Chronic effects of antidepressants on serotonin release in rat raphe slice cultures: high potency of milnacipran in the augmentation of serotonin release

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Abstract
Most clinically-used antidepressants acutely increase monoamine levels in synaptic clefts, while their therapeutic effects often require several weeks of administration. Slow neuroadaptive changes in serotonergic neurons are considered to underlie this delayed onset of beneficial actions. Recently, we reported that sustained exposure of rat organotypic raphe slice cultures containing abundant serotonergic neurons to selective serotonin (5-HT) reuptake inhibitors (citalopram, fluoxetine and paroxetine) caused the augmentation of exocytotic serotonin release. However, the ability of other classes of antidepressants to evoke a similar outcome has not been clarified. In this study, we investigated the sustained actions of two tricyclic antidepressants (imipramine and desipramine), one tetracyclic antidepressant (mianserin), three 5-HT and noradrenaline reuptake inhibitors (milnacipran, duloxetine and venlafaxine) and one noradrenergic and specific serotonergic antidepressant (mirtazapine) on serotonin release in the slice cultures. For seven of nine antidepressants, sustained exposure to the agents at concentrations of 0.1–100 μM augmented the level of increase in extracellular serotonin. The rank order of their potency was as follows: milnacipran>duloxetine>citalopram>venlafaxine>imipramine>fluoxetine>desipramine. Neither mirtazapine nor mianserin caused any augmentation. The highest augmentation by sustained exposure to milnacipran was partially attenuated by an α1-adrenoceptor antagonist, benoxathian, while the duloxetine-, venlafaxine- and citalopram-mediated increases were not affected. These results suggest that inhibition of the 5-HT transporter is required for the enhancement of serotonin release. Furthermore, the potent augmentation by milnacipran is apparently due to the accompanied activation of the α1-adrenoceptor.

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Introduction
Major depressive disorder (MDD) is a mood disorder characterized by sadness, hopelessness and suicidal thoughts, and its lifetime incidence is about 10–20% (Belmaker and Agam, 2008; Kessler et al., 2003). Because most currently employed antidepressants increase monoamine (i.e. serotonin (5-HT), noradrenaline (NE) and/or dopamine) levels in synaptic clefts by inhibiting 5-HT and NE reuptake (Bel and Artigas, 1996, 1999; Jordan et al., 1994), dysfunction of the monoaminergic system is considered to be associated with the pathophysiology of depression (Schildkraut, 1995; Slattery et al., 2004).

Among antidepressants, the selective 5-HT reuptake inhibitors (SSRIs) and the 5-HT and NE reuptake inhibitors (SNRIs) are widely prescribed all over the world (Kennedy et al., 2011). However, tricyclic antidepressants (TCAs), tetracyclic antidepressants (TeCAs), and noradrenergic and specific serotonergic antidepressants (NaSSAs) are also frequently used in the treatment of MDD (Gorman, 1999; Millan, 2006; Sartorius et al., 2007). Even though a single injection of these
antidepressants can increase extracellular monoamine levels, several weeks of administration are usually required for the drugs to exert their maximal therapeutic effects (Mendels et al., 1999; Millan, 2006; Sartorius et al., 2007). The delayed onset of remedial action is thought to be due to slow neuroadaptive changes in the monoaminergic system, although the underlying mechanism is unclear.

Lines of research on antidepressants have been conducted by using in vitro techniques, such as behavioural experiments, microdialysis and electrophysiological analysis. However, in vitro techniques are also necessary to investigate the detailed mechanisms of antidepressant actions. For example, several in vitro studies have revealed the acute and chronic effects of antidepressants on cell lines and dissociated primary neurons and astrocytes (Iceta et al., 2007; Ohno et al., 2007; Pákáski et al., 2005). However, to the best of our knowledge, there are few in vitro techniques that can evaluate the neuroadaptive changes involved in 5-HT release from raphe serotonergic neurons after sustained exposure to antidepressants. Furthermore, few reports have compared the chronic effects of a series of antidepressants on extracellular 5-HT levels.

