our preliminary data showed that acute treatment with the α_1-adrenoceptor agonist phenylephrine (3 μM) increased 5-HT release in the presence of citalopram, suggesting that the raphe 5-HTergic neurons in the slice cultures retain responsiveness to noradrenergic inputs. However, it is noted that all experiments in the present study were performed under the conditions in which raphe 5-HTergic neurons were not firing at physiological rates, because of the absence of tonic noradrenergic input.

The major finding of the present study is that sustained exposure of slice cultures to SSRI facilitated 5-HT release. The augmentation of 5-HT release required sustained exposure for several days. Consistent with these results, several *in vivo* microdialysis studies have shown that chronic treatment with SSRIs induced augmentation of 5-HT release in the frontal cortex (Bel and Artigas, 1993; Rutter *et al.*, 1994; Invernizzi *et al.*, 1996). However, to our knowledge, this is the first demonstration that sustained SSRI exposure can induce delayed augmentation of 5-HT release *in vitro*, although it cannot fully mimic the time scale of the delayed clinical therapeutic efficacy of antidepressants in humans (2–6 weeks).

The concentration-dependent responses of citalopram and paroxetine were bell-shaped, and the maximum effect was observed at a concentration of 1 μM. Possible explanations of these observations include the autoregulation of 5-hydroxytryptaminergic neurons by activation of 5-HT_1 autoreceptors, depletion of intravesicular 5-HT by excess 5-HT release or non-specific toxic effects of the highest concentration of SSRIs (10 μM). The augmentation of 5-HT release induced by fluoxetine was concentration dependent, but weaker in magnitude than those caused by citalopram or paroxetine exposure. These results are consistent with the lower affinity and efficacy of fluoxetine for SERT than citalopram and paroxetine (Owens *et al.*, 1997; Mantovani *et al.*, 2009). We observed that sustained exposure to 100 μM fluoxetine, as well as citalopram and paroxetine, induced lower augmentation of 5-HT release than that of 10 μM fluoxetine (Supporting Information Figure S4).

It has been reported that chronic treatment with SSRIs causes up-regulation of TPH expression and 5-HT synthesis *in vivo* and *in vitro* (Kim *et al.*, 2002), which may be involved in long-term alterations in 5-HTergic transmission, although this is still a matter of debate (Spurlock *et al.*, 1994; Abumaria *et al.*, 2007). However, the augmentation of 5-HT release is unlikely to be due to increases in 5-HT and 5-HIAA tissue contents and TPH expression in raphe slice cultures, because sustained citalopram exposure did not affect these characteristics. Extracellular 5-HT levels are regulated by SERT, which is the primary initial target for SSRIs. Although many studies have investigated the effects of chronic treatment with SSRIs and other antidepressants on SERT expression and ligand-binding activity, these results were controversial, and have variously shown an increase (Hrdina and Vu, 1993; López *et al.*, 1994), decrease (Lesch *et al.*, 1993; Benmansour *et al.*, 1999) or no change (Spurlock *et al.*, 1994). Furthermore, a recent report suggested that citalopram induces SERT internalization in cultured cells (Lau *et al.*, 2008). However, in the present study, SERT membrane expression was not affected by sustained citalopram exposure, suggesting that the augmentation of 5-HT release was unlikely to be due to the changes in the 5-HT uptake system.

Neurotransmitters including 5-HT are released from the presynaptic compartment to the synaptic cleft via Ca^{2+}- and action potential-dependent exocytosis. Our results using Ca^{2+}-free conditions and TTX treatment indicate that the augmentation of 5-HT release following sustained citalopram exposure is mediated through increased Ca^{2+}- and action potential-dependent exocytosis. These results suggest that sustained exposure to SSRIs increases extracellular 5-HT levels not only by blockade of SERT, but also by facilitation of depolarization-dependent exocytotic 5-HT release. Consistent with our results, Chaput *et al.* (1986) reported that chronic administration of citalopram increased the reduced firing rate of dorsal raphe 5-hydroxytryptaminergic neurons by acute administration of citalopram.

