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Title:

Differential effects of the 5-HT_{1A} receptor inverse agonists Rec 27/0224 and Rec 27/0074 on electrophysiological responses to 5-HT_{1A} receptor activation in rat dorsal raphe nucleus and hippocampus *in vitro*.

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Running title: Differential antagonism by 5-HT_{1A} receptor inverse agonists

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Words:

Abstract	249
Introduction	664
Discussion	1392

Abbreviations: 5-CT, 5-Carboxamidoytryptamine; 8-OH-DPAT, 8-Hydroxy-dipropylaminotetralin; DRN, dorsal raphe nucleus; GPCR, G-protein coupled receptor; GTPγS, Guanosine 5'-O-(3-thiotriphosphate); Rec 15/3079, N-{2-[4-(2-Methoxyphenyl)-1-piperazinyl]ethyl}-N-(2-nitrophenyl) cyclohexanecarboxamide ; Rec 27/0074, Cyclohexanecarboxylic acid (2-methoxy-phenyl)-{2-[4-(2-methoxyphenyl)-piperazin-1-yl]ethyl}amide; Rec 27/0224, Cyclohexanecarboxylic acid {2-[4-(2-bromo-5-methoxybenzyl)piperazin-1-yl]ethyl}-(2-trifluoromethoxyphenyl)amide; R_{in} , input resistance; r.m.p., resting membrane potential; SSRI, selective serotonin re-uptake inhibitor; WAY 100635, N-{2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl}-N-2-pyridinylcyclo-hexanecarboxamide; V_m, membrane potential; CHO, Chinese hamster ovary; aCSF, artificial cerebrospinal fluid

Recommended section : Neuropharmacology

Abstract

The pharmacological properties of Rec 27/0224 (Cyclohexanecarboxylic acid {2-[4-(2-bromo-5-methoxybenzyl)piperazin-1-yl]ethyl}-(2-trifluoromethoxyphenyl)amide) and Rec 27/0074 (Cyclohexanecarboxylic acid (2-methoxy-phenyl)-{2-[4-(2-methoxyphenyl)-piperazin-1-yl]ethyl}amide) were characterized using radioligand displacement and [^{35}S]-GTPγS binding assays, as well as electrophysiological experiments, in rat hippocampal and dorsal raphe nucleus (DRN) slices. Both compounds showed a high affinity (K_i~1 nM) and selectivity (>70 fold) at human 5-HT$_{1A}$ receptors vs other 5-HT receptors. In [^{35}S]-GTPγS binding assays on HeLa cells stably expressing human 5-HT$_{1A}$ receptors, Rec 27/0224 and Rec 27/0074 inhibited basal [^{35}S]-GTPγS binding by 44.8 ±1.7% (pEC$_{50}$=8.58) and 25 ±2.5% (pEC$_{50}$=8.86), respectively. In intracellularly recorded CA1 pyramidal cells, 5-HT$_{1A}$ (hetero)receptor-mediated hyperpolarization, elicited by 100 nM 5-carboxamidoytryptamine (5-CT), was partially antagonized by Rec 27/0224 (~50%; IC$_{50}$=18.0 nM) and Rec 27/0074 (74%; IC$_{50}$=0.8 nM). In extracellularly recorded DRN serotonergic neurons, Rec 27/0224 and Rec 27/0074 fully antagonized the inhibition of firing caused by the activation of 5-HT$_{1A}$ (auto)receptors by 30 nM 5-CT with an IC$_{50}$ of 34.9 nM and 16.5 nM, respectively. The antagonism had a slow time-course, reaching a steady state within 60 min. Both compounds also antagonized the citalopram-elicited, endogenous 5-HT-mediated inhibition of cell firing. In conclusion, Rec 27/0224 and Rec 27/0074 exhibited inverse agonism in [^{35}S]-GTPγS binding assays and differential antagonistic properties on 5-HT$_{1A}$ receptor-mediated responses in the hippocampus, but not in the DRN. Whether this differential effect is causally related to inverse agonist activity is unclear. The qualitatively different nature of the antagonism in the hippocampus versus the DRN clearly distinguishes the compounds from neutral antagonists, such as WAY 100635 (N-{2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl}-N-2-pyridinylcyclohexanecarboxamide).

Introduction

Impairment of 5-HT neurotransmission is involved in major neuropsychiatric pathological states, e.g. depression, anxiety, schizophrenia and Parkinson's disease. Considerable effort has been consistently made to understand the functioning of the 5-HT system and to develop new selective drugs for 5-HT receptor subtypes.

The 5-HT$_{1A}$ receptor has been extensively studied due to the early discovery of a selective agonist (Gozlan et al., 1983) and receptor cloning (Kobilka et al., 1987; Albert et al., 1990). 5-HT$_{1A}$ receptors are G-protein coupled receptors (GPCRs) expressed throughout the CNS (Pompeiano et al., 1992). Stimulation of 5-HT$_{1A}$ receptors activates G$_{i/o}$ proteins leading to at least two different cellular responses: inhibition of adenylate cyclase and opening of inwardly rectifying K$^+$ channels (Andrade and Nicoll, 1987; Fargin et al., 1989, Penington et al., 1993). 5-HT$_{1A}$ receptors mediate the hyperpolarization of neurons in almost all brain regions (see Barnes and Sharp, 1999), thereby exerting an inhibitory action on cell discharge, including the rhythmic firing of serotonergic cells in the raphe nuclei.

The 5-HT$_{1A}$ receptors expressed in raphe serotonergic cells (5-HT$_{1A}$ autoreceptors) display differential characteristics when compared to 5-HT$_{1A}$ receptors from other, particularly cortical, regions (5-HT$_{1A}$ heteroreceptors). For instance, 5-HT$_{1A}$ receptor agonist-evoked hyperpolarization of dorsal raphe nucleus (DRN) serotonergic neurons, but not of CA1 pyramidal cells, strongly desensitize following treatment with antidepressant drugs, namely selective serotonin re-uptake inhibitors (SSRIs) (Le Poul et al., 2000). It has been suggested that coupling to specific G proteins may be responsible for region-specific differences in 5-HT$_{1A}$ receptor desensitization (Li et al., 1997; Le Poul et al., 2000; Mannoury La Cour et al., 2001; see Hensler, 2003). Furthermore, raphe serotonergic cells have a 5-HT$_{1A}$ receptor reserve (Cox et al., 1993) and respond to weak partial agonists with a higher efficacy than cortical neurons. Thus, 8-Hydroxy-dipropylaminotetralin (8-OH-DPAT) acts as a full agonist in the DRN (Williams et al., 1988), but behaves as a partial agonist in the CA1 hippocampal region (Andrade and Nicoll, 1987; Beck et al., 1992).

The classical criteria of agonism and antagonism based on the theory of occupancy are clearly inadequate to explain these cell-type specific effects of drugs acting at 5-HT$_{1A}$ receptors and more complex models of ligand-G protein coupled receptor interaction should be applied.

According to the extended ternary complex model (Samama et al., 1993), GPCRs exist in at least two states, inactive (R) and active (R*), and ligands that preferentially bind to one of these states, modify their equilibrium. Inverse agonists preferentially bind to R, and when co-applied with agonists, compete for the receptor's binding site, exerting variable degrees of antagonism on the agonists' effect (Christopoulos and Kenakin, 2002, Kenakin, 2004). In addition, some inverse agonists may promote the change in GPCR conformation from R* to R through an allosteric transition (see Neubig et al., 2003). Recently, it was found that as much as 80 % of the receptor ligands acting as antagonists show inverse agonist activity when tested in recombinant receptor systems (Kenakin, 2004). Consistently, the [^{35}S]- Guanosine 5'-O-(3-thiotriphosphate) (GTPγS) binding assays in cells transfected with 5-HT$_{1A}$ receptors revealed that many of the "classical", non-selective 5-HT$_{1A}$ receptor antagonists (e.g. methiothepine, spiperone) are in fact inverse agonists (e.g. Newman-Tancredi et al., 1997). However, several newer compounds, such as N-{2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl}-N-2-pyridinylcyclo-hexanecarboxamide (WAY 100635) (Newman-Tancredi et al., 1997; Testa et al., 1999) and N-{2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl}-N-(2-nitrophenyl) cyclohexanecarboxamide (Rec 15/3079) (Leonardi et al., 2001) have been found to be neutral antagonists.

Although the [^{35}S]-GTPγS binding assay may disclose the inverse agonist nature of ligands, the functional consequences of inverse agonism in native tissue not overexpressing receptors, remains largely speculative.

