Sustained administration of trazodone enhances serotonergic neurotransmission: *In vivo* electrophysiological study in the rat brain

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Abbreviations, 5-HT, 5-hydroxytryptamine (serotonin); NE, norepinephrine; SSRI, selective serotonin reuptake inhibitor; WAY100,635, N-[2-[4(2-methoxyphenyl)-1-piperazinyl]ethyl]-N-(2-pyridinyl) cyclohexanecarboxamide trihydrochloride; LSD, lysergic acid diethylamide; 8-OH-DPAT, 8-hydroxy-2-(di-n-propylamino) tetralin.

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ABSTRACT

Background: Despite its clinical use of more than two decades, the mechanisms by which trazodone acts as an antidepressant are not clear since it has affinity for a variety of serotonin (5-HT) receptors and for the 5-HT transporter. This study examined effects of sustained trazodone administration on 5-HT neurotransmission. Methods: Electrophysiological recordings were conducted in anesthetized rats. Subcutaneously implanted minipumps delivered vehicle or trazodone (10 mg/kg/day) for 2 and 14 days. Results: A 2-day trazodone administration suppressed the firing rate of raphe 5-HT neurons, which recovered to baseline following 14 days. This was attributable to 5-HT₁A autoreceptor desensitization since the suppressant effect of the 5-HT autoreceptor agonist LSD was dampened in 14-day trazodone-treated rats. Prolonged trazodone administration did not change the sensitivity of postsynaptic 5-HT₁A and α₂-adrenergic receptors in hippocampus, but enhanced synaptic 5-HT levels since the 5-HT₁A antagonist WAY 100,635 enhanced hippocampal firing in treated, but not in controls. Trazodone administration for 14 days increased the RT₅₀ value, an index of 5-HT transporter blockade in vivo, and decreased the inhibitory function of terminal 5-HT₁B autoreceptors on the electrically-evoked release of 5-HT. The agonistic action of trazodone at 5-HT₁A receptors was characterized as being full because it did not attenuate the inhibitory action of 5-HT when co-applied locally. Conclusion: The enhanced 5-HT neurotransmission by trazodone is due in part to reuptake blockade as well as activation of postsynaptic 5-HT₁A receptors which may account for its effectiveness in major depression.
INTRODUCTION

Since its introduction in the 1980’s trazodone hydrochloride, a triazolopyridine derivative, has been used in the treatment of major depression (Cunningham et al., 1994), and as a bedtime sedative. The structure of trazodone, unrelated to those of other major classes of antidepressants, results in a low toxicity profile (Al-Yassiri et al., 1981). Its mechanism of action in the treatment of depression has not been fully elucidated, largely in part to its affinity for a variety of receptors which may contribute to its clinical actions (Table 1). Although the antidepressant action of trazodone is partially attributed to blockade of serotonin (5-HT) reuptake sites, its potency to inhibit 5-HT transporters (5-HTT) is considerably less than that of selective serotonin reuptake inhibitors (SSRIs) such as citalopram and fluoxetine (Owens et al., 1997). The action of SSRIs on 5-HT neurotransmission has been established over the years. Short-term administration of SSRIs attenuates the firing rate of dorsal raphe (DR) 5-HT neurons, due to overactivation of somatodendritic 5-HT1A autoreceptors since SSRIs promptly inhibit 5-HT uptake, leading to an enhancement of 5-HT levels in the vicinity of the cell body. Prolonged administration of SSRIs, however, leads to complete recovery of 5-HT firing rate. Such a phenomenon is attributable to desensitization of 5-HT1A autoreceptors (Piñeyro and Blier, 1999). Indeed, desensitization of somatodendritic and terminal autoreceptors, following chronic administration of SSRIs, enhances 5-HT levels in the synaptic cleft (Piñeyro and Blier, 1999). The gradual adaptive changes of 5-HT neuronal elements, which correspond to the delayed therapeutic action, is perhaps due to alterations in receptor gene transcription and modifications of G-protein coupled to the 5-HT1A autoreceptor (Hensler, 2003).

In addition to its inhibiting action at 5-HTT, trazodone shows moderate potency at blocking 5-HT2A/2C receptors (Table 1) which may contribute to the mechanism of action of trazodone. Microdialysis studies have consistently shown that 5-HT2C antagonists enhance dopamine (DA) concentration in prefrontal cortex (Di Matteo et al., 1999; Gobert and Millan, 1999), while the effect of 5-HT2A antagonists on DA levels remains controversial (Schmidt and Fadayel, 1995; Gobert and Millan, 1999). The active metabolite of trazodone meta-chlorophenyl piperazine (mCPP) is, however, a potent 5-
HT$_{2C}$ agonist which would tend to counteract 5-HT$_{2C}$ antagonism of the parent compound. More importantly, pharmacological experiments have shown that blockade of 5-HT$_{2A}$ receptors restores inhibited locus coeruleus norepinephrine (NE) neuronal activity produced by SSRI (Dremencov et al., 2007). Given the interactions between the monoaminergic neurons in vivo, the antagonism of trazodone at 5-HT$_{2A}$ receptors may help compensate for a lower 5-HT inhibitory action at 5-HTT.

Another interesting feature of trazodone is its moderate affinity at 5-HT$_{1A}$ receptors (Table 1). Indeed, Odagaki and colleagues (2005) showed that trazodone acts as an agonist at human 5-HT$_{1A}$ receptors. The agonistic action of trazodone at 5-HT$_{1A}$ receptors may contribute to the modulation of the 5-HT system, in addition to the inhibition of 5-HT uptake. An in vivo electrophysiological study showed that systemic administration of trazodone potently suppressed the firing rate of DR 5-HT neurons (Scuvée-Moreau and Dresse, 1982), although this effect could be due to the inhibition of 5-HTT or its potent $\alpha_1$-adrenoceptor antagonist action (Table 1). Nevertheless, it is expected that the combined action of trazodone at 5-HTT and 5-HT$_{1A}$ receptors may enhance 5-HT neurotransmission. In line with this, previous studies have shown that 5-HT$_{1A}$ agonists enhance the tonic activation of postsynaptic 5-HT$_{1A}$ receptors due to desensitization of somatodendritic 5-HT$_{1A}$ autoreceptors as well as direct activation of normosensitive postsynaptic 5-HT$_{1A}$ receptors in hippocampus (Blier and de Montigny, 1990).