Recently, we reported that a rat organotypic slice culture containing the raphe nuclei is a useful tool for investigating the function of serotonergic neurons (i.e. 5-HT release). Moreover, sustained exposure (4 d) of this slice culture to SSRIs (citalopram, paroxetine or fluoxetine) caused the augmentation of 5-HT release. In addition, we showed that this augmentation is not due to the desensitization of 5-HT1A/1B autoreceptors, but rather to an activation of alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA)-type glutamate receptors (Nagayasu et al., 2010a, b). However, it has not yet been clarified whether other classes of antidepressants can mimic this effect. In the present study, therefore, we investigated the actions of several classes of antidepressants such as TCAs, TeCAs, SNRIs and NaSSA on the function of serotonergic neurons in rat raphe slice cultures. Here, we show that sustained exposure to antidepressants that inhibit the serotonin transporter (SERT) causes the augmentation of 5-HT release. Among these antidepressants, milnacipran, an SNRI, was found to have the highest potency. Finally, we investigated the role of α1-adrenoceptors in the sustained effect of SNRIs.

**Materials and methods**

**Materials**

Citalopram was purchased from LKT Laboratories, Inc. (USA). Benoxathian, desipramine, fluoxetine, imipramine, mianserin, milnacipran, mirtazapine, phenylephrine, prazosin and venlafaxine were purchased from Sigma-Aldrich (USA). Duloxetine was purchased from Santa Cruz Biotechnology, Inc. (USA). Tetrodotoxin (TTX) was purchased from Wako Pure Chemical Industries (Japan). Drugs were dissolved in phosphate-buffered saline (PBS), saline, water, or dimethyl sulfoxide (DMSO), and then stock solutions were stored at −20 °C until use. On the day of treatment, stock solutions were thawed and diluted in PBS, saline, water, or DMSO to the concentration of 100× the final concentration. Next, the 100× solutions (7 μl) were added to 0.7 ml buffer or culture medium.

NE and 5-HT were purchased from Nacalai Tesque (Japan). Oligodeoxynucleotide primers were purchased from Thermo Fisher Scientific (GeneRuler 100 bp DNA ladder; Waltham, USA). [3H]prazosin was purchased from Perkin-Elmer (3.156 TBq/mmol; Waltham, USA).

**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. Rat raphe serotonergic slice cultures were prepared as previously described (Higuchi et al., 2008; Nagayasu et al., 2010a, b). Briefly, Wistar/ST rats at postnatal day 2–3 (Nihon SLC, Japan) were anesthetized by hypothermia and decapitated, and the brain was isolated. Coronal sections (350 μM thickness) containing dorsal raphe nuclei, median raphe nuclei, part of locus coeruleus and the A5 NE cells were prepared by using a tissue chopper (Narishige, Japan), in accordance with a published brain atlas (refer to P0-53-60; Paxinos et al., 1991). Four slices were placed on each 30 mm Millicell-CM insert (pore size 0.4 μm; Millipore, USA), and the inserts were transferred into a six-well culture plate. Slice cultures were maintained at the liquid/air interface for 14–16 d in an incubator at 37 °C and an atmosphere of 5% CO2 and were subsequently used in experiments.

**Measurement of extracellular 5-HT levels and tissue 5-HT content**

Measurements of extracellular 5-HT levels and tissue 5-HT content were performed as previously described (Higuchi et al., 2008; Nagayasu et al., 2010a, b). 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 MgCl2, 1.2 mM CaCl2, 10 mM D-glucose, 15 mM HEPES, 5 mM HEPES-Na, 0.2 mM ascorbic acid; pH 7.4) for 15 min. They were then transferred into 0.7 ml KRH buffer containing drugs and incubated for 30 min. After incubation, the conditioned KRH buffer was collected, and 1 M acetic acid (50 μl) was immediately added to protect 5-HT from degradation. 5-HT (in 25 μl samples) was immediately analysed by high performance liquid chromatography with an electrochemical detector (HPLC-ECD) (Eicom, Japan).