In the present work, we characterized the pharmacological properties of two novel compounds synthesized toward the 5-HT$_{1A}$ receptors, Rec 27/0074 (Cyclohexanecarboxylic acid (2-methoxy-phenyl)-{2-[4-(2methoxyphenyl)-piperazin-1-yl]ethyl}amide) and Rec 27/0224 (Cyclohexanecarboxylic acid {2-[4-(2-bromo-5-methoxybenzyl)piperazin-1-yl]ethyl}-(2-trifluoromethoxyphenyl)amide). The [^{35}S]-GTPγS binding was used as a parameter for studying the functional consequences of drug binding at the 5-HT$_{1A}$ receptor level, while electrophysiological responses in brain slices were used to characterize cell responses to drug actions on serotonergic cells in the DRN and on pyramidal neurons in the CA1 region of hippocampus.

Methods

All animal manipulations were performed according to the European Community guidelines for animal care (DL 116/92, application of the European Communities Council Directive 86/609/EEC) and approved by the Committee for Animal Care and Experimental Use of the University of Florence.

Radioligand binding assays

Radioligand binding studies for a number of receptors were performed using experimental procedures previously described in detail. Binding to 5-HT$_{1A}$ serotonin receptors was performed on membrane homogenates of HeLa cells stably transfected with human 5-HT$_{1A}$ serotonin receptors (Testa et al., 1999) using [^{3}H]8-OH-DPAT as the radioligand. Binding to human cloned α_1-adrenoceptors was performed on membrane homogenates of Chinese hamster ovary (CHO) cells transfected with DNA expressing the gene encoding each α_1 adrenoceptor subtype (Testa et al., 1995) and labelled with [^{3}H]prazosin. Binding studies on native α_2 adrenoceptors and D$_2$ dopamine receptors (Leonardi et al., 1994) were carried out in membranes of rat cerebral cortex taken from male Sprague Dawley rats (200-300 g, Charles River, Italy), using [^{3}H]Rauwolscine or [^{3}H]spiperone, respectively.

Binding of Rec 27/0224 and Rec 27/0074 to rat 5-HT$_{1B}$, bovine 5-HT$_{1D}$, and human recombinant 5-HT$_{2A}$, 5-HT$_{2B}$, 5-HT$_{2C}$, 5-HT$_3$, 5-HT$_{4c}$, 5-HT$_{4d}$, 5-HT$_{4e}$, 5-HT$_{5A}$, 5-HT$_6$ and 5-HT$_7$ serotonin receptor subtypes was carried out by Cerep at their Poitiers Laboratories (Le bois l'Evêque, 86600 Celle l'Evescault, France) using standardized operating procedures (ISO 9001:2000) fully described and available on line at the website: http://www.cerep.fr/Cerep/Users/pages/catalog/assay/catalog.asp.

[^{35}S]GTPγS binding at 5-HT$_{1A}$ receptors.

The effects of the different compounds tested on [^{35}S]GTPγS binding were evaluated as previously described (Testa et al., 1999) with minor modifications. Cell membranes from HeLa cells transfected with human cloned 5-HT$_{1A}$ receptors were resuspended in buffer containing 20 mM HEPES, 3 mM $MgCl_2$ and 120 mM NaCl (pH 7.4). The membranes were incubated with 10 µM GDP and decreasing concentrations of test drugs (from 10 µM to 0.01 nM) or decreasing concentrations of 5-HT (from 100 µM to 0.1 nM, reference curve, data not shown) for 20 min at 30°C in a final volume of about 0.25 ml. [^{35}S]GTPγS (200 - 250 pM in 10 µl) was added to the samples and incubated for a further 30 min at 30°C. Non-specific binding was determined in the presence of 10 µM GTPγS. The incubation was stopped by the addition of ice-cold HEPES buffer and rapid filtration through Unifilter GF/C filters, using a Filtermate cell harvester (Perkin-Elmer Inc., USA). The filters were washed four times with a total of 1.2 ml of the same buffer. Radioactivity was counted by liquid scintillation spectrometry with an efficiency > 90 % (TopCount Packard, Perkin-Elmer Inc., USA).

The inhibition of specific binding by the compounds was analyzed to estimate the IC$_{50}$ value by the non-linear curve-fitting using Prism 3.02 software (GraphPad Software, San Diego, CA, USA). The IC$_{50}$ value was converted to K$_i$ using the equation by Cheng and Prusoff (1973). Similar fitting procedures were used for the data obtained with the [^{35}S]-GTPγS binding.

Electrophysiological recordings

Methods for obtaining slices and recordings in the two preparations were essentially those previously reported in detail (Corradetti et al., 1996; Corradetti et al., 1998) with minor modifications.

Male Wistar rats (Harlan Italy, Udine, Italy) were anesthetized with ether and decapitated with a guillotine. The brain was quickly removed and cooled in partially frozen oxygenated artificial cerebrospinal fluid (aCSF) and was bubbled with a 95% O_2 / 5% CO_2 gas mixture.

Preparation of slices from DRN and hippocampus. Using a vibratome (T1000, DSK, Japan), a block of tissue containing the dorsal raphe was cut into sections (350-400 µm thick) while immersed in ice-cold aCSF with the following composition (mM): NaCl 120, KCl 3.5, NaH_2PO_4 1.2, $MgCl_2$ 1.3, $CaCl_2$ 2, $NaHCO_3$ 25, D-glucose 11 (pH 7.3). The hippocampi were rapidly isolated and placed on ice-cold oxygenated aCSF with the following composition (mM): NaCl 124, KCl 3.0, NaH_2PO_4 1.25, $MgSO_4$ 1.4, $CaCl_2$ 2, $NaHCO_3$ 25, D-glucose 11 (pH 7.4). Hippocampal slices from the dorsal hippocampus (400 µm thick) were cut using a McIlwain tissue chopper (Gomshall, U.K.).

After sectioning, slices were kept in oxygenated aCSF for at least 1 h at room temperature (20-23°C). A single slice was then placed on a nylon mesh and completely submerged in a small chamber and superfused with oxygenated aCSF (32-34°C) at a constant flow rate of 2-3 ml min^{-1}. The drugs were administered through a three-way tap and a complete exchange of the chamber volume occurred in 1 min.

In some experiments, the DRN slices were incubated in Rec 27/0224 for over 240 min in oxygenated aCSF (32-34°C), while the controls were incubated in similar conditions in the presence of the solvent used for solubilizing Rec 27/0224

Extracellular recording of serotonergic cell firing in DRN slices. Extracellular recordings were made with glass microelectrodes filled with 2 M NaCl (12-15 MΩ). Firing was facilitated by adding the α-adrenoceptor agonist phenylephrine (3 µM) to the superfusing aCSF (Vandermaelen and Aghajanian,1983). Cells were identified as serotonergic neurons according to the following criteria: bi- or triphasic action potentials of 2-3 ms duration, slow (0.5-2.5 Hz) and regular pattern of discharge and inhibition of cell firing produced by 5-HT_{1A} receptor activation. Electric signals were fed into a high-input impedance amplifier (NL 102G, Neurolog, Digitimer Ltd., U.K.), an oscilloscope and an electronic rate meter (D130, Digitimer Ltd., U.K.), triggered by individual neuronal action potentials and connected to an

A/D converter and a personal computer. Using dedicated software, the integrated firing rate was recorded, computed, and displayed on a chart recorder as consecutive 10 s samples.

Intracellular recording from CA1 pyramidal cells. CA1 pyramidal neurons were recorded in current-clamp mode with 3 M KCl- (35-50 MΩ) or 2 M K-methylsulphate- (45-80 MΩ) filled electrodes. Electrical signals were amplified with an Axoclamp 2A (Axon Instruments, Union City, CA, USA) and displayed on an oscilloscope and chart recorder (2800 Gould, Valley View, OH, USA). Traces were stored on a digital tape (DTR 1200, BioLogic, Claix, France; sampling frequency 48 kHz) and on a computer using pClamp software (pClamp 6.02, Axon Instruments) for off-line analysis.

Several criteria were used for choosing cells for the experiments as follows: stable resting membrane potential (r.m.p.) of at least -60 mV and with no spontaneous action potential firing; no sudden drops in the cell membrane input resistance (R_{in}), indicating cell damage; constant amplitude of the spike ($\geq$ 80 mV) obtained by direct activation of the cell during the control period prior to tetrodotoxin application.