Several lines of evidence suggest that long-term administration of SSRIs induces down-regulation and/or desensitization of 5-HT_1 autoreceptors, which leads to the disinhibition of 5-HTergic neurons and thus increases 5-HT release (Blier and de Montigny, 1983; Invernizzi *et al.*, 1994; 1996;

Rutter *et al.*, 1994; Le Poul *et al.*, 1995; Dremencov *et al.*, 2000; Hensler, 2002; Ceglia *et al.*, 2004; Newman *et al.*, 2004). However, there are conflicting reports that the 5-HT_{1A} and 5-HT_{1B} autoreceptors are not down-regulated or desensitized and still remain functional even after long-term administration of SSRIs (Hjorth and Auerbach, 1994; 1999; Bosker *et al.*, 1995; Rueter *et al.*, 1998; Rossi *et al.*, 2006). In the present study, [^{35}S]GTPγS binding assays using raphe slice cultures revealed that sustained citalopram exposure induced desensitization of 5-HT_{1B} receptors, while it had no effect on 5-HT_{1A} receptors. These findings are further supported by experiments using 5-HT_1 receptor agonists, demonstrating that the 5-HT_{1A} receptor agonist was sufficient to inhibit the augmented 5-HT release following sustained citalopram exposure, while the 5-HT_{1B} receptor agonist was not able to inhibit this increase. However, although sustained co-treatment with the 5-HT_{1B} receptor antagonist and citalopram prevented the desensitization of 5-HT_{1B} receptors, it had no effect on the augmentation of 5-HT release, together suggesting that the desensitization of 5-HT_{1B} receptors is unlikely to contribute to the induction of augmentation of 5-HT release. Consistent with our study, Gardier's study with 5-HT_{1B} receptor knockout mice showed that the increase of extracellular 5-HT levels was induced by long-term administration of SSRIs in spite of the absence of 5-HT_{1B} receptors (Gardier *et al.*, 2003). Taken together, these results suggest that the autoregulatory function of 5-HT_{1A} receptors persisted, even following sustained exposure to citalopram, while hypofunction of 5-HT_{1B} autoreceptors was not involved in the augmentation of 5-HT release, at least in our raphe slice cultures. However, the present results do not contradict the current theory for the down-regulation and/or desensitization of 5-HT_1 autoreceptors, which is supported by many *in vivo* and electrophysiological studies. For example, the firing activity of the dorsal raphe 5-HTergic neurons is also regulated by postsynaptic 5-HT_{1A} receptors located in the medial prefrontal cortex (Hajós *et al.*, 1999). Our raphe slice culture system was not able to examine the involvement of such postsynaptic feedback from the 5-hydroxytryptaminergic projecting areas. Furthermore, recent studies have shown that 5-HT_{2C} receptors, which are $G_{q/11}$-coupled to phospholipase C activation and Ca^{2+} mobilization, are involved in autoregulation of 5-HTergic neurons in response to acute SSRIs (Sotty *et al.*, 2009). In the dorsal and medial raphe nuclei, 5-HT_{2C} receptors are located on GABAergic interneurons, but not on 5-HTergic neurons (Serrats *et al.*, 2005). Whether other 5-HT autoreceptors including 5-HT_{2C} receptors could be involved in the augmentation of 5-HT release following sustained SSRI exposure would need further investigation.

Many reports indicate that the glutamatergic system is involved in bringing about the therapeutic effects of antidepressants (Trullas and Skolnick, 1990; Martinez-Turrillas *et al.*, 2002). In the raphe, 5-HTergic neurons receive glutamatergic innervation (Groenewegen and Uylings, 2000; Roche *et al.*, 2003), and the local application of glutamate, AMPA or kainate increases the firing activity of 5-HTergic neurons and subsequent 5-HT release in the areas to which these neurons project, via AMPA/kainate receptors (Tao *et al.*, 1997; Celada *et al.*, 2001; Harsing, 2006; Gartside *et al.*, 2007). Indeed, we have found that acute treatment with AMPA or kainate caused 5-HT release in our raphe slice cultures (Nagayasu *et al.*, unpubl. data). In the present study, activation of AMPA/kainate receptors appeared to contribute to the augmentation of 5-HT release following sustained citalopram exposure. Several lines of evidence have indicated that AMPA/kainate potentiators have an antidepressant-like effect *in vivo* (Li *et al.*, 2001; Maeng *et al.*, 2008; Machado-Vieira *et al.*, 2009) and these would be compatible with our findings that AMPA/kainate receptors are involved in the long-term effect of SSRIs. It has been shown that chronic treatment with antidepressants including SSRIs increases phosphorylation of AMPA receptor subunit GluR1 at Ser-831 and Ser-845 (Svenningsson *et al.*, 2002; Du *et al.*, 2007) and trafficking of GluR1 to synaptic sites (Martinez-Turrillas *et al.*, 2002), which positively regulate the function of GluR1 channels. Potentiation of AMPA receptor function by phosphorylation, insertion onto the neuronal surface and movement into synapses following sustained SSRI exposure may be involved in the augmentation of 5-HT release. Furthermore, the present results suggest that raphe slice cultures contain a neural network constituting the link between the 5-HTergic and glutamatergic systems, which might *trans*-synaptically contribute to the augmentation of 5-HT release. On the other hand, the NMDA receptor antagonist MK-801 failed to attenuate the augmentation of 5-HT release, suggesting that NMDA receptors were not required for this effect.