For measurement of tissue 5-HT content, slices were collected, homogenized, and sonicated in 100 μl ice-cold 0.1 M HClO4 containing 10 mM Na2S2O5 and 1 mM ethylenediaminetetraacetic acid (EDTA). The sonicated homogenate was placed on ice for 15 min. Protein concentrations were measured by using the Bradford protein assay (Bio-Rad, USA). Homogenates were centrifuged at 18000 g for 15 min at 4°C, and supernatants were analysed with HPLC-ECD. The measured 5-HT concentration was normalized against the total protein concentration for samples collected from each culture insert (4 slices/insert). The detection limits of the HPLC system used in the present study for 5-HT and NE were 0.5 fmol/25 μl and 2.5 fmol/25 μl, respectively.

**Sustained drug exposure protocol**

Slice cultures were incubated with culture medium containing drugs for 4 d. Immediately prior to drug exposure (day 0), extracellular 5-HT levels were measured by incubating the cultures in KRH buffer containing drugs for 30 min, following a 15 min pre-incubation in KRH alone. For sustained exposure, slices were maintained with culture medium containing drugs for 4 d, whereupon slice cultures were pre-incubated in KRH buffer for 15 min and treated with the drugs for 30 min, followed by measurement of extracellular 5-HT levels.

[^3H]Prazosin binding assay

For the [^3H]prazosin binding assay, slice cultures that were exposed to drugs for 4 d were collected in isotonc buffer and homogenized. Homogenates were centrifuged at 600 g for 10 min at 4°C, and the supernatants were centrifuged at 40000 g for 20 min. The resulting pellets were resuspended in reaction buffer (50 mM Tris–HCl, 10 mM MgCl2; pH 7.4) and stored at -80°C until use. Membrane suspensions from slice cultures (25 μg protein) and [^3H]prazosin dissolved in reaction buffer (0.003–3 nM final concentration, 3.156 TBq/mmol) were mixed and incubated for 60 min in a shaking water bath at 37°C. Reactions were terminated by rapid filtration through glass fibre filters (GF/C, Whatman, UK), followed by 10 washes with reaction buffer. Radioactivity was measured by liquid scintillation counting. Non-specific binding was defined as binding in the presence of 30 μM cold prazosin. The data were analysed by non-linear regression using Prism version 5.0 (GraphPad Software, USA) to determine the maximum number of binding sites (B_{max}).

**Statistical analysis**

Data are presented as the means±S.E.M. Differences between two groups were compared by Student’s t-test. Data with more than two groups were compared by the one-way analysis of variance (ANOVA). The time-course data were analysed by two-way ANOVA for repeated measures. Unless otherwise noted, post-hoc comparisons were performed by Bonferroni correction. Differences of p<0.05 were considered statistically significant.

**Results**

**Effects of acute treatment with and sustained exposure to antidepressants on extracellular 5-HT levels in raphe slice cultures**

First, we investigated the effects of acute treatment with various classes of antidepressants on extracellular 5-HT levels in raphe slice cultures (Fig. 1). Slice cultures were acutely treated with citalopram, fluoxetine (SSRIs), desipramine, imipramine (TCAs), duloxetine, venlafaxine, milnacipran (SNRIs), mianserin (TeCA), and mirtazapine (NaSSA) at concentrations of 0.1–100 μM for 30 min, and then extracellular 5-HT levels were measured. Acute treatments with fluoxetine (100 μM), desipramine (100 μM), imipramine (10 and 100 μM), duloxetine (100 μM), venlafaxine, (100 μM) and milnacipran (100 μM) significantly increased extracellular 5-HT levels compared with the vehicle-treated group. Acute treatment with citalopram did not significantly affect the extracellular 5-HT levels, although it had a tendency to increase extracellular 5-HT levels. However, acute treatment with mirtazapine or mianserin had no effect. Furthermore, we evaluated the effects of tetrodotoxin (TTX, 1 μM) or removal of calcium on the basal level of extracellular 5-HT in the absence of any SERT inhibitor, and found that extracellular 5-HT levels were markedly decreased by TTX treatment or removal of calcium to below the limit of detection, whereas extracellular 5-HT levels after PBS treatment were 0.189±0.005 nM (Supplementary Fig. 1), suggesting the observed low basal extracellular...
5-HT level is dependent on spontaneous firing of 5-HT neurons.