When the cells appeared to have reached stable membrane potentials, pulses of hyperpolarizing current (200-400 pA, 400 ms, 0.05-0.1 Hz) were delivered through the recording electrode to monitor changes in R_{in} during drug application.

Concentration-response curves and data analysis

5-Carboxamidoytryptamine (5-CT) was used for to evoke 5-HT$_{1A}$ receptor-mediated responses in CA1 pyramidal cells and DRN serotonergic cells because it has a fast and almost full agonist action in CA1, while other 5-HT$_{1A}$ receptor agonists (e.g. 8-OH-DPAT; Beck 1992) produce much slower and/or partial responses in this region.

The lack of selectivity of 5-CT for 5-HT$_{1A}$ vs 5-HT$_7$ or 5-HT$_{1B}$ receptors should not be a drawback in our experimental conditions because both the responses evoked by 5-CT, cell membrane hyperpolarization in the CA1 and inhibition of cell firing in the DRN, depend solely on 5-HT$_{1A}$ receptor activation. In fact, effect of 5-CT is fully blocked by WAY 100635 in both regions (Corradetti et al, 1996; 1998).

Equiactive 5-CT concentrations producing about 90% of the maximum response in CA1 pyramidal cells (100 nM, Beck et al., 1992; Corradetti et al., 1996) and in the DRN (30 nM, Williams et al., 1988; Corradetti et al., 1998) were used for testing the effects of Rec 27/0224 and Rec 27/0074 on electrophysiological responses to 5-HT$_{1A}$ receptor activation.

In the CA1 region, the effect of 5-CT was measured as the maximal hyperpolarizing response obtained with drug application. In pilot experiments and from previous experience (Corradetti et al., 1998; Corradetti et al., 1996), the application of 5-CT for 3-5 min elicited a steady-state response.

To obtain the IC$_{50}$ of Rec 27/0074 or Rec27/0224 for a given concentration of 5-CT, the effect of 5-CT was tested in control aCSF and in the presence of various concentrations of the drug ($\geq$ 30 min), gradually increased using a cumulative protocol. The magnitude of the response obtained in the presence of each concentration of Rec 27/0074 or Rec 27/0224, normalized vs that obtained in control aCSF, was fitted to a hyperbolic function: $E=E_{max}/[1+(IC_{50}/[D])^n]$, where E was the response produced by the drug under study at the concentration [D], E_{max} was the maximal response, and n was the slope index. A non-linear regression fitting was carried out using Prism 3.02.

In the DRN, a baseline firing rate of at least 5 min (5-15 min) was recorded before the application of 5-CT that was superfused until the maximal response was obtained (2.5-3.5 min). In each experiment, drug application time was kept constant and the interval for measurement of the drug effect, chosen on the basis of time elapsed after 5-CT application (corrected for dead space time) to 50 % recovery of the firing rate, was kept constant for the following applications in the presence of the various drugs under study. This procedure was favored vs the measurement of peak responses to 5-CT because it also took the duration of 5-CT effects into consideration and allowed for better measurements of smaller responses.

The number of action potentials discharged during control 5-CT application was measured and compared to that expected in the same interval of time calculated on the basis of the average firing rate recorded for 2 min immediately preceding 5-CT application. All responses to 5-CT obtained in the presence of the inverse agonists were normalized vs the response to 5-CT in the control.

In experiments in which citalopram effects were tested, steady-state responses were measured at the last min of drug application (10 min) for each concentration.

Drugs

The compounds coded as Rec and WAY 100635 were synthesized in the Recordati Chemical Department (Recordati S.p.A, Milan, Italy) according to the methods described in the following US patents: Rec 27/0074, Rec 27/0224 and Rec 15/3079 in US 6,399,614; WAY 100635 in US 6,127,357. [^{3}H]8-OH-DPAT, [^{3}H]prazosin, [^{3}H]Rauwolscine, [^{3}H]Spiperone and [^{35}S]-GTPγS were obtained from NEN Life Science Products (Milan, Italy). For receptor binding studies, the compounds were dissolved in absolute alcohol or de-ionized water according to their solubility.

Spiperone hydrochloride and 5-CT maleate were from Research Biomedical Inc. (Natick, USA); GDP, GTPγS and phenylephrine hydrochloride were from Sigma-Aldrich (Milan, Italy); tetrodotoxin was from Alomone Labs (Jerusalem, Israel). Citalopram was from Recordati S.p.A.

In the electrophysiological experiments, Rec 27/0224 was dissolved (25 mM) in dimethylformamide and stored in a refrigerator at 4°C. Aliquots (10 µl) were taken the day of the experiment and added with 240 µl Tween-80/H_2O (1:1000 vol/vol) to obtain an intermediate stock solution of 1 mM which was further diluted > 1000-fold in aCSF and used for the experiments. In control experiments (n=3), the application (90 min) of the solvent at a concentration 3-fold higher than that used for the present experiments did not affect the response to 5-CT.

Statistical analysis

Numerical data are given as the means ± s.e. mean. Wilcoxon or Mann-Whitney tests were used for statistical analysis, as appropriate; a value of $P<0.05$ was considered statistically significant.

Results

The activity at 5-HT$_{1A}$ receptors of Rec 27/0224 and Rec 27/0074 were investigated using radioligand and [^{35}S]-GTPγS binding methods. In these assays spiperone, a known inverse agonist at 5-HT$_{1A}$ receptors (Newmann-Tancredi, 1997) was used for comparison.

Radioligand binding studies

Rec 27/0224 and Rec 27/0074 showed high-affinity binding at human 5-HT$_{1A}$ receptors expressed in HeLa cells with Ki values for displacing 1.04 nM (pK$_i$ = 8.98) and 0.9 nM (pK$_i$ = 9.05) of [^{3}H]8-OH-DPAT, respectively (Table 1). Both compounds were highly selective for 5-HT$_{1A}$ receptors showing a moderate affinity for h5-HT$_{2B}$ (pK$_i$ = 7.0) and h5-HT$_7$ (pK$_i$ = 7.15), and pK$_i$ values ≤ 6.5 for rat 5-HT$_{1B}$, bovine 5-HT$_{1D}$, human recombinant 5-HT$_{2A}$, 5-HT$_{2B}$, 5-HT$_{2C}$, 5-HT$_3$, 5-HT$_{4c}$, 5-HT$_{4d}$, 5-HT$_{4e}$, 5-HT$_{5A}$, 5-HT$_6$ and 5-HT$_7$ serotonin receptor subtypes. Rec 27/0224 was 200-10,000-fold more selective for 5-HT$_{1A}$ receptors vs human α_{1a}-, α_{1b}-, α_{1d}- adrenoceptors, rat α_2-adrenoceptors and D$_2$ receptors. Rec 27/0074 showed a lower (4-10-fold) selectivity vs human α_1-adrenoceptor subtypes. For comparison, binding data for spiperone and for two structurally related 5-HT$_{1A}$ receptor antagonists, WAY 100635 and Rec 15/3079 are included in Table 1.

Functional studies - [^{35}S]-GTPγS binding

The functional intrinsic activity of Rec 27/0224, Rec 27/0074 and spiperone was measured with [^{35}S]-GTPγS binding studies carried out in HeLa cells stably expressing 5-HT$_{1A}$ receptors. All compounds concentration-dependently inhibited basal binding of [^{35}S]-GTPγS, an effect typical of inverse agonism (Figure 1).

The pEC$_{50}$ and maximal inhibition of [^{35}S]-GTPγS binding, calculated from concentration-binding curves, for Rec 27/0224 was 8.58 (95% C.L.: 8.80-8.36) and 44.8 ± 1.7 %, respectively. This effect of Rec 27/0224 was comparable to that of spiperone for which a

pEC_{50} value of 8.17 (95% C.L.: 8.21-8.12) and a maximum [^{35}S]-GTPγS binding inhibition value of 40.2 ± 0.4 % were found (not shown). Rec 27/0074 produced a smaller (25 ± 2.5 %) inhibition of [^{35}S]-GTPγS binding (pEC_{50}: 8.86; 95% C.L.: 8.96-8.77).

Functional studies - Electrophysiology

Experiments were carried out in 83 DRN and 32 CA1 hippocampal slices obtained from 74 rats.

Effects of Rec 27/0224 and Rec 27/0074 on 5-HT$_{1A}$ receptor-mediated hyperpolarization in CA1 pyramidal cells.