The various 5-HT agonist-antagonist properties of trazodone may, therefore, contribute to the net therapeutic benefits of this antidepressant. The present in vivo electrophysiological experiments were thus undertaken to examine the effects of sustained administration of trazodone on 5-HT neuronal elements in the DR and hippocampus of the rat brain.
METHODS

Experimental preparations

All the electrophysiological experiments were carried out in male Sprague-Dawley (Charles River, St. Constant, QC, Canada) rats, weighing between 250 and 350 g, at the time of recordings. The animals were kept under standard laboratory conditions (12:12 light-dark cycle with access to food and water ad libitum). Under isoflurane anesthesia, the rats were implanted subcutaneously with an osmotic Alzet minipump (Alza, Palo Alto, Calif., USA) to ensure slow and steady release of 5, 10 and 20 mg/kg/day of trazodone hydrochloride or the vehicle (hydroxy propyl-betha-cyclodextrin 20%, used to dissolve the drug) for 2 or 14 days. On the day of recording, the rats were anesthetized with chloral hydrate (400 mg/kg, i.p.) and mounted in a stereotaxic frame (David Kopf Instruments, Tujunga, CA). Additional doses of chloral hydrate were given to maintain constant anesthesia. Body temperature was maintained at 37°C throughout the experiment utilizing a thermistor-controlled heating pad. For systemic injection of pharmacological agents a catheter was inserted in a lateral tail vein, prior to the electrophysiological recordings. The electrophysiological experiments were carried out with the minipumps in place. All the experiments were approved by the local Animal Care Committee and conducted in accordance with the Canadian Council on Animal Care, for the care and use of laboratory animals.

In vitro determination of [\(^3\)H]5-HT uptake

To determine the degree of 5-HT reuptake inhibition in hippocampus, following a 2-day administration of trazodone (5, 10 and 20 mg/kg/day) and the SSRI escitalopram (10 mg/kg/day; used as a positive control), hippocampal slices were incubated in oxygenated Krebs’ solution at 37°C to determine in vitro [\(^3\)H]5-HT uptake. After a 3-minute stabilization period, the slices were incubated with 20 nM of [\(^3\)H]5-HT for 3 minutes. The uptake process was stopped by transferring the slices into 5 ml ice-cold Krebs’ solution. The slices were then solubilized in 0.5 ml of Soluene 350. A parallel experiment was carried out at 0°C as a control for passive diffusion. The radioactivity in the media and tissue was determined by liquid scintillation spectrometry (Beckman Coulter, CA, USA).
The reuptake activity was assessed by determining the tissue-medium ratio of radioactivity, using the following formula: \((R_{C}-R_{T})/(R_{C}-R_{0})\), where \(R_{C}\) is the ratio of tissue to medium for the control slice, \(R_{T}\) is the ratio of tissue for the treated slice, and \(R_{0}\) is the ratio of tissue to medium for the control slice at 0°C. For the in vitro determination of \(^{3}\text{H}\)5-HT uptake, each set of experiment consisted of 5 test tubes each containing 2 hippocampus slices from a single rat, and a total of 3 – 6 rats were used per group.

**Extracellular unitary recording of DR 5-HT neurons**

**In vivo** extracellular recordings of 5-HT neurons were obtained using single-barrel glass micropipettes. The impedance of the electrodes was between 4 and 6 MΩ. After securing the rats on the stereotaxic apparatus, a burr hole was drilled 1 mm anterior to lambda. The microelectrodes were positioned 0.9 – 1.2 mm anterior to lambda on the midline and lowered into the DR. The 5-HT neurons were encountered over a distance of 1 mm starting immediately below the ventral border of the Sylvius aqueduct. These neurons were identified by their slow (0.5 - 2.5 Hz), regular firing rate and a long duration (0.8 - 1.2 ms) positive action potential (Aghajanian, 1978).

**Assessment of sensitivity of DR 5-HT\(_{1A}\) autoreceptor**

To assess the sensitivity of 5-HT\(_{1A}\) autoreceptor, dose-response curves for the alteration of 5-HT neuronal firing activities were constructed using systemic administration of the 5-HT autoreceptor agonist lysergic acid diethylamide (LSD) in control and treated rats. LSD is a more reliable probe than the 5-HT\(_{1A}\) agonist 8-OH-DPAT for 5-HT\(_{1A}\) autoreceptor since the latter also acts on 5-HT\(_{1A}\) receptors of cortical neurons feeding back to DR 5-HT neurons. Furthermore, the responsiveness of dorsal raphe 5-HT neurons to microiontophoretic-application of LSD, 8-OH-DPAT and systemic injection of LSD is decreased, whereas that to systemic 8-OH-DPAT is not altered following chronic administration of the 5-HT\(_{1A}\) receptor agonist gepirone (Blier and de Montigny, 1990). The potent and selective 5-HT\(_{1A}\) receptor antagonist WAY 100,635 (Khawaja et al., 1995) was injected, following the LSD-induced inhibition, to reverse the 5-HT firing activity. This also served to validate the use of the agonist which is not entirely selective for 5-HT\(_{1A}\) receptors. To avoid the drug residual effects, the dose-response curves were
obtained using only the last neuron in each rat. It is worth noting that the firing rate of all 5-HT neurons used in this set of experiments was within their average firing rate, and that a 2-minute period was allowed to obtain a stable baseline before the systemic injection of LSD. The rate and pattern of firing rate were typical for 5-HT neurons as previously described (Aghajanian, 1978).