Following the acute treatments, the slice cultures were exposed to the same antidepressants (0.1–100 μM) in culture medium for 4 d. Next, the slice cultures were challenged with the same antidepressants at the same concentrations for 30 min, and the extracellular 5-HT levels were again measured. As previously reported (Nagayasu et al., 2010b), sustained exposure to citalopram and fluoxetine significantly augmented their increasing effects on the extracellular 5-HT level ($F_{1,11} = 57.6, p < 0.001$ and $F_{1,21} = 5.60, p < 0.05$, respectively: two-way repeated measures ANOVA). Significant increases were observed by sustained exposure to citalopram at concentrations of 1–100 μM and by fluoxetine at concentrations of 1 and 10 μM, compared with acute treatment (Fig. 1a, b). However, sustained exposure to 100 μM fluoxetine significantly decreased the extracellular 5-HT level compared with acute treatment.

Similarly, sustained exposure to desipramine ($F_{1,25} = 35.9, p < 0.001$), imipramine ($F_{1,25} = 9.90, p < 0.01$), duloxetine ($F_{1,26} = 82.8, p < 0.001$), venlafaxine ($F_{1,22} = 68.6, p < 0.001$), and milnacipran ($F_{1,10} = 41.5, p < 0.001$)
serotonin release augmentation by antidepressants

Comparison of augmented increases in extracellular 5-HT level induced by sustained exposure to antidepressants

To compare the potency of antidepressants in the augmented increases in the extracellular 5-HT level, slice cultures were exposed to the antidepressants at their optimal concentrations for 4 d, and then the extracellular 5-HT levels were measured. The optimal concentrations were defined as the concentration that caused the largest increase in the extracellular 5-HT level after a 4 d exposure (see Fig. 1). The optimal concentration of each antidepressant was as follows: citalopram (1 μM), fluoxetine (10 μM), desipramine (10 μM), imipramine (10 μM), duloxetine (10 μM), venlafaxine (100 μM), milnacipran (100 μM), mianserin (0.1 μM), and mirtazapine (10 μM).

Extracellular 5-HT levels were normalized by the average extracellular 5-HT level of citalopram-exposed slice cultures in the same dissection batch to minimize the differences among batches. The rank order of antidepressant potency in increasing the extracellular 5-HT level was as follows: milnacipran > duloxetine > citalopram > imipramine > venlafaxine > fluoxetine > desipramine > mianserin = mirtazapine (Fig. 2). The extracellular 5-HT level after sustained exposure to milnacipran (100 μM) was significantly higher than it was after exposure to citalopram (1 μM).

Effect of sustained exposure to milnacipran on tissue 5-HT content and high-K⁺-evoked increase in extracellular 5-HT level

Next, we investigated the changes in tissue 5-HT content after sustained exposure to milnacipran or citalopram. Following sustained exposure to vehicle, milnacipran (100 μM) or citalopram (1 μM), the slice cultures were collected, and the tissue 5-HT contents were measured. Sustained exposure to milnacipran or citalopram did not alter the tissue 5-HT content (Fig. 3a). Furthermore, we determined whether there is a difference in the quantity of 5-HT released by depolarization in the presence of milnacipran or

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**Fig. 2.** Comparison of the augmentation of serotonin (5-HT) release induced by sustained exposure to various antidepressants. Slice cultures were exposed to antidepressants at their optimal concentrations for 4 d. Following sustained exposure, the slice cultures were challenged with the same antidepressants for 30 min, and the extracellular 5-HT levels were measured. Extracellular 5-HT levels of each sample were normalized against those of the citalopram (1 μM)-exposed group in the same dissection batch. Values represent the means ± S.E.M. **p < 0.01 vs. citalopram group. n = 3.