To investigate the effects of inverse agonists at the level of postsynaptic cells, we tested Rec 27/0224 and Rec 27/0074 on the 5-HT$_{1A}$ receptor-mediated hyperpolarization produced by 5-CT in hippocampal CA1 pyramidal neurons. Intracellular recordings from CA1 pyramidal cells (r.m.p. –63.5 ± 0.9 mV; R_{in} 48.2 ± 1.8 MΩ; n=32) were performed in the presence of tetrodotoxin (1 µM) to functionally isolate postsynaptic effects of the drugs. Rec 27/0224 and Rec 27/0074 (1-300 nM) did not significantly affect cell r.m.p. and R_{in} (Wilcoxon test), although a slight depolarizing trend was observed in the presence of Rec 27/0224. After 60-90 min application of 300 nM Rec 27/0224, or at the end of cumulative concentration-response curves, the change in membrane potential (ΔVm) was 1.8 ± 0.9 mV, while R_{in} was 103±3 % of the control (n=9). Similarly, a ΔVm of 0.5 ± 0.9 V_m, and a R_{in} of 102 ± 4 % (n=5) were found for 300 nM Rec 27/0074. The lack of hyperpolarizing effects of the two compounds indicates the absence of agonist action at 5-HT$_{1A}$ receptors.

Both compounds decreased 5-HT$_{1A}$ receptor-mediated hyperpolarization produced by 5-CT (100 nM) in a concentration-dependent manner (Figure 2), but were unable to completely block 5-CT responses within the range of concentrations tested (1-300 nM).

The IC_{50} of Rec 27/0224, obtained from the curve relating the effects of 5-CT in the presence of increasing concentrations of Rec 27/0224, was 18.0 nM (95 % C.L.: 3.2 - 90.2 nM) and the maximal block of 5-CT induced hyperpolarization was 53.4 % (95 % C.L.: 38.4 - 76.4 %). In five of these experiments, concentration-response curves could also be obtained for individual cells leading to an average IC_{50} value of 18.2 ± 14.5 nM and a calculated maximal effect of 54.5 ± 4.5 %. In additional experiments, a single concentration of Rec 27/0224 (300 nM), corresponding to the highest used in cumulative curves, was applied for 60-90 min. The resulting block of 5-CT-induced hyperpolarization (47.2 ± 15.8 %, n=4) was not statistically different (Mann-Whitney test) from that observed with concentration-response experiments.

The other inverse agonist, Rec 27/0074, appeared more potent in antagonizing 5-CT-induced hyperpolarization of CA1 pyramidal cells with an IC_{50} of 0.8 nM (95 % C.L.: 0.5 - 1.3 nM) and maximal block of 74.1 % (95 % C.L.: 64.2 - 84.0 %).

Effects of Rec 27/0224 and Rec 27/0074 on the 5-HT$_{1A}$ receptor-mediated response of DRN serotonergic cells

Extracellular recordings of DRN serotonergic cell firing were used to investigate the effects of inverse agonists on 5-HT$_{1A}$ autoreceptors. In these experiments, multiple applications of the 5-HT$_{1A}$ receptor agonist 5-CT were used to reversibly inhibit cell firing in control conditions and in the presence of a single concentration of an inverse agonist.

Application of Rec 27/0224 or Rec 27/0074 (10-300 nM), *per se*, increased the cell firing rate in about 80 % of the recordings with no change or small (<10 %) decreases in the remaining cells.

It is noteworthy that the firing rate of serotonergic cells is dependent on activation of α_1-adrenoceptors for which Rec 27/0224 and Rec 27/0074 have a moderate affinity (Table 1). However, since in our experimental conditions, α_1-adenoceptors are maximally activated by phenylephrine and the two compounds did not decrease the cell firing rate, the contribution of α_1-adrenoceptor antagonism to their effects can be ruled out.

The increase in cell firing rate often developed slowly, reaching a steady state within 30-40 min of application (e.g. Figure 4B). At the highest concentration tested (300 nM, 60 min), Rec 27/0224 and Rec 27/0074 increased the firing rate of serotonergic cells by 21.0 ± 6% (n=6) and 22.5 ± 8.3 % (n=7), respectively.

Rec 27/0224 antagonized the 5-CT-mediated inhibition of cell firing in a time- and concentration-dependent manner. The threshold concentration of Rec 27/0224 for observing a depression in the response to 30 nM 5-CT was 10 nM (Figure 3A). The effect of Rec 27/0224 was detectable after 30 min of application and increased during the first hour. In order to assess whether the effect of Rec 27/0224 had reached a steady state within 1 h of drug application, in a separate set of experiments, the slices were pre-incubated for 240-280 min in the presence of the compound. As shown in Figure 3B,c, the pre-incubation with 10 nM Rec 27/0224 decreased the response to 5-CT by 27.8 ± 8.9 % (n=9), a reduction no different from that obtained at 60 min of superfusion (24.7 ± 7.1 %, n=6, Mann-Whitney test). The antagonism of responses to 5-CT developed with a similar time-course with higher concentrations of Rec 27/0224 (Figure 4A). These results show that Rec 27/0224 had reached a steady state within 60 min of application. Furthermore, repetitive applications of 5-CT were not responsible for the slow time-course of Rec 27/0224 effects. Indeed, a single application of 5-CT after superfusion of 100 nM Rec 27/0224 for 60 min (Figure 4B) produced a response very similar to that observed in experiments where 5-CT was applied three times during Rec 27/0224 application (29.3 ± 5.4, n=5 and 29.4 ± 7.4, n=5, respectively, Mann-Whitney test).

Concentration-response relationships for the inhibition of 5-CT responses by Rec 27/0224 and Rec 27/0074 at an application of 60 min are shown in Figure 4C. The IC_{50} values obtained from curves for Rec 27/0224 and Rec 27/0074 were 34.9 nM (95 % C.L.: 22.5-54.3 nM) and 16.5 nM (95 % C.L.: 11.9-22.8 nM), respectively.

In contrast with the results obtained in CA1 pyramidal cells, in serotonergic cells, both compounds apparently fully antagonized the 5-CT-evoked inhibition of the firing rate.

To compare the effects of inverse agonists in the two regions with those of orthosteric antagonists, we re-analyzed raw data obtained with WAY 100635 in our previous work (Corradetti et al., 1996; Corradetti et al., 1998). The IC_{50} values of WAY 100635 were 0.98 ± 0.09 nM (n=5) and 3.27 ± 03 (n=6) for the effect of 100 nM 5-CT on pyramidal cells and 30 nM 5-CT on serotonergic cells, respectively. Therefore, it appears that inverse agonists, particularly Rec 27/0224 and the neutral antagonist WAY 100635, have opposite region-potency relationships.

In a final set of experiments, we investigated whether inverse agonists could block the effects of endogenous 5-HT on the firing rate of serotonergic cells (Figure 5).

To activate 5-HT_{1A} receptors with endogenous 5-HT and thereby inhibit serotonergic cell firing, we used citalopram, which elicits an increase in extracellular 5-HT levels through the selective block of 5-HT transporters (Arborelius et al., 1995).

Superfusion with increasing concentrations of citalopram caused a concentration-dependent inhibition of serotonergic cells with an IC_{50} value of 59.9 nM (95 % C.L.: 58.7-61.1 nM; n=6; Figure 5B). Pre-treatment of DRN slices with Rec 27/0224 (100 nM, 60 min, n=5) potently antagonized citalopram effects (Figure 5A,B). Rec 27/0074 exerted a similar effect on citalopram-elicited inhibition of serotonergic cell firing (Figure 5C). Therefore, the two inverse agonists were able to antagonize the inhibitory effect of endogenous 5-HT on the firing of serotonergic cells.

Discussion

Our data demonstrate that two selective 5-HT$_{1A}$ receptor inverse agonists display differential antagonism of electrophysiological responses at native 5-HT$_{1A}$ receptors expressed by serotonergic cells of DRN and CA1 pyramidal neurons. These results provide the first electrophysiological characterization of the functional effects of 5-HT$_{1A}$ receptor inverse agonists in brain slices and show that a region-specific block of 5-HT$_{1A}$ receptor-mediated responses can be achieved using this class of 5-HT$_{1A}$ receptor ligands.

Rec 27/0224 and Rec 27/0074, are high-affinity ligands at 5-HT$_{1A}$ receptors with a high degree of selectivity for this 5-HT receptor subtype (Table1).