**Extracellular recording and microiontophoresis of CA3 dorsal hippocampus pyramidal neurons**

Extracellular recording and microiontophoresis of CA3 pyramidal neurons were performed with five-barreled glass micropipettes. The central barrel, used for the unitary recording, was filled with a 2 M NaCl solution, and the impedance of these electrodes ranged from 2 to 4 MΩ. The side barrels were filled with the following solutions: 5-HT creatinine sulfate (10 mM in 200 mM NaCl, pH 4), (±)-NE bitartrate (10 mM in 200 mM NaCl, pH 4) or trazodone hydrochloride (20 mM in 200 mM NaCl, pH 4), quisqualic acid (1.5 mM in 200 mM NaCl, pH 8), and the last barrel was filled with a 2 M NaCl solution used for automatic current balancing. The micropipettes were lowered into the dorsal hippocampus CA3 region using the following coordinates; 4 mm anterior to lambda and 4.2 mm lateral (Paxinos and Watson, 1998). A small current of quisqualate +2 to –5 nanoampere (nA) was used to activate the pyramidal neurons within their physiological firing range (10 to 15 Hz; Ranck, 1975) because these neurons do not discharge spontaneously in chloral hydrate anesthetized rats. The hippocampus CA3 pyramidal neurons were found at a depth of 4.0 ± 0.5 mm below the surface of the brain, and identified by their large amplitude (0.5 – 1.2 mV) and long-duration (0.8 – 1.2 ms) simple action potentials, alternating with complex spike discharges (Kandel and Spencer, 1961). 5-HT and NE were microiontophoretically applied for 50 seconds. The duration of local application of the 5-HT and the ejection currents (nA) were kept constant before and after each i.v. injection of 5-HT\textsubscript{1A} antagonist WAY 100,635. The responsiveness of CA3 pyramidal neurons to the microiontophoretic application of 5-HT and NE, prior to and following i.v. injections, was assessed by determining the number of spikes suppressed per nA for the 50-sec ejection period.
Assessment of the tonic activation of postsynaptic 5-HT\(_{1A}\) receptors in hippocampus

The degree of tonic activation of hippocampus CA3 5-HT\(_{1A}\) receptors was assessed using systemic injection of the selective 5-HT\(_{1A}\) antagonist WAY 100,635. Such disinhibition of the neuronal activity is best assessed when the firing rate is low. Indeed, a low stable firing baseline was obtained by lowering the ejection current of quisqualate. The baseline firing was recorded for at least 2 minutes before the administration of WAY 100,635. WAY 100,635 (100 µg/kg) was systemically administered in incremental doses of 25 µg/kg at time intervals of 2 minutes to detect the changes in the firing activity of hippocampus pyramidal neurons in rats treated with vehicle and trazodone (10 mg/kg/day) for 14 days. Such curves represent stable changes in the firing rate of CA3 pyramidal neurons as percentages of baseline firing following each systemic drug administration. In order to avoid residual drug effects, only one neuron in each rat was studied.

**In vivo determination of 5-HT uptake**

In order to assess the relative degree to which trazodone blocks the 5-HTT, the RT\(_{50}\) values were determined after microiontophoretic application of 5-HT in hippocampus CA3 region. The RT\(_{50}\) values correspond to the time in seconds elapsed from the cessation of microiontophoretic application of 5-HT to obtain a 50% recovery of the initial firing rate (de Montigny *et al*., 1980). It is a reliable index of 5-HT reuptake process *in vivo*. Indeed, previous experiments showed that acute systemic injection of the SSRI paroxetine significantly increased RT\(_{50}\) values. Furthermore, this phenomenon was also observed in rats after the lesion of 5-HT neurons, thereby eliminating 5-HTT (Piñeyro *et al*., 1994).

**The 5-HT pathway stimulation**

The CA3 region of hippocampus receives extensive projections from DR 5-HT neurons. In order to electrically stimulate the ascending 5-HT pathway a bipolar electrode (NE-100, David Kopf, Tujunga, CA, USA) was implanted 1 mm anterior to lambda on the midline with a 10° backward angle in the ventromedial tagmentum and 8.0 ± 0.2 mm
below the surface of the brain. Two hundred square pulses with duration of 0.5 ms were delivered by a stimulator (S48, Grass Instruments, West Warwick, RI, USA) at an intensity of 300 µA and a frequency of 1 and 5 Hz. The stimulation of 5-HT pathway induces a brief suppressant period due to the release of 5-HT in the synapse. The effects of stimulation of ascending 5-HT pathway were assessed using 1 and 5 Hz, on the same neuron, to determine the function of terminal 5-HT\textsubscript{1B} autoreceptors (Chaput \textit{et al}., 1986). The two series of stimulations, 1 and 5 Hz, were carried out because previous studies showed that activation of terminal 5-HT\textsubscript{1B} autoreceptors decreases the 5-HT release in the terminal areas and that increasing the frequency of stimulation from 1 to 5 Hz induces a greater activation of 5-HT\textsubscript{1B} autoreceptors, consequently a greater negative feedback on the release of 5-HT (Chaput \textit{et al}., 1986). As a result, the smaller release of the neurotransmitter in the synapse, obtained at 5 Hz, induces a smaller period of suppression compared to that of the 1 Hz stimulation. The stimulation pulses and the firing activity were analyzed by computer using Spike 2 (Cambridge Electronic Design Limited, UK). Peristimulus time histograms of CA3 pyramidal neurons were generated to determine the suppression of firing measured in absolute silence (SIL) value in ms. The SIL value corresponds to the duration of a total suppression of the CA3 hippocampal pyramidal neuron. This parameter was calculated by computer by dividing the total number of events, suppressed by the stimulation, by the frequency of firing of the recorded neuron.

**Quantification of trazodone and mCPP in the brain**

**Standards preparation**

Trazodone, mCPP and domperidone were initially prepared at 1 mg/mL in DMSO and subsequently diluted in acetonitrile. For analysis in brain, trazodone and mCPP were added to 100 mg/mL control brain homogenate (5–2500 pg/mg tissue) and extracted as described below. Domperidone was prepared at 1 µg/mL in acetonitrile to be used as an internal standard.

**Sample preparation**

Tissue samples were homogenized at 100 mg/mL in deionized water and 100 µL of tissue homogenate was used for the extraction. To each sample, 10 ng (10 µL of 1 µg/mL of
domperidone was added followed by 1 mL of ethyl acetate containing 0.1% (v/v) ammonium hydroxide (30%, w/v) and extraction by vortexing for 10 min. Organic and aqueous layers were separated by centrifugation (10 min, 20,000 x g) and the organic layer removed, evaporated by vacuum centrifugation at room temperature and reconstituted in 200 µL of beginning mobile phase (20% acetonitrile: 80% 0.1% formic acid in water) for LC/MS/MS analysis.