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significant augmented their increasing effects on the extracellular 5-HT level (two-way ANOVA). Significant increases were observed by desipramine at 1 and 10 μM, by imipramine at 10 μM, by duloxetine at 1–100 μM, by venlafaxine at 10 and 100 μM, and by milnacipran at 10 and 100 μM compared with acute treatment (Fig. 1c–g). The dose-response curves of desipramine, imipramine and duloxetine were all bell-shaped. Sustained exposure to 100 μM desipramine significantly decreased the extracellular 5-HT level compared with acute treatment. There was no significant difference between acute treatment with and sustained exposure to 100 μM imipramine. On the other hand, sustained exposure to mianserin or mirtazapine had no effect on the extracellular 5-HT levels (Fig. 1h, i).
Involvement of the α1-adrenoceptor in the enhanced augmentation of increase in extracellular 5-HT level induced by milnacipran

Because the largest difference between milnacipran and citalopram from the pharmacological point of view is that milnacipran acts on noradrenergic signaling via an inhibition of NE reuptake (Vaishnavi et al., 2004), we hypothesized that changes in noradrenergic signaling are underlying the larger augmentation of increases in extracellular 5-HT level induced by milnacipran vs. citalopram. First, we investigated whether the raphe slice cultures employed herein contain noradrenergic cells. RT-PCR analysis showed that dopamine-β-hydroxylase (DBH) and NET mRNA were present in the slice cultures (Supplementary Fig. 3). Furthermore, HPLC analysis revealed that the tissue content of NE in the slice cultures was 2.20±0.05 pmol/mg protein.

In the dorsal raphe nuclei (DRN), α1-adrenoceptor agonists positively regulate the firing rate of serotonergic neurons in vitro and the extracellular 5-HT level in vivo (Padovkina et al., 2003; Vandermaelen and Aghajanian, 1983). To determine whether the α1-adrenoceptor is functionally associated with 5-HT release in the current culture system, slice cultures were treated with an α1-adrenoceptor agonist, phenylephrine, for 30 min. Citalopram was concurrently added to the cultures to inhibit 5-HT reuptake. Acute treatment with phenylephrine (3 μM) plus citalopram significantly increased the extracellular 5-HT level, compared to citalopram alone-treated group. The acute phenylephrine-induced increase in extracellular 5-HT level was significantly antagonized by co-treatment with a selective α1-adrenoceptor antagonist, benoxathian (10 μM), although benoxathian itself had no effect on the extracellular 5-HT level (Fig. 4a).

Following sustained exposure to citalopram, the slice cultures were challenged with phenylephrine in the presence of citalopram for 30 min. Phenylephrine (0.3 and 3 μM) significantly increased the citalopram-augmented increase in extracellular 5-HT level in a concentration-dependent manner (F2,12=8.85, p<0.01), and the significant increase was observed at 3 μM phenylephrine (Fig. 4b).

Next, we investigated the effect of benoxathian on the augmented increases in extracellular 5-HT level induced by three SNRIs (milnacipran, duloxetine, and venlafaxine) and citalopram. Following sustained exposure to the optimal concentrations of milnacipran (100 μM), duloxetine (10 μM), venlafaxine (100 μM), or...
citalopram (1 μM) for 4 d, the slice cultures were challenged with the same antidepressant in the presence or absence of phenylephrine (0.3 and 3 μM), and then extracellular 5-HT levels were measured. **p<0.01. n=3.

Next, we examined whether milnacipran directly stimulates α₁-adrenoceptors and induce further increase in extracellular 5-HT levels, or not. Chinese hamster ovary (CHO-K1) cells stably expressing hADRA1A (generous gift from Dr Tsujimoto; Obika et al., 1995) were preloaded with Rhod 2-AM and stimulated by phenylephrine (1 μM) with and without benoxathian (10 μM) or milnacipran (0.1–100 μM) (Supplementary Fig. 4). Phenylephrine (1 μM) significantly increased Rhod 2 fluorescence (p<0.001), and this increase was not observed in the presence of benoxathian (10 μM), suggesting that hADRA1A is sufficiently and functionally expressed. In this condition, milnacipran (0.1–100 μM), however, did not affect Rhod 2 fluorescence, indicating that α₁-adrenoceptor dependent 5-HT increase after sustained exposure to milnacipran in slice cultures is not due to direct stimulation of α₁-adrenoceptors by milnacipran.