Both compounds have significant inverse agonist activity in [^{35}S]-GTPγS assays, qualitatively similar to that of spiperone, a recognized 5-HT$_{1A}$ receptor inverse agonist (Newman-Tancredi et al., 1997). In the same conditions, a structurally related compound, Rec 15/3079, acted as a neutral antagonist in [^{35}S]-GTPγS binding assays (Leonardi et al., 2001), as well as WAY 100635 (Newman-Tancredi et al., 1997; Testa et al., 1999). The binding affinities of Rec 27/0224 and Rec 27/0074 at 5-HT$_{1A}$ receptors were similar, whereas their efficacy in inhibiting [^{35}S]-GTPγS binding differed significantly, indicating that other intrinsic properties of the two compounds, and not affinity for 5-HT$_{1A}$ receptors, were responsible for their differences in efficacy.

One of the functional consequences of inverse agonism at a given receptor is antagonism of the responses elicited by agonists. Accordingly, Rec 27/0224 and Rec 27/0074 behaved as antagonists in electrophysiological studies. However, comparison of their effects with those of the orthosteric antagonist WAY 100635 revealed peculiar pharmacological characteristics of inverse agonists.

Characteristics of 5-HT$_{1A}$ receptor antagonism by inverse agonists

In a substantial fraction of the recorded serotonergic cells, the firing rate was increased by Rec 27/0224 and Rec 27/0074. This effect developed slowly and had a variable magnitude. The increase in firing rate by Rec 27/0224 and Rec 27/0074, however, cannot directly be interpreted as a phenotypic manifestation of inverse agonism in electrophysiological assays, analogue to the decrease in [^{35}S]-GTPγS binding. In fact, WAY 100635, a neutral antagonist at 5-HT$_{1A}$ receptors, also produces variable increases in the cell firing rate. This effect has been ascribed to relief from the inhibition of cell firing exerted by endogenous 5-HT at 5-HT$_{1A}$ receptors (Craven et al., 1994; Gartside et al., 1995; Corradetti et al., 1996; Mundey et al., 1996; Fornal et al., 1996) and the variability of responses likely results from different levels of extracellular 5-HT in individual *in vitro* preparations. Notably, the effect of WAY 100635 was much faster than that of inverse agonists.

A characteristic that more clearly differentiates Rec 27/0224 and Rec 27/0074 from neutral antagonists is the slow kinetics to reach steady-state antagonism of 5-HT$_{1A}$ receptor-mediated responses. In DRN, the fact that the effect of 10 nM Rec 27/0224 reached its plateau at 60 min and progressed no further at 240 min, allows the conclusion that the effect was concentration-dependent, but that the mechanisms of action of the drug needed time to fully develop its effects. A low diffusion rate in the tissue was unlikely, since Rec 27/0224 is much more lipophilic than Rec 27/0074, but both needed more than 40 min to reach a steady state response. The possibility that slow cellular mechanisms were involved (e.g. disappearance of receptors through internalization or persistent uncoupling from G proteins) is also unlikely because, in that case, prolonged application of the drugs would have produced continuous increases in the drug effect.

In contrast, the relatively long time required by Rec 27/0224 and Rec 27/0074 for achieving their full effects could be explained by an allosteric action typical of inverse agonists, comprising a slow shift of the receptors toward inactive conformation(s).

Several further differences characterize Rec 27/0224 and Rec 27/0074 effects in comparison to those of neutral antagonists.

For instance, in the same experimental conditions used in the present work, WAY 100635 fully blocked the effects of 5-CT in the CA1 pyramidal cells at lower concentrations than those needed in DRN serotonergic cells (Corradetti et al., 1996; Corradetti et al., 1998). In contrast, inverse agonists, and in particular Rec 27/0224, were more effective in the DRN than in the hippocampus.

More interestingly, inverse agonists did not fully antagonize the 5-HT$_{1A}$ receptor-mediated cell membrane hyperpolarization in CA1 pyramidal cells. In individual experiments, a substantial fraction (20-50 %) of the response to 5-CT was left unaffected by both compounds at concentrations fully effective in serotonergic cells. A similar region-specific antagonism of 5-HT$_{1A}$ electrophysiological responses was found *in vivo* using the inverse agonist spiperone (Blier et al., 1993).

Considering that Rec 27/0224 and Rec 27/0074 can completely displace 8-OH-DPAT in binding assays, the expected effect of these inverse agonists in electrophysiological experiments in the hippocampus was full antagonism of 5-CT actions. However, the binding studies were done on cell fragments, while the functional studies were performed on intact cells. The partial antagonism of electrophysiological responses in hippocampus by Rec 27/0224 and Rec 27/0074 suggests the existence of two functional subpopulations of 5-HT$_{1A}$ receptors in intact CA1 pyramidal cells. One possibility is that in these neurons 5-HT$_{1A}$ receptors can take on a conformation fully responsive to agonists, but not to inverse agonists. An alternative explanation, although thermodynamically unfavorable, is that these compounds *induce* conformational changes of 5-HT$_{1A}$ receptors leading to forms with a low affinity for inverse agonists, but still responsive to agonists.

Molecularly identical 5-HT_{1A} receptors may take on conformations differentially responsive to ligands when located in specific subcellular microenvironment (e.g. somatic vs dendritic spines or trunk) or in different neurons. This difference can be explained on the basis of the expected properties of the interaction of inverse agonists with GPCRs. In fact, the shift between active and inactive receptor conformations that an inverse agonist ligand can produce is constrained by equilibrium constants proper of that GPCR. On the other hand, these constants depend on the microenvironment surrounding the GPCR (Christopoulos and Kenakin, 2002). This encompasses the type of G-protein coupled, the "tightness" of coupling, the number of receptors and/or phosphorylation etc., and therefore may be different for the same receptor protein in different neurons. Thus coupling of 5-HT_{1A} receptors to different G-proteins in CA1 pyramidal cells and serotonergic cells might *per se* explain differential receptor behavior in the two brain regions, as suggested by Hensler (2003). It has consistently been reported (Mannoury La Cour et al., 2001) that 5-HT_{1A} receptors are coupled to Gαo proteins in the hippocampus and to Gαi3 proteins in the DRN. Interestingly, inverse agonists have been found to inhibit Gαi3 activation by 5-HT_{1A} receptors expressed in CHO cells (Newman-Tancredi et al., 2002).

In summary, regardless of the exact molecular mechanism of action of these compounds in the hippocampus, the pharmacological properties of Rec 27/0224 and Rec 27/0074 support the notion that these ligands acted through a mechanism different than orthosteric antagonism and that a region-specific block of 5-HT_{1A} receptors can be achieved by these ligands.

Possible therapeutic implications of region-specific antagonism at 5-HT_{1A} receptors.

Administration of SSRIs causes an immediate increase in brain serotonin level, which appears to be necessary for the subsequent development of the antidepressant action. The increase in serotonin level, at the same time, activates 5-HT_{1A} somatodendritic autoreceptors leading to inhibition of serotonergic cell firing. This negative feedback is believed to contribute to the slow onset of the therapeutic effect of SSRIs (2 to 3 weeks). 5-HT$_{1A}$ receptor antagonists block the inhibition of serotonergic cell discharge caused by the SSRI-elicited increase in 5-HT levels (Arborelius et al., 1995; Romero and Artigas, 1997), suggesting the potential utility of 5-HT$_{1A}$ receptor antagonists in hastening the therapeutic effect of antidepressant drugs (see Artigas et al., 1996). For this purpose, an ideal antagonist should *selectively* block raphe 5-HT$_{1A}$ autoreceptors without affecting postsynaptic 5-HT$_{1A}$ heteroreceptors. However, the currently available antagonists appear devoid of regional selectivity (Corradetti et al., 1996, 1998).

The observed block of the citalopram-induced inhibition of DRN cell firing by Rec 27/0224 and Rec 27/0074, and their limited effects at hippocampal 5-HT$_{1A}$ receptors suggest that inverse agonists might accelerate the onset of SSRI therapeutic actions *in vivo* (Gartside et al., 1995; see Artigas et al., 1996), with minimal interference at postsynaptic 5-HT$_{1A}$ receptors.

In conclusion, we demonstrate that two 5-HT$_{1A}$ receptor inverse agonists display a considerable degree of region selectivity and are effective in blocking the effects of SSRIs in the raphe. As to whether or not these characteristics are relevant to therapeutic effects needs to be seen in *in vivo* testing.

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Figure 1

Rec 27/0224 and Rec 27/0074 inhibit basal [^{35}S]-GTPγS binding to HeLa cell membranes expressing human 5-HT$_{1A}$ receptors.