**Mass spectrometry**

Positive ion electrospray ionization (ESI) mass spectra were obtained with an AB Sciex 3200 QTRAP™ triple quadrupole mass spectrometer (Foster City, CA) with a turbo V™ ion source interfaced to a Shimadzu HPLC system. Samples were chromatographed with a Waters Sunfire C18, 2.5 µm, 50 mm × 4.6 mm column (Milford, MA). The LC was a gradient elution utilizing 100% acetonitrile as the organic phase and 0.1% formic acid in water as the aqueous as follows: 20% acetonitrile for 0.1 min, linearly ramp to 90% acetonitrile at 1.5 min, hold at 90% for 30 sec, return to 20% acetonitrile over 30 sec and equilibrate column for 30 sec at 20% acetonitrile. The flow rate was 1 ml/min and sample injection volume of 60 µL. The analysis time was 3 min. The mass spectrometer settings were: temperature, 550°C; spray needle, 5500 V; curtain gas, 10; collision gas, N₂ (CAD), 3; ion source gas 1 and 2; 55 and 45, respectively. The compound dependent settings for trazodone, mCPP and domperidone were as follows, respectively: declustering potential, 57, 24 and 63; excitation potential, 4, 10, and 9; collision cell entrance potential, 21, 26, and 19; collision energy, 33, 26 and 36; and collision cell exit potential, 3, 3 and 2. Samples were quantified by the internal standard reference method in the MRM mode by monitoring the transition m/z 372→176 for trazodone, m/z 197→154 for mCPP, m/z 426→175 for the internal standard domperidone. Each ion transition was integrated for 250 ms. Quantitation of trazodone and mCPP in the brain were based on standard curves in spiked matrix using the ratio of either analyte peak area to domperidone peak area using 1/x² weighting for both analytes.

**Drugs**
The following drugs were used: trazodone HCl (Labopharm, Montreal, QC, Canada) was dissolved in hydroxy propyl-betha-cyclodextrin 20%. Escitalopram (Lundbeck, Copenhagen, DK), LSD (Health Canada), WAY 100,635, 5-HT creatinine sulfate, (±)-NE bitartrate and quisqualic acid were purchased from Sigma (Sigma, St. Louis, MO, USA) and dissolved in distilled water. [3H]5-HT was purchased from PerkinElmer Life Sciences (Boston, MA, USA). For quantification experiments trazodone, mCPP and domperidone were obtained from Sigma (St. Louis, MO). All other chemicals and solvents were of reagent or higher grade and obtained from Fisher Scientific (Pittsburgh, PA).

**Statistical analysis**

All data are reported as mean values ± SEM. The n values represent the number of neurons recorded, unless otherwise indicated. Data were obtained from 3 to 8 rats per experimental group. Statistical comparisons were carried out using a one-way analysis of variance (treatment as the main factor) and the Bonferroni post hoc analysis was conducted when significant ANOVA results were obtained (GraphPad Software Inc, La Jolla, CA). Dose-response curves were constructed using linear regression analysis GraphPad Prism 5 software. Statistical comparisons were carried out using the two-tailed Student’s t test when a parameter was studied in control and treated rats. The effects of changing the frequency of stimulation from 1 to 5 Hz on the SIL value of the same neuron were assessed using the paired Student’s t test. Analysis of covariance was used to assess statistical significance of the difference in the degree of reduction in the response of hippocampus neurons when the frequency of stimulation was increased from 1 to 5 Hz in control and trazodone-treated rats. Statistical significance was taken as p < 0.05.
RESULTS

Effects of 2- and 14-day administration of trazodone on the firing rate of DR 5-HT neurons

In comparison to the vehicle group, the 2-day administration of trazodone at the dose of 10 and 20, but not 5, mg/kg/day significantly decreased the spontaneous firing rate of DR 5-HT neurons by about 40% and 37%, respectively (vehicle: 1.11 ± 0.08 Hz, p < 0.01 for both trazodone 10 and 20 mg/kg/day; Fig 1A). Although trazodone at the dose of 20 mg/kg/day inhibited the 5-HT firing rate to the extent of 10 mg/kg/day, it significantly reduced the number of spontaneously active 5-HT neurons by about 65% after 2 days once compared to the vehicle group (p < 0.01; Table 2). Therefore, the average firing rate for the 2-day trazodone 20 mg/kg/day is an underestimated value since this regimen markedly decreased the number of spontaneously active 5-HT neurons. Interestingly, systemic administration of the potent and selective 5-HT<sub>1A</sub> receptor antagonist WAY 100,635 (100 µg/kg) normalized the 5-HT firing-inhibition induced by trazodone 10 and 20 mg/kg/day (Fig. 1A and B), and restored the number of spontaneously active neurons in rats treated with trazodone 20 mg/kg/day (Table 2). It is important to note that previous studies in our laboratory as well as others have shown that systemic administration of WAY 100,635 does not significantly alter the average spontaneous firing rate of DR 5-HT neurons in naive rats (Gartside et al., 1995; Haddjeri et al., 2004).

Since trazodone at the dose of 10 mg/kg/day induced a marked physiological action on 5-HT neurons, this dose was used to study the effects of 14-day administration of trazodone on the 5-HT system. In contrast to the 2-day regimen, the firing rate of DR 5-HT neurons completely recovered to the baseline following 14 days of trazodone administration (10 mg/kg/day; p > 0.05; Fig. 1C).

Brain concentration of trazodone and m-CPP following 2-day regimen
The steady-state concentrations of trazodone and its major metabolite m-CPP were 25 ± 2.7 ng/ml and 4 ± 0.25 ng/ml, respectively, following 2-day administration of trazodone (10 mg/kg). These levels are in the same range as those reported by DeVane et al (1999).