Finally, to determine whether sustained exposure to milnacipran caused an increase in the expression of α₁-adrenoceptors, the number of α₁-adrenoceptors in the slice cultures was measured by a radioligand-binding experiment. Slice cultures were exposed to vehicle, citalopram (1 μM), or milnacipran (100 μM) for 4 d, and then [3H]prazosin binding to the membrane fraction prepared from the slice cultures was measured by a saturation binding assay. The Bmax values of [3H]prazosin binding to the membrane fractions prepared from vehicle-, citalopram-, and milnacipran-exposed slice cultures were 218.3±50.9, 190.6±22.9, and 212.7±78.7 nmol/mg protein, respectively. There was no significant difference between the three conditions (F2,11=0.067, p=0.94).

Discussion

The rat raphe slice cultures employed in this study contain an abundant number of raphe serotonergic neurons. These cultures also retain neural and synaptic serotonergic functions that enable the analysis of the mechanisms of antidepressant actions over a relatively long period of time in vitro (Higuchi et al., 2008; Nagayasu et al., 2010b). Furthermore, the raphe slice cultures also possess noradrenergic neurons containing NE, DBH, and NET, suggesting that this culture model is a suitable tool for analysing the effects of antidepressants on serotonergic-noradrenergic interactions. In the present study, we employed the raphe slice culture model to assess acute and chronic effects of various classes of antidepressants on extracellular 5-HT levels.

Consistent with our previous reports (Higuchi et al., 2008; Nagayasu et al., 2010b), acute treatment with SSRIs (citalopram and fluoxetine), SNRIs (duloxetine, venlafaxine, and milnacipran) and TCAs (desipramine and imipramine) induced a small increase in extracellular 5-HT levels. Because SSRIs, SNRIs, and TCAs commonly block SERT (Mantovani et al., 2009; Vaishnavi et al., 2004), this result is likely due to an acute inhibition of 5-HT reuptake by the blockade of the transporter. In vivo microdialysis studies have demonstrated that acute administration of SSRIs, SNRIs, or TCAs elicits a pronounced increase in 5-HT levels in the raphe, prefrontal cortex, striatum, hypothalamus and hippocampus (Bel and Artigas, 1999; Gartside et al., 1995; Invernizzi et al., 1997; Matos et al., 1990). In contrast to these studies, the acute increase in extracellular 5-HT levels in the raphe slice cultures in our study was not so large, suggesting...
weak spontaneous activity of raphe serotonergic neurons in slice cultures under normal conditions, although basal 5-HT levels were still dependent on both action potentials and exocytosis.

In addition to the blockade of SERT, SNRIs and TCAs also inhibit the reuptake of NE by the blockade of NET (Sparatore et al., 1982; Mantovani et al., 2009). Therefore, it is possible that an acute inhibition of NET by SNRIs or TCAs may induce an activation of serotonergic neurons through α2-adrenoceptors. However, extracellular 5-HT levels after acute treatment with SNRIs or TCAs were similar to those with SSRIs, whereas acute treatment with the α2-adrenoceptor agonist phenylephrine increased the extracellular 5-HT level. These data suggest that acute NET inhibition does not sufficiently increase the extracellular NE level enough to induce the activation of α2-adrenoceptors in raphe slice cultures.

TCAs, such as imipramine and desipramine, also bind to H1-histamine, muscarinic, and α1-adrenergic receptors (Cusack et al., 1994). Under our culture conditions, however, there was apparently no difference among the acute effects of TCAs, SSRIs and SNRIs on extracellular 5-HT levels. This suggests that these receptors do not play a key role in maintaining the extracellular 5-HT level in the raphe slice cultures.

Unlike SSRIs and SNRIs, mianserin and mirtazapine preferentially inhibit presynaptic α2-adrenoceptors and increase extracellular 5-HT and NE levels (de Boer et al., 1988, 1996). Moreover, because mirtazapine has a low affinity for α2-adrenoceptors (de Boer et al., 1988), increased NE may stimulate α2-adrenoceptors existing on serotonergic neurons, resulting in a facilitation of serotonergic transmission (de Boer et al., 1994, 1996). In vivo microdialysis studies revealed that acute administration of mirtazapine increases extracellular 5-HT levels (de Boer et al., 1996). However, we could not observe any effect of mianserin or mirtazapine on extracellular 5-HT levels in this slice culture system, suggesting that inhibition of serotonergic terminals through α2-adrenoceptor activation is not sufficient to elevate extracellular 5-HT levels, at least in the absence of other antidepressants.