In summary, the present study using the raphe slice cultures showed that sustained exposure to SSRIs caused augmentation of 5-HT release without affecting 5-HT tissue content, TPH expression or the 5-HT uptake system. The augmentation of 5-HT release is caused by facilitated 5-HTergic activity mediated through AMPA/kainate receptor activation, but is independent of disinhibition of 5-HTergic neurons by 5-$HT_{1A/B}$ autoreceptor hypofunction, at least, in these slice cultures. To our

knowledge, few culture systems have functional 5-HTergic neurons and other neuronal networks and are also available for long-term analysis *in vitro*. Therefore, our raphe slice culture system may be used as a new *in vitro* model for investigating the neural and molecular mechanisms underlying the antidepressant effects of SSRIs and other antidepressants and suggesting whether and how the glutamate/AMPA receptors/Ca^{2+}-signalling pathway can be a target for novel antidepressants. To elucidate them, further investigations will be needed in our raphe slice culture system in combination with electrophysiological techniques and selective gene expression system in 5-HTergic neurons (Benzekhroufa *et al.*, 2009).

Acknowledgements

This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan and from the Japan Society for the Promotion of Science.

Conflicts of interest

None.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Figure S1 Effects of a selective serotonergic neurotoxin, 5,7-DHT, on the SERT expression in the membrane fraction. Slice cultures were treated with 5,7-DHT (200 μM) for 2 days. Then, the slices were collected and membrane fractions were prepared as described in Materials and Methods. The expression of SERT in the membrane fraction was determined by Western blot analysis. Na^+/K^+-ATPase was used as a loading control. Upper panel shows representative blots of SERT and Na^+/K^+-ATPase. Lower graph shows densitometric analysis of SERT expression. SERT level was normalized against Na^+/K^+-ATPase. $n = 5$.

Figure S2 Effects of sustained exposure to a 5-HT_{1A} receptor agonist, 8-OH-DPAT, on the 8-OH-DPAT-stimulated specific [^{35}S]GTP©S binding. Following sustained exposure to 8-OH-DPAT (10 μM) in culture medium for 4 days, slices were collected and membrane suspensions were prepared as described in Materials and Methods. [^{35}S]GTPγS binding stimulated by 8-OH-DPAT (10 μM) was quantified. Values represent the means of percent stimulation of [^{35}S]GTPγS binding normalized to basal binding. $n = 3$.

Figure S3 Effects of co-treatment with citalopram and a 5-HT_{1B} receptor agonist, SB224289, on the CGS12066A-stimulated specific [^{35}S]GTPγS binding. Following sustained exposure to vehicle or citalopram (1 μM) with SB224289 (10 μM) in culture medium for 4 days, slices were collected, and membrane suspensions were prepared. CGS12066A-stimulated [^{35}S]GTPγS binding was quantified. Values represent the means of percent stimulation of [^{35}S]GTPγS binding normalized to basal binding. $n = 4$–7. $^*P < 0.05$.

Figure S4 Effects of sustained exposure to higher concentrations of fluoxetine on the extracellular 5-HT levels in raphe slice cultures. Following sustained exposure to fluoxetine (10 and 100 μM) in culture medium for 4 days, slice cultures were treated with 10 and 100 μM fluoxetine for 30 min on Day 4, respectively, and extracellular 5-HT levels determined. Values represent the means of the 5-HT concentration ± S.E.M. $n = 3$.

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