**Assessment of sensitivity of somatodendritic 5-HT$_{1A}$ autoreceptors**

The responsiveness of somatodendritic 5-HT$_{1A}$ autoreceptor, following 14-day trazodone (10 mg/kg/day) regimen, was assessed using the 5-HT autoreceptor agonist LSD probe (Blier and de Montigny, 1990). The firing of DR 5-HT neurons in all the naive rats were completely inhibited at the dose of 20 µg/kg (ED$_{50}$ = 9.0 ± 1.0 µg/kg; Fig. 2A). In contrast, the effect of LSD at this dose was markedly attenuated in suppressing the neuronal activity of 5-HT neurons in rats treated with trazodone for 14 days. In fact, higher doses of the LSD were needed to induce a complete suppression of the 5-HT firing (ED$_{50}$ = 16.0 ± 1.0 µg/kg; Fig. 2B). A complete dose-response relationship between the suppression of DR 5-HT firing rate and different doses of LSD showed a significant two-fold shift to the right in the 14-day trazodone treated rats. In addition to the altered ED$_{50}$ values, the slopes of the two dose-response curves were significantly different (5.2 ± 0.5 versus 2.3 ± 0.1 for the vehicle and trazodone groups, respectively; F(1, 18) = 5.46, p < 0.05; Fig. 2C), indicating that the long-term trazodone regimen resulted in 5-HT$_{1A}$ autoreceptor desensitization.

**Effects of sustained administration of trazodone for 14 days on the responsiveness of dorsal hippocampus CA$_3$ pyramidal neurons to exogenous 5-HT and NE**

Microiontophoretic application of 5-HT and NE suppressed the firing rate of dorsal hippocampal CA$_3$ pyramidal neurons in the vehicle and trazodone-treated rats (Fig. 3A and B, respectively). The inhibitory action of 5-HT and NE is mediated via postsynaptic 5-HT$_{1A}$ and $\alpha_2$-adrenergic receptors, respectively, since administration of 5-HT$_{1A}$ antagonist WAY 100,635 and $\alpha_2$-adrenoceptor antagonist idazoxan readily blocks the suppressant action of the agonists (Chaput and de Montigny, 1988; Curet and de Montigny, 1988). Microiontophoretic application of 5-HT and NE in the dorsal hippocampus of rats administered with trazodone for 14 days revealed that the number of
spikes suppressed/nA was not significantly different from that of the vehicle group (p > 0.05 for both 5-HT and NE; Fig. 3C and D), suggesting that 5-HT$_{1A}$ and α$_2$-adrenergic receptors in the CA$_3$ region remain normosensitive following prolonged administration of trazodone.

**In vivo assessment of the degree of 5-HT reuptake inhibition by trazodone**

The function of hippocampus 5-HTT was determined using the recovery time (RT$_{50}$) of firing rate of pyramidal neurons following a complete suppression of the firing activity induced by local application of 5-HT. Sustained administration of trazodone (10 mg/kg/day) for 14 days significantly increased the RT$_{50}$ value compared to that of the vehicle group (p < 0.01; Fig. 3E), indicating that trazodone blocks 5-HT reuptake process in vivo.

**In vitro determination of [3H]5-HT uptake by trazodone**

Following the 2-day regimens, the ex vivo experiments from hippocampal slices revealed that trazodone, in a dose-dependent manner, at the dose of 5 and 10 mg/kg/day significantly blocked the 5-HT reuptake process by 57% (p < 0.05) and 62% (p < 0.01), respectively. It is worth noting that trazodone at the dose of 20 mg/kg/day also markedly inhibited 5-HTT in hippocampus by 70% (p < 0.01). The SSRI escitalopram, used as a positive control, at the dose of 10 mg/kg/day significantly inhibited the 5-HT reuptake process by 81% (p < 0.001; Fig. 4).

**Assessment of the overall serotonergic tone following 14-day administration of trazodone as determined with the tonic activation of postsynaptic 5-HT$_{1A}$ receptors**

As illustrated in figure 5, cumulative administration of 5-HT$_{1A}$ antagonist WAY 100,635 failed to modify the firing activity of dorsal hippocampus CA$_3$ pyramidal neurons in the vehicle group (p > 0.05; Fig. 5). On the other hand, the second dose and subsequent doses of systemic injection of WAY 100,635 markedly enhanced the firing activity in rats administered with trazodone for 14 days (p < 0.05 for all doses except the first one; Fig. 5). Indeed, the final injection of WAY 100,635 increased the neuronal activity in the CA$_3$
region by about 160% compared to control rats. The marked increment in the firing activity of pyramidal neurons in the trazodone-treated rats reflects the degree to which WAY 100,635 disinhibits neuronal activity due to the tonic activation of postsynaptic 5-HT<sub>1A</sub> receptors by extracellular 5-HT in the hippocampus. It is important to emphasize that the inhibitory effect of microiontophoretic application of 5-HT was significantly blocked in all rats after the last injection of WAY 100,635 (vehicle: pre-WAY 100,635: 55 ± 5 spikes suppressed/nA, post-WAY 100,635: 29 ± 4 spikes suppressed/nA, n = 6, p < 0.001; 14-day trazodone: pre-WAY 100,635: 46 ± 3, post-WAY 100,635: 12 ± 2, n = 8, p < 0.001; Fig. 5), confirming that the inhibitory action was mediated by 5-HT<sub>1A</sub> receptors.

**Effects of sustained administration of trazodone for 14 days on the efficacy of the electrical stimulation of the 5-HT afferent fibers to the hippocampus**

In order to assess the amount of 5-HT released per electrical impulse in the 5-HT pathway, the 5-HT afferent fibers to the hippocampus were electrically stimulated. The responsiveness of terminal 5-HT<sub>1B</sub> autoreceptors was also evaluated by increasing the frequency of stimulation from 1 to 5 Hz on the same neuron. The stimulation of the 5-HT pathway at 1 Hz enhanced the period of suppression in rats administered with trazodone for 14 days by 20% once compared to the corresponding SIL value in vehicle group (p < 0.01; Fig. 6). Increasing the frequency of stimulation from 1 to 5 Hz significantly reduced the period of suppression in the vehicle group by 25% (p < 0.01; Fig. 6), an effect due to greater degree of activation of terminal 5-HT<sub>1B</sub> autoreceptors (Chaput et al., 1986). Furthermore, contrary to the vehicle group, the decremental effect obtained by enhancing the frequency of stimulation from 1 to 5 Hz was abolished in rats treated with trazodone and the period of suppression at 5 Hz was greater than the corresponding value in the vehicle group (p < 0.01; Fig. 6), indicating that the inhibitory function of terminal autoreceptor was diminished.