Several lines of evidence suggest that repeated injection or chronic treatment of antidepressants causes an increase in basal extracellular 5-HT levels, in 5-HT release after antidepressant challenge in vivo, or in electrically evoked 5-HT release from slices (Bel and Arfagas, 1993; Blier and Bouchard, 1994; Kihara and Ikeda, 1994; Kreiss and Lucki, 1995). Consistent with these studies, sustained exposure to SSRIs (citalopram and fluoxetine), SNRIs (duloxetine, venlafaxine and milnacipran) and TCAs (desipramine and imipramine) caused the augmentation of 5-HT release in the raphe slice cultures. By contrast, sustained exposure to TeCAs (mianserin) and NaSSA (mirtazapine) did not induce the augmentation of 5-HT release. The former class of antidepressants, which induced the augmentation of 5-HT release in this study, commonly inhibit SERT, while the latter do not (Bolden-Watson and Richelson, 1993; Mantovani et al., 2009; Thomas et al., 1987). Furthermore, the order of optimal concentrations of SSRIs and TCAs determined from Fig. 1 is almost the same as the order of Ki values for SERT, as previously reported (Bolden-Watson and Richelson, 1993; Thomas et al., 1987). Among SNRIs,
there seems to be a correlation between the optimal concentration and $K_i$ values for SERT (Mantovani et al., 2009; Bolden-Watson and Richelson, 1993), indicating that an inhibition of SERT is required to cause the augmentation of 5-HT release.

To our knowledge, this is the first in vitro report that compares the effects of a number of antidepressant classes on extracellular 5-HT levels, especially after chronic exposure to these agents. The concentration-dependent responses to fluoxetine, desipramine, imipramine and duloxetine were bell-shaped, which may be due to the depletion of intravesicular 5-HT by excess 5-HT release and inhibition of 5-HT reuptake, or to non-specific toxic effects of the highest concentration of the antidepressants employed. We cannot rule out the possibility that the decreased tissue 5-HT contents in response to high concentrations of these antidepressants may affect the potency of their augmentation. One may have concern about the high concentrations of drugs we used in the present study. However, we must note that the rat raphe slice cultures, and presumably other organotypic slice cultures, usually require relatively high drug concentrations (Strassburger et al., 2008) for the drugs to penetrate the glial cell layer which proliferates after 14–16 d in culture, and the actual concentrations, which can access the serotonergic neurons in the presence of this thick cellular layer, will be far less than those which can access isolated neurons in culture. Indeed, $IC_{50}$ values of citalopram or milnacipran for prevention of 5,7-dihydroxytryptamine (200 $\mu M$, 2 d)-induced toxicity to 5-HT tissue content in the rat raphe slice cultures were 4.38 and 7.45 $\mu M$, respectively (Supplementary Fig. 5), which are 100–1000 times higher than those for inhibition of 5-HT reuptake in synaptosomal preparations (Mantovani et al., 2009).

The relative potency of the SNRIs in our study in regard to augmentation of 5-HT release was in the same order as the selectivity for NE rather than 5-HT reuptake inhibition (Bolden-Watson and Richelson, 1993; Mantovani et al., 2009; Stahl et al., 2005). These data indicate that the addition of noradrenergic signaling to SERT inhibition contributes to the higher augmentation potency of SNRIs. On the other hand, tissue 5-HT content and releasable 5-HT storage were not affected by sustained milnacipran exposure, suggesting that higher potency of milnacipran is due to a further facilitation of 5-HT neuronal activity.