Basal [^{35}S]GTPγS binding was taken as 100 % and determined in the absence of the compound. Data were expressed as a percent of the basal [^{35}S]-GTPγS and are mean values ± S.E.M.from three individual experiments, each performed in duplicate. Curves are fitted by a four-parameter logistic equation (see Methods) and [^{35}S]-GTPγS minimum binding values obtained with Rec 27/0224 and Rec 27/0074 were 55.1 ± 1.7 and 74.9 ± 2.5 %, respectively.

Figure 2

Rec 27/0224 and Rec 27/0074 partially inhibit 5-HT$_{1A}$ receptor-mediated hyperpolarization in CA1 pyramidal cells.

(A) Chart recordings of CA1 pyramidal cell membrane responses to 5-CT (100 nM, bar) elicited in controls (left trace) and after 90 min application of 300 nM Rec 27/0224 (right trace). Downward deflections are electrotonic cell membrane responses to constant current steps (-200 pA, 400 ms, 0.05 Hz) injected through the recording electrode to monitor R_{in}. TTX (1 μM) was present throughout the experiment. Calibrations: 5 mV, 3 min. K-methylsulphate-filled electrode, r.m.p.: –63 mV, R_{in} 49 MΩ. (B) Concentration-response curves for the antagonism exerted by Rec 27/0224 and Rec 27/0074 on 5-HT$_{1A}$ receptor-mediated hyperpolarization. Symbols represent mean values ± S.E.M.(n= 5-9). Curves are fitted by a four-parameter logistic equation. Note that neither Rec 27/0224 nor Rec 27/0074 completely antagonized the response to 5-CT.

Figure 3

The effect of Rec 27/0224 develops slowly and reaches a steady state within one hour of application.

(A) Application of 5-CT (30 nM, bars) reversibly decreased the firing rate of recorded DRN cells contributing to characterization as serotonergic cells. Subsequent applications of 5-CT in the presence of Rec 27/0224 (at 10, 30 and 60 min drug applications) resulted in increasingly smaller responses. The firing rate is expressed as the number of action potentials discharged per 10 s (Spikes 10 s^{-1}). Note the small increase in neuron firing rate during superfusion of Rec 27/0224. (B) Time-course of the response to 5-CT application (30 nM, 3 min, bar) obtained in slices pre-incubated for $\geq$ 240 min with 10 nM Rec 27/0224 (open circles, n=9) or with the solvent (filled circles, n=9). Symbols represent the means ± S.E.M.of the normalized firing rate, expressed as a percent of the baseline firing rate computed over the 5 min preceding 5-CT application in each experiment. (C) Inhibition of serotonergic cell firing rate by 5-CT at different time points of Rec 27/0224 (10 nM) application . 5-CT effects in the presence of Rec 27/0224 is expressed as a percent of the response to 5-CT in the controls before application of the inverse agonist.

Figure 4

Rec 27/0224 and Rec 27/0074 inhibit serotonergic cell responses to 5-CT in a concentration-dependent manner.

(A,B) The effect of Rec 27/0224 did not depend on repeated agonist (5-CT, 30 nM, bars) applications. After 60 min of Rec 27/0224 superfusion, the decreases in response to 5-CT were similar irrespective of the multiple (A) or single (B) application protocols used. In (A) and (B) the firing rate is expressed as the number of action potentials discharged per 10 s (Spikes 10 s^{-1}). (C) Concentration-response curves for the antagonism exerted by Rec 27/0224 or Rec 27/0074, applied for 60 min, on 5-CT-elicited inhibition of serotonergic cell firing. Symbols represent the means ± S.E.M.(n= 5-10) of 5-CT effects expressed as a percent of the control 5-CT response obtained in each preparation before inverse agonist application. Curves are fitted by a four-parameter logistic equation. Note that the response to 5-CT could be completely antagonized by both Rec 27/0224 or Rec 27/0074.

Figure 5

The inhibition of serotonergic cell firing elicited by citalopram through an increase in extracellular 5-HT levels is antagonized by Rec 27/0224 and Rec 27/0074.

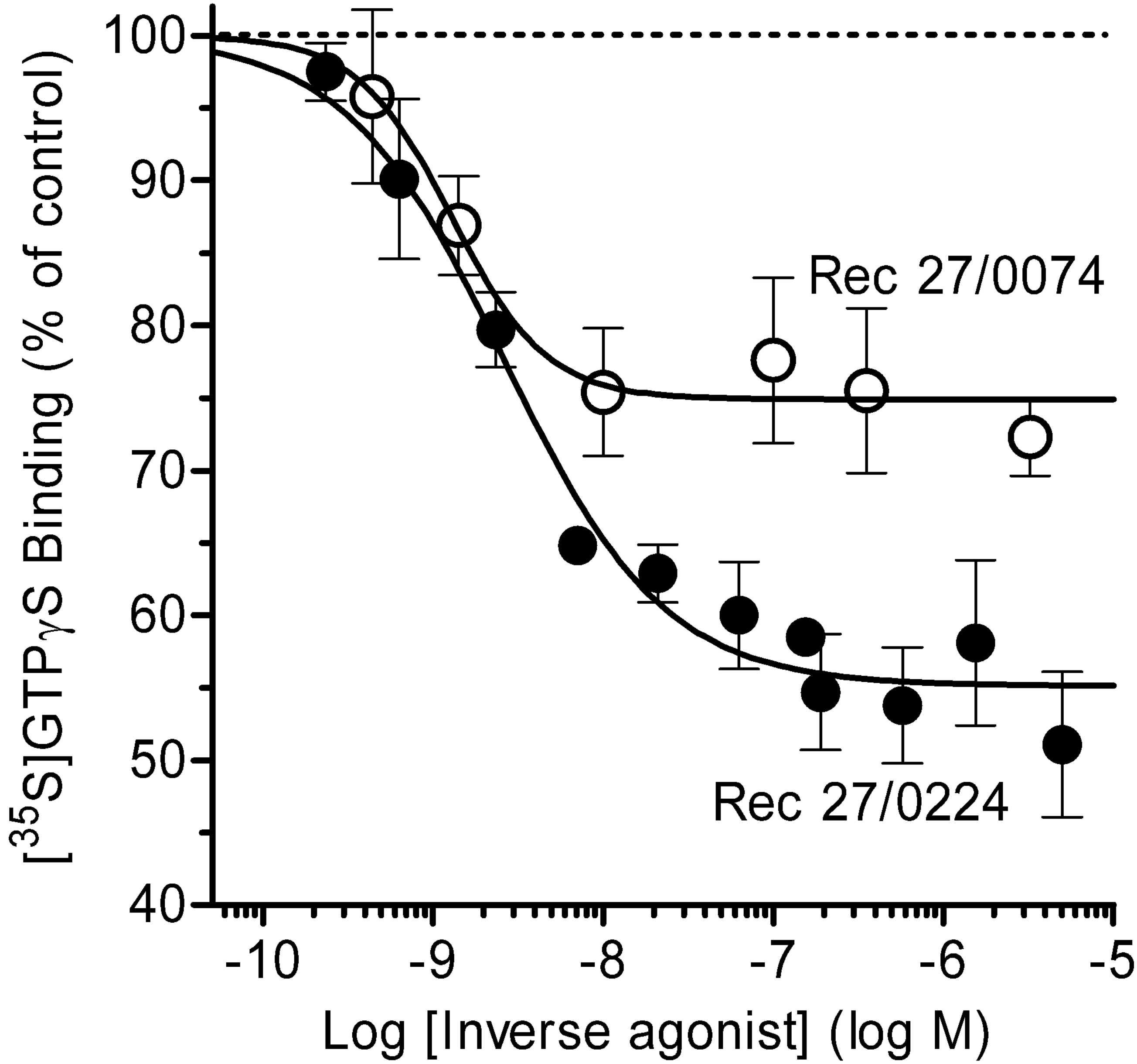
(A) Single cell recording from a serotonergic cell. Increasing concentrations of citalopram (0.3-10 µM, staircase bar), after 60 min superfusion of 100 nM Rec 27/0224, slightly decreased the firing rate of the recorded serotonergic cell. The cell was sensitive to 5-CT application before the addition of Rec 27/0224 (not shown). (B) Concentration-response curves comparing the effects of citalopram obtained in the presence of Rec 27/0224 (100 nM, n=5) and in control aCSF (n=6) show that the inverse agonist almost completely blocked the effect of citalopram. Symbols represent the means ± S.E.M.of firing rate values expressed as a percent of the firing rate recorded before citalopram application. Curves are fitted by a four-parameter logistic equation. (C) Application of 300 nM citalopram after 60 min superfusion of Rec 27/0074 (100 nM) did not decrease the firing rate of a serotonergic cell. The cell was sensitive to 5-CT application before the addition of Rec 27/0074 (not shown). Similar results were obtained in three further cells. In (A) and (C), the firing rate is expressed as the number of action potentials discharged per 10 s (Spikes 10 s^{-1}).