**Effects of microiontophoretic application of trazodone on hippocampus CA<sub>3</sub> pyramidal neurons**
Trazodone was locally applied in the dorsal hippocampus CA3 region to characterize its effects at postsynaptic 5-HT$_{1A}$ receptors. The firing activity of pyramidal neurons was markedly reduced by local application of 5-HT (Fig. 7A and B). Microiontophoretic-application of trazodone also markedly inhibited the CA3 neuronal activity (Fig. 7A and C). The inhibitory effects of both 5-HT and trazodone were significantly blocked following the systemic administration of WAY 100,635 (100 µg/kg), indicating that these inhibitory actions were mediated by 5-HT$_{1A}$ receptors (p < 0.01; Fig. 7A, C and D). The agonistic action of trazodone at postsynaptic 5-HT$_{1A}$ receptors was further characterized as being full because it did not attenuate the inhibitory action of the endogenous agonist 5-HT when co-applied by microiontophoresis in hippocampus (p > 0.05, Fig. 7A and B).
The present data showed that trazodone potently suppressed the firing rate of DR 5-HT neurons after 2 days of sustained administration. This firing rate, however, recovered to baseline following administration of trazodone for 14 days. The complete recovery of 5-HT firing is attributable to decreased sensitivity of 5-HT\textsubscript{1A} autoreceptors since the suppressant effect of the 5-HT autoreceptor agonist LSD was decreased by the long-term trazodone regimen. With respect to 5-HT neurotransmission in the forebrain, although the sensitivity of postsynaptic 5-HT\textsubscript{1A} and \textalpha\textsubscript{2}-adrenergic receptors was not changed following its prolonged administration, trazodone increased tonic activation of postsynaptic 5-HT\textsubscript{1A} receptors in hippocampus, as indicated by disinhibition of neuronal activity by WAY 100,635. This study, therefore, indicates that sustained administration of trazodone enhances 5-HT neurotransmission, at least in part, by desensitizing the inhibitory function of 5-HT\textsubscript{1A} and 5-HT\textsubscript{1B} autoreceptors that are present on the cell body and terminals, respectively, in the presence of 5-HT reuptake inhibition.

The \textit{in vitro} data showed that trazodone inhibited 5-HTT in a dose-dependent manner in the hippocampus; a phenomenon that was also observed with the SSRI escitalopram (Fig. 4), suggesting that trazodone modulates the 5-HT system, in part, by inhibiting 5-HT reuptake sites. Although trazodone at the dose of 5 mg/kg/day significantly blocked the 5-HTT, it did not alter the firing rate of 5-HT neurons after 2 days (Fig. 1), indicating that the degree of blockade was below the threshold to induce a net physiological action. This result is akin to what has been reported in humans: an 80\% occupancy of 5-HTT is required to obtain an antidepressant effect with a variety of reuptake inhibitors (Meyer \textit{et al.}, 2004). Trazodone 10 and 20 mg/kg/day, on the other hand, suppressed the firing rate of 5-HT neurons following a 2-day administration. In parallel to the 2-day trazodone regimen, acute administration of trazodone dose-dependently reduced the firing rate of 5-HT neurons (Scuvée-Moreau and Dresse, 1982), perhaps due to its blocking property at 5-HTT and/or \textalpha\textsubscript{1}-adrenoceptors (Table 1). Although the 2-day administration of trazodone at the dose of 20 mg/kg/day reduced the firing rate of 5-HT neurons to the same extent as 10 mg/kg/day, the number of spontaneously active 5-HT neurons was only reduced by the highest dose. It is well established that short-term administration of SSRI
potently suppresses 5-HT neurons due to activation of somatodendritic 5-HT$_{1A}$ autoreceptors by enhancing 5-HT levels in the vicinity of the cell body. In line with this, systemic administration of 5-HT$_{1A}$ antagonist WAY 100,635 reversed the trazodone-induced suppression of 5-HT firing rate to their baseline (Fig.1), and normalized the number of spontaneously active 5-HT neurons (Table 2), indicating that the suppressant effect may be mediated via 5-HT$_{1A}$ receptors and not by blocking $\alpha_1$-adrenoceptors. In contrast to the 2-day effect, the firing rate of 5-HT neurons showed a full recovery following 14 days of trazodone administration. The recovery of 5-HT firing activity was concurrent with desensitization of 5-HT$_{1A}$ autoreceptors (Fig. 2) which may account for firing normalization, a physiological adaptation that was consistently observed with long-term SSRI administration (Piñeyro and Blier, 1999).

Even though long-term administration of trazodone did not alter the sensitivity of postsynaptic 5-HT$_{1A}$ and $\alpha_2$-adrenergic receptors (Fig. 3), it induced a robust tonic activation of postsynaptic 5-HT$_{1A}$ receptors in the hippocampus after 14 days, as revealed by disinhibition of the neuronal firing activity induced by WAY 100,635 (Fig. 5). Enhanced tonic activation is consistent with previous microdialysis experiments showing increased extracellular concentration of 5-HT in terminal brain areas following administration of antidepressants with serotonergic action (Romero et al., 1996). It has been shown that the presumed enhanced 5-HT levels in hippocampus may partially be due to the inhibition of 5-HTT by prolonged, but not a 2-day, administration of the SSRI paroxetine (Besson et al., 2000). The observation that prolonged administration of trazodone considerably increased the overall net effect of 5-HT in hippocampus suggests similar mechanism of action as the SSRIs. In line with this, microdialysis experiments showed that administration of trazodone markedly enhanced the extracellular 5-HT concentration in frontal cortex of freely moving rats (Pazzagli et al., 1999), an effect that was higher than that of the SSRI sertraline (Garrone et al., 2009). Vilazodone, which like trazodone is a SSRI/5-HT$_{1A}$ agonist, enhances extracellular 5-HT concentration in the rat frontal cortex more than the SSRI paroxetine alone (Hughes et al., 2005).
Previous studies have reported that long-term administration of agents that potently inhibit 5-HTT, such as citalopram, fluoxetine, paroxetine and fluvoxamine, induce desensitization of 5-HT_1B autoreceptors on 5-HT terminals (Piñeyro and Blier, 1999). Thus, it was deemed crucial to assess the sensitivity of terminal autoreceptors since these autoreceptors exert an inhibitory role on the 5-HT release (Chaput et al., 1986). When compared to the vehicle group, stimulation of the 5-HT bundle at 1 Hz increased the effectiveness of stimulation in rats administered with trazodone for 14 days, as for the abovementioned SSRIs. Increasing the frequency of stimulation from 1 to 5 Hz reduces the period of suppression, in the vehicle group, due to greater activation of terminal 5-HT_1B autoreceptors, as reported previously (Chaput et al., 1986). In trazodone-treated rats, however, this decremental effect was abolished when the frequency of stimulation was increased from 1 to 5 Hz, suggesting that the inhibitory action of terminal autoreceptors was diminished (Fig. 6). In addition, long-term administration of YM992, which like trazodone blocks 5-HTT and 5-HT_2A receptors, desensitized terminal 5-HT autoreceptors (Dong et al., 1999). Desensitization of terminal 5-HT_1B autoreceptors may thus be the driving force for the enhanced 5-HT release in the synapse following 14-day administration of trazodone. Indeed, 5-HT reuptake blockade per se could not explain the increased 5-HT tone in hippocampus since acute administration of the SSRIs citalopram or fluoxetine does not enhance the effectiveness of stimulation (Piñeyro and Blier, 1999). Furthermore, Groß and colleagues (1987) showed that trazodone increased the electrically-induced [³H]5-HT release from cortical slices, an effect that was independent of 5-HT reuptake inhibition. Moreover, it is worth mentioning that desensitization of terminal 5-HT_1B autoreceptor following long-term administration of trazodone is not likely due to the agonistic action of its major metabolite m-CPP on these receptors nor to its potent antagonistic effect at 5-HT_3 receptors, since its concentration is low in the brain, as previously reported at the steady-state level (DeVane et al., 1999).