Raphe serotonergic neurons receive excitatory noradrenergic inputs from the A1, A2, A5 and A6 regions (Peyron et al., 1996), and noradrenergic signaling positively regulates the activity of serotonergic neurons (Vandermaelen and Aghajanian, 1983). Positive regulation of the raphe serotonergic neurons by noradrenergic input is mediated through the activation of $\alpha_1$-adrenoceptor on the raphe serotonergic neurons (Bortolozzi and Artigas, 2003; Pudovkina et al., 2002, 2003). Consistent with these studies, the present study showed that acute stimulation of the $\alpha_1$-adrenoceptor increased extracellular 5-HT levels, suggesting that the $\alpha_1$-adrenoceptor is functionally associated with 5-HT release in the raphe slice cultures. Furthermore, phenylephrine enhanced the citalopram-augmented 5-HT release, suggesting that SSRI-induced augmentation can be further potentiated by stimulation of the $\alpha_1$-adrenoceptor. On the other hand, the $\alpha_1$-adrenoceptor antagonist, benoxathian, attenuated the milnacipran-augmented 5-HT release to the same extent as citalopram, suggesting that the augmentation induced by sustained exposure to milnacipran is partially mediated by activation of the $\alpha_1$-adrenoceptor.

Consistently, some studies suggest that 5-HT release stimulated by SNRIs is mediated through both the blockade of terminal SERT and the activation of raphe serotonergic neurons by noradrenergic inputs following NET inhibition (Dawson et al., 1999; Koch et al., 2003; Yamauchi et al., 2012). In some reports, serotonin–noradrenergic antidepressants were suggested to have a superior efficacy to SSRIs in the treatment of MDD (Papakostas et al., 2007). In addition to the effect of increasing NE levels, the augmented 5-HT release by milnacipran via $\alpha_1$-adrenoceptors might contribute to its efficacy in a treatment of MDD.

In contrast to milnacipran, the attenuation of 5-HT release by benoxathian was not observed when the slices were exposed to other SNRIs, duloxetine or venlafaxine. Lines of evidence suggest that NET inhibition by duloxetine and venlafaxine are relatively weak when compared to SERT inhibition, while milnacipran inhibits both NET and SERT with almost equal affinity to them (Bolden-Watson and Richelson, 1993; Mantovani et al., 2009; Stahl et al., 2005). Furthermore, venlafaxine is reported to preferentially increase extracellular 5-HT over NE in vivo (Koch et al., 2003). Taken together, it is suggested that potent NET inhibition by milnacipran enhances the augmentation of 5-HT release. Another possible explanation for this discrepancy is that milnacipran may directly activate or increase the expression of the $\alpha_1$-adrenoceptor. However, we showed that milnacipran did not stimulate $\alpha_1$-adrenoceptors in the present study, which is consistent with previous researches reporting that milnacipran has no affinity for this receptor (Mochizuki et al., 2002; Moret et al., 1985). Furthermore, milnacipran did not change the expression of $\alpha_1$-adrenoceptors in the slice cultures. Another possible
explanation is that an indirect pathway, such as a changing affinity for an agonist, may be involved in the effect of milnacipran, rather than direct effects on the α1-adrenoceptor. In support of this hypothesis, there is a report suggesting that milnacipran enhances the ability of α1-adrenoceptor agonists to compete for the [3H]prazosin binding site without changing the number of binding sites or the affinity for [3H]prazosin in the rat brain cortex (Maj et al., 2000). However, further studies into this issue will be needed. In addition, considering the high concentrations in the present study, it might be possible that the effects of antidepressants we observed may be partly due to intracellular mechanisms induced by applied antidepressants, even though their actual concentrations in the slices is suggested not to be so high.

In conclusion, the present study demonstrated that inhibition of the 5-HT transporter is required to cause the augmentation of 5-HT release in the rat raphe slice cultures. Among nine antidepressants examined, milnacipran had the highest potency in terms of augmenting 5-HT release, which was partially mediated by the α1-adrenoceptor. Although no statistically significant differences in therapeutic antidepressive effects were observed when comparing milnacipran with SSRIs or TCAs (Nakagawa et al., 2009), several studies have suggested that high doses of milnacipran are more efficacious than conservative doses (Kanemoto et al., 2004; von Frenckell et al., 1990). The comparatively high potency of milnacipran in the augmentation of 5-HT release may therefore reflect its high-dose efficacy.

Supplementary material

For supplementary material accompanying this paper, visit http://dx.doi.org/10.1017/S1461145713000771.

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Statement of Interest

None.

References