TABLE 1
Inverse agonist and antagonist binding affinities at selected receptors

	Receptor					
Ligand	5-HT$_{1A}$	α_{1a}	α_{1b}	α_{1d}	α_2	D$_2$
Rec 27/0224	8.98	6.65	6.19	6.14	< 5.0	5.32
Rec 27/0074	9.05	8.29	8.99	8.38	< 5.0	6.50
Spiperone	7.48	7.69	8.51	8.08	6.87	9.22
Rec 15/3079 (a)	9.70	7.09	6.98	8.24	< 5.0	6.06
WAY 100635 (b)	9.48	7.73	6.18	8.34	5.90	6.46

Human 5-HT$_{1A}$ receptors were expressed in HeLa cells and labelled using [^{3}H]8-OH-DPAT , human α_1 receptor subtypes expressed in CHO cells were labelled with [^{3}H]prazosin. The binding at α_2 receptors was performed in rat cortex membranes using [^{3}H]Rauwolscine as the radioligand and D$_2$ receptors were labelled with [^{3}H]Spiperone in rat striatum membranes. Data are expressed as the mean of pK$_i$ (- log Ki) values obtained from at least three determinations in triplicate. Values for Rec 15/3079 and WAY 100635 are taken from Leonardi et al., 2001(a) and Testa et al., 1999 (b), respectively.

Figure 1



100
90
80
70
60
50
40
$[^{35}S]GTP\gamma S$ Binding (% of control)
Rec 27/0074
Rec 27/0224
-10
-9
-8
-7
-6
-5
Log [Inverse agonist] (log M)

Figure 2

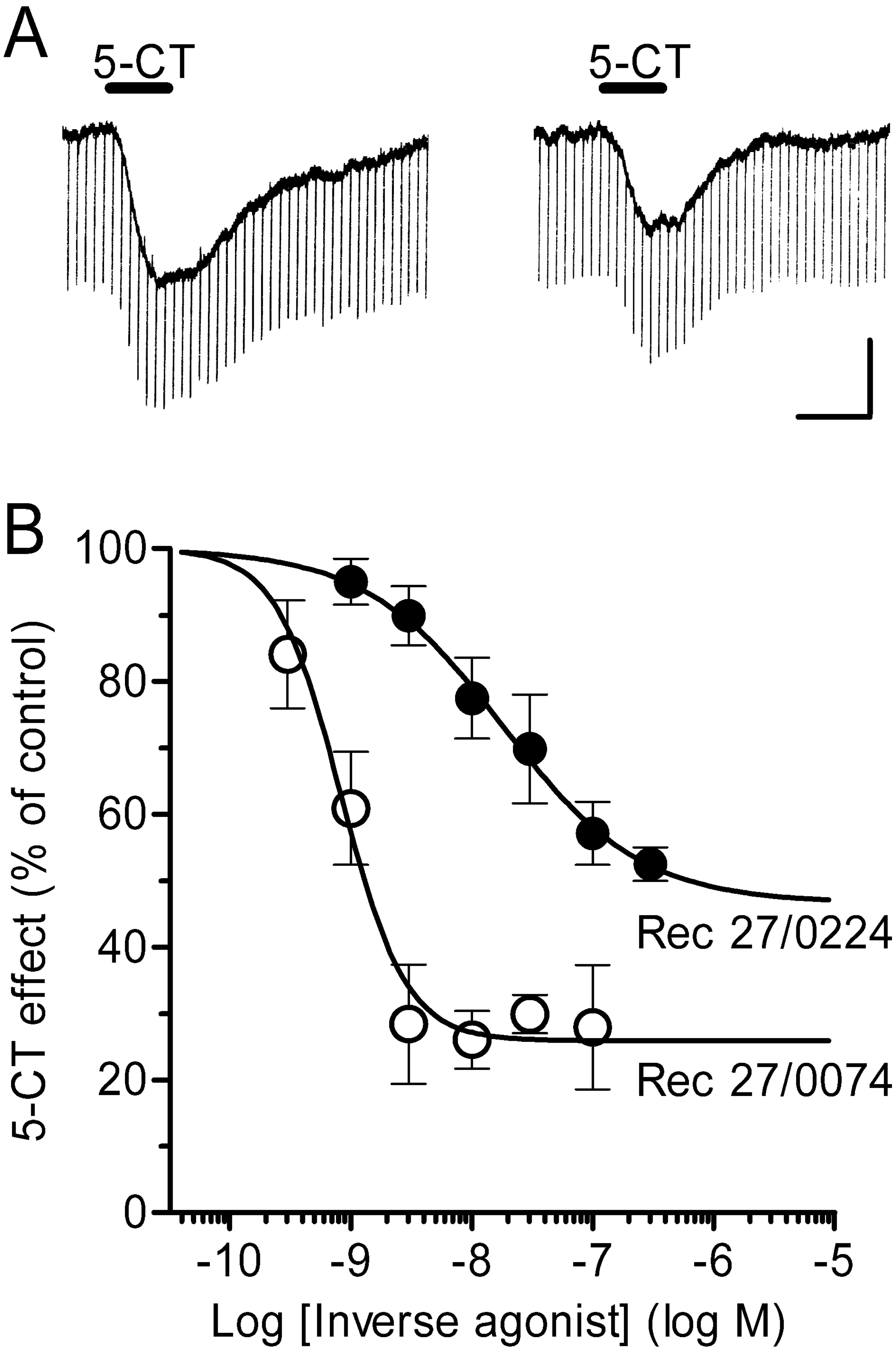

A
5-CT
5-CT
B
100
80
60
40
20
0
5-CT effect (% of control)
-10
-9
-8
-7
-6
-5
Log [Inverse agonist] (log M)
Rec 27/0224
Rec 27/0074