Microiontophoretic application of trazodone showed that, similar to 5-HT, it acts as an agonist at postsynaptic 5-HT_1A receptors in the hippocampus CA3 region since administration of the 5-HT_1A receptor antagonist WAY 100,635 markedly blocked the inhibitory actions of both 5-HT and trazodone (Fig. 7). The agonistic action of trazodone
at 5-HT$\textsubscript{1A}$ receptors was further characterized as being full because it did not antagonize the inhibitory action of the endogenous agonist 5-HT when co-applied by microiontophoresis in hippocampus (Fig. 7). In addition, when trazodone was administered for 14 days and the responsiveness to 5-HT was examined with the minipump in the animal delivering the drug, there was no alteration in the sensitivity of the pyramidal neurons to 5-HT (Fig. 3). On the other hand, the 5-HT$\textsubscript{1A}$ partial agonist gepirone (Blier and de Montigny, 1990) significantly offsets the inhibitory effect of 5-HT on the hippocampus firing rate when co-applied locally. Thus far, BAY x 3702 is the only 5-HT$\textsubscript{1A}$ agonist that acts as a full agonist, like trazodone, at 5-HT$\textsubscript{1A}$ receptors in the CA$\textsubscript{3}$ region (Dong et al., 1998). Even though both agents behave as full agonists at these postsynaptic 5-HT$\textsubscript{1A}$ receptors, they did not alter the sensitivity of these receptors following prolonged administration, in contrast to that of the somatodendritic autoreceptors. Furthermore, previous in vitro studies showed that trazodone has moderate affinity at 5-HT$\textsubscript{1A}$ receptors in the frontal cortex of human brain as well as rat hippocampus (Cusack et al., 1994; Owens et al., 1997), and that it acts as an agonist at human 5-HT$\textsubscript{1A}$ receptors (Odagaki et al., 2005). The agonistic action of trazodone at 5-HT$\textsubscript{1A}$ receptors, in addition to its modulatory action on 5-HT neurotransmission, may also contribute to enhance DA release in medial prefrontal cortex (Chung et al., 2004).

Enhanced 5-HT transmission by prolonged administration of trazodone may be clinically relevant. Indeed, trazodone similar to other classes of antidepressants including tricylics, MAOIs, SSRI$s$, mirtazapine, gepirone, and electroconvulsive shocks shares the property of enhancing the overall 5-HT neurotransmission in hippocampus following long-term administration (Haddjeri et al., 1998). The therapeutic effect of trazodone in the treatment of major depression may thus be, at least in part, due to the enhancement of 5-HT neurotransmission via 5-HTT inhibition and 5-HT$\textsubscript{1A}$ receptor agonism. This medication however, has not been used as first-line treatment for depression because of its daytime sedative action. The latter effect is likely due to a combination of H$\textsubscript{1}$, 5-HT$\textsubscript{2A}$ and $\alpha$-adrenergic receptor antagonism (Table 1), properties that can individually contribute to hypnotic and/or sedative effects. The development of a slow release preparation of trazodone thereby eliminating sharp plasma/brain peaks has, however,
helped diminish day-time drowsiness, while maintaining the therapeutic benefit on depressed mood (Sheehan et al., 2009).

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FOOTNOTES

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LEGENDS

Figure 1. The effects of 2-day administration of vehicle, trazodone 5, 10 and 20 (mg/kg/day) on the firing rate of DR 5-HT neurons, and the effects of systemic administration of 5-HT1A antagonist WAY 100,635 (100 µg/kg) on the 5-HT firing-inhibition induced by trazodone 10 and 20 (mg/kg/day; A). The lower panel represents the integrated histogram of the firing activity of a 5-HT neuron (upper panel), that was inhibited by 2-day administration of trazodone (10 mg/kg/day), in response to the systemic administration WAY 100,635 (B). The effects of 2- and 14-day administration of vehicle and trazodone 10 (mg/kg/day) on the firing rate of DR 5-HT neurons (C). ** p < 0.01 when compared to the vehicle group. † Indicates statistical significance compared to prior i.v. injection of WAY 100,635. The numbers at the bottom of each column indicates the number of neurons recorded for the given group.

Figure 2. Representative integrated firing rate histograms of DR 5-HT neurons illustrating the effect of intravenous administration of 5-HT autoreceptor agonist LSD in suppressing neuronal activity of rats treated with vehicle (A) and trazodone (10 mg/kg/day; B) for 14 days, and the relationship between the degree of suppression of DR 5-HT firing activity and doses of LSD administered intravenously in vehicle and trazodone-treated rats. Outer lines represent the standard error of the regression line (C).

Figure 3. Integrated firing rate histogram of dorsal hippocampus CA3 pyramidal neuron illustrating microiontophoretic application of 5-HT and NE in rats treated with vehicle (A) and trazodone (Traz, 10 mg/kg/day; B), and responses of dorsal hippocampus CA3 pyramidal neurons to microiontophoretic application of 5-HT (C), NE (D), and the duration of the 50% recovery, RT50, of pyramidal neurons from microiontophoretic application of 5-HT following 14-day regimens (E). Each bar corresponds to 50-sec application of the agonists, and the number above each bar corresponds to the ejection current in nA. The number of neurons recorded is displayed in each box. ** p < 0.01

Figure 4. The tissue/medium ratios of radioactivity illustrating the effects of 2-day administration of vehicle, trazodone (Traz, 5 and 10 mg/kg/day) and escitalopram (Escit,
10 mg/kg/day) on the inhibition of hippocampus 5-HTT ex vivo. Data were obtained from 3 to 6 rats per experimental group. * p < 0.05, ** p < 0.01, *** p < 0.001

Figure 5. Integrated firing rate histograms of dorsal hippocampus CA3 pyramidal neurons illustrating systemic administration of incremental doses of 25 µg/kg of WAY 100,635 in vehicle (A) and 14-day trazodone (10 mg/kg/day; B), and the overall effect of systemic administration of WAY 100,635 on baseline firing in vehicle and trazodone-treated rats (E). Each bar corresponds to 50-sec application of the agonists, and the number above each bar corresponds to the ejection current in nA. Each arrow indicates a single injection of 25 µg/kg of WAY 100,635. * p < 0.05

Figure 6. Integrated firing rate histograms illustrating effects of stimulation of the ascending 5-HT pathway, with 1 and 5 Hz, on the firing activity of CA3 pyramidal neurons in vehicle (A) and 14-day trazodone-treated rats (B), and the suppressing effect of 5-HT pathway stimulations (1 and 5 Hz) on the firing activity of hippocampal neurons in rats administrated with vehicle and trazodone (C). The numbers in the columns correspond to the number of recorded neurons. * Indicates p < 0.01, comparing the mean SIL value to that of the corresponding value obtained at 1 Hz in the vehicle group. † Indicates statistical significance comparing the mean SIL values obtained at 5 Hz of the trazodone group to that of the corresponding value in the vehicle group by analysis of covariance.

Figure 7. Integrated firing rate histogram of dorsal hippocampus CA3 pyramidal neuron illustrating microiontophoretic application of trazodone (Traz) and 5-HT (A), and responses of dorsal hippocampus CA3 pyramidal neurons to microiontophoretic co-application of 5-HT and trazodone (B), trazodone (C), and 5-HT (D) prior to and following i.v. injection of 5-HT1A antagonist WAY 100,635. The number above each bar corresponds to the ejection current in nA. The number of neurons recorded is displayed in each box. ** p < 0.01
Tables:

Table 1: Affinities (Kᵢ, nM) of trazodone at various binding sites†

<table>
<thead>
<tr>
<th>Binding Site</th>
<th>Rat</th>
<th>Human</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-HT₂A</td>
<td>20ᵃ</td>
<td>25ᶜ</td>
</tr>
<tr>
<td>H₁</td>
<td>32ᵃ,ᵉ</td>
<td>1100ᶜ</td>
</tr>
<tr>
<td>α₁</td>
<td>40ᵃ</td>
<td>42ᶜ</td>
</tr>
<tr>
<td>5-HT₁A</td>
<td>42ᵇ</td>
<td>96ᶜ</td>
</tr>
<tr>
<td>5-HTT</td>
<td>126ᵃ</td>
<td>160ᵈ</td>
</tr>
<tr>
<td>5-HT₂C</td>
<td>251ᵃ</td>
<td>N.D.</td>
</tr>
</tbody>
</table>

ᵃ Millan, 2006;ᵇ Owens et al., 1997;ᶜ Cusack et al., 1994;ᵈ Tatsumi et al., 1997.ᵉ guinea pig. N.D. not determined.
Table 2. Average number of serotonergic neurons per electrode descent in the dorsal raphe\textsuperscript{c}

<table>
<thead>
<tr>
<th>Groups</th>
<th>2 Days</th>
<th>Post-WAY100,635</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>4.9 ± 0.5</td>
<td>(15)</td>
</tr>
<tr>
<td>Trazodone (5 mg/kg)</td>
<td>5.5 ± 0.6</td>
<td>(14)</td>
</tr>
<tr>
<td>Trazodone (10 mg/kg)</td>
<td>4.2 ± 0.7</td>
<td>(10)</td>
</tr>
<tr>
<td>Trazodone (20 mg/kg)</td>
<td>1.7 ± 0.3*</td>
<td>(11)</td>
</tr>
<tr>
<td></td>
<td>4.7 ± 0.7</td>
<td>(7)</td>
</tr>
<tr>
<td></td>
<td>4.2 ± 0.8</td>
<td>(6)</td>
</tr>
</tbody>
</table>

\textsuperscript{c} A descent consisted of lowering a single-barrelled electrode over a 1 mm distance from the floor of the sylvius aqueduct and recording spontaneously firing 5-HT neurons. The numbers in the parenthesis correspond to the number of descents in each group. * p<0.01
Figure 2

A. Vehicle

B. Trazodone

C. Dose-response curve
Figure 5

**A. Vehicle**

- NE: NE 10 10 20
- 5-HT: 10 10

**B. Trazodone**

- NE: NE 10 20 10 20
- 5-HT: 10 20

**C. 5-HT tone**

% increase in firing of CA3 pyramidal neurons

- Vehicle, n = 6
- Trazodone, n = 8

Cumulative WAY 100,635 (µg/kg)

* indicates significant difference.
Figure 7

A. Graph showing spikes per 10 seconds over time (Sec) with different treatments: Quis, 5-HT, and Traz. The graph includes a timeline with bars indicating the application of 5-HT and Traz at specific time points.

B. Bar graph showing the number of spikes suppressed per nA for 5-HT and 5-HT + Traz.

C. Bar graph showing the number of spikes suppressed per nA for Traz and Traz + WAY 100,635.

D. Bar graph showing the number of spikes suppressed per nA for 5-HT and 5-HT + WAY 100,635.

WAY 100,635 (100 μg/kg, i.v.)