Figure 3

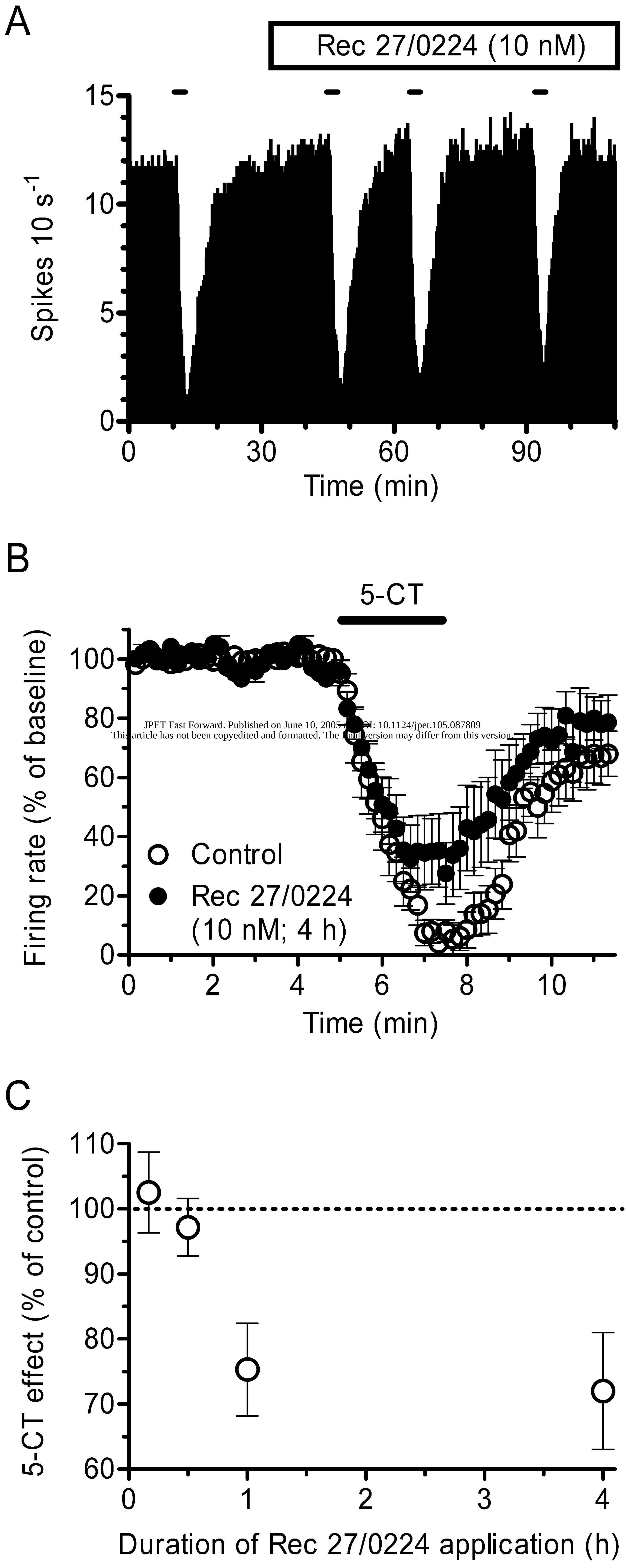
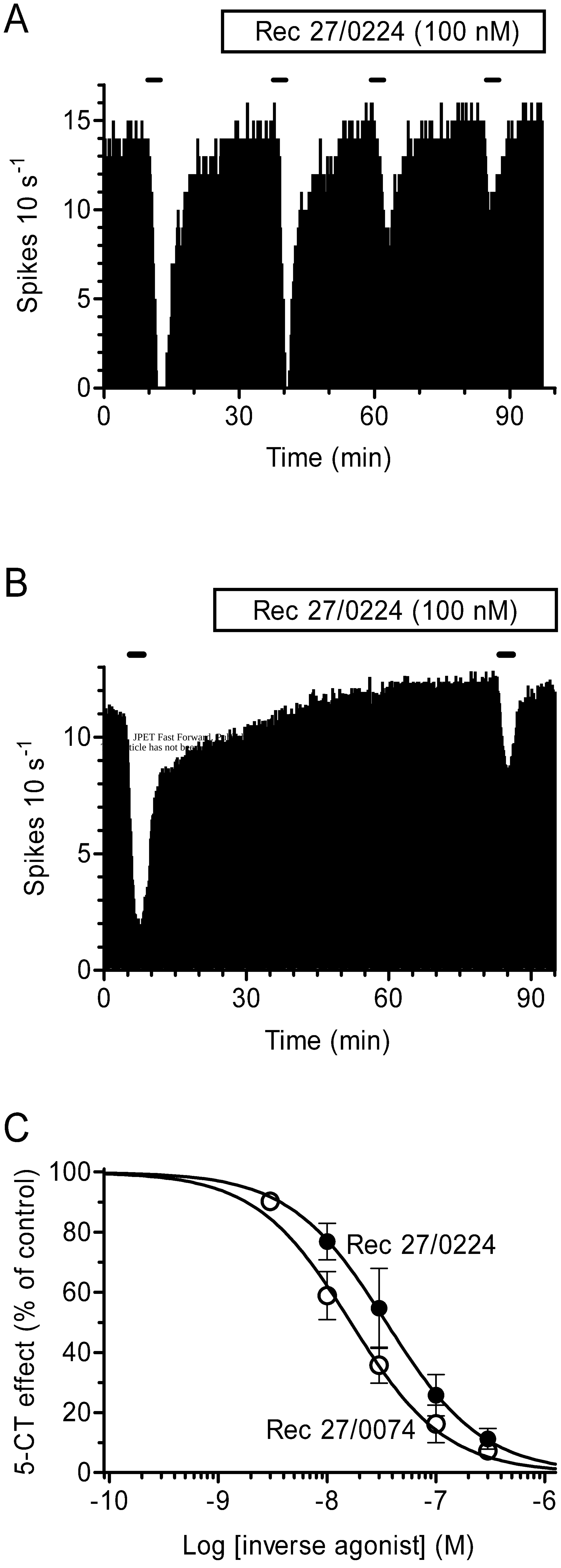
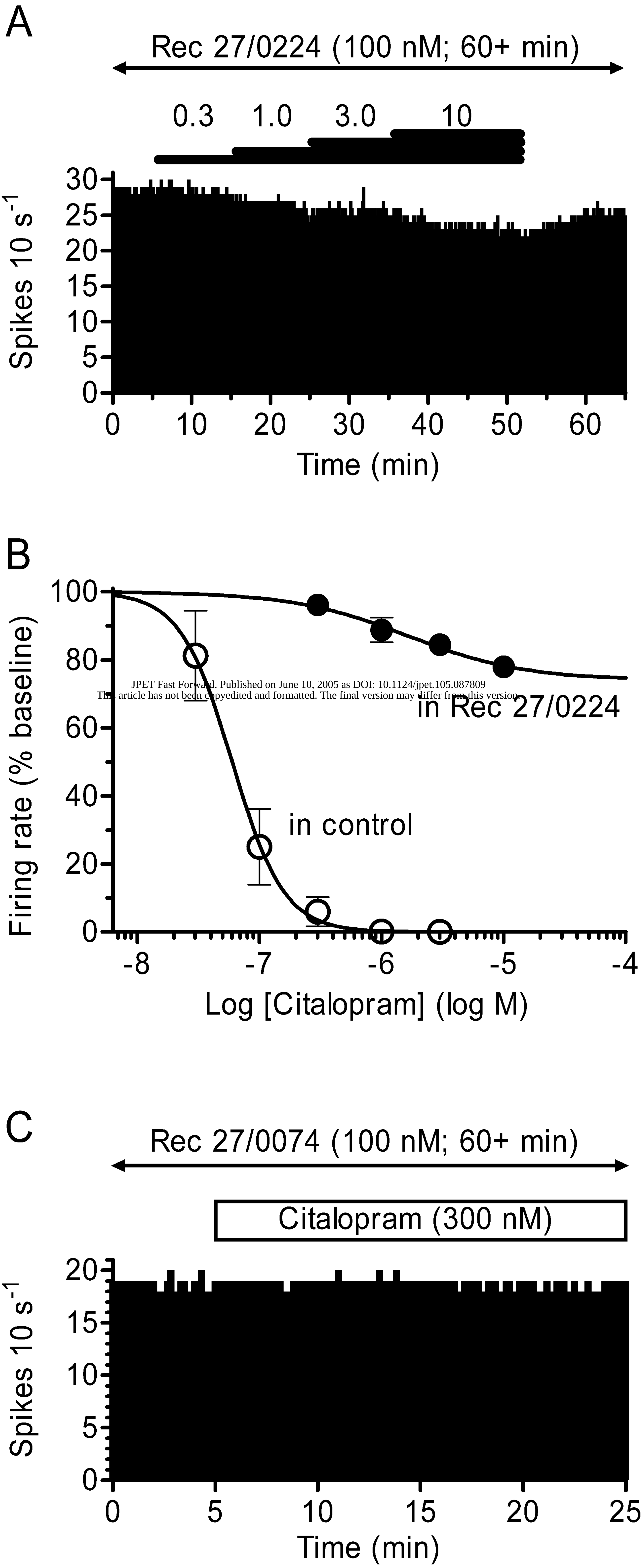

A
Rec 27/0224 (10 nM)
Spikes 10 s^{-1}
15
10
5
0
0
30
60
90
Time (min)
B
5-CT
Firing rate (% of baseline)
100
80
60
40
20
0
Control
Rec 27/0224
(10 nM; 4 h)
0
2
4
6
8
10
Time (min)
C
5-CT effect (% of control)
110
100
90
80
70
60
0
1
2
3
4
Duration of Rec 27/0224 application (h)

Figure 4



A
Rec 27/0224 (100 nM)
Spikes 10 s^{-1}
0
5
10
15
0
30
60
90
Time (min)
B
Rec 27/0224 (100 nM)
Spikes 10 s^{-1}
0
5
10
0
30
60
90
Time (min)
C
5-CT effect (% of control)
0
20
40
60
80
100
Rec 27/0224
Rec 27/0074
-10
-9
-8
-7
-6
Log [inverse agonist] (M)

Figure 5


A
Rec 27/0224 (100 nM; 60+ min)
0.3
1.0
3.0
10
Spikes 10 s-1
30
25
20
15
10
5
0
0
10
20
30
40
50
60
Time (min)
B
Firing rate (% baseline)
100
80
60
40
20
0
in Rec 27/0224
in control
-8
-7
-6
-5
-4
Log [Citalopram] (log M)
C
Rec 27/0074 (100 nM; 60+ min)
Citalopram (300 nM)
Spikes 10 s-1
20
15
10
5
0
0
5
10
15
20
25
Time (min)