



The effect of SB-269970, a 5-HT₇ receptor antagonist, on 5-HT release from serotonergic terminals and cell bodies

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1 The presence of 5-HT₇ receptor mRNA and protein in 5-HT neurons suggests that this receptor may act as a 5-HT autoreceptor. In this study, the effect of the 5-HT₇ receptor antagonist, SB-269970 ((R)-1-[3-hydroxy phenylsulfonyl]-2-[2-(4-methyl-1-piperidinyl)ethyl]pyrrolidine), was investigated on 5-HT release in the guinea-pig and rat cortex and the rat dorsal raphe nucleus (DRN), using the techniques of *in vitro* [³H]-5-HT release or fast cyclic voltammetry, respectively.

2 Cortical slices were loaded with [³H]-5-HT and release was evoked by electrical stimulation. 5-CT inhibited the evoked release of [³H]-5-HT in a concentration-dependent manner. SB-269970 had no significant effect on [³H]-5-HT release while the 5-HT_{1B} receptor antagonist, SB-224289 significantly potentiated [³H]-5-HT release. In addition, SB-269970 was unable to attenuate the 5-CT-induced inhibition of release while SB-224289 produced a rightward shift of the 5-CT response, generating estimated pK_B values of 7.8 and 7.6 at the guinea-pig and rat terminal 5-HT autoreceptors respectively.

3 Rat DRN slices were electrically stimulated and the evoked 5-HT efflux detected by voltammetric analysis. 8-OH-DPAT inhibited evoked 5-HT efflux and was fully reversed by WAY 100635. SB-269970 had no effect on either 5-HT efflux *per se* or 8-OH-DPAT-induced inhibition of 5-HT efflux. In addition, 5-CT inhibited 5-HT efflux in a concentration-dependent manner. SB-269970 was unable to attenuate the 5-CT-induced inhibition of 5-HT efflux.

4 In conclusion, we were unable to provide evidence to suggest a 5-HT autoreceptor role for 5-HT₇ receptors. However, investigations with more selective 5-HT₇ receptor agonists are needed to confirm the data reported here.

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Abbreviations: a. CSF, artificial cerebrospinal fluid; CF_e, carbon fibre electrode; DRN, dorsal raphe nucleus; FR, fractional release

Introduction

5-Hydroxytryptamine (5-HT) receptors have been classified into seven families (5-HT_{1–7}) based on their structural, functional and pharmacological characteristics (Hoyer *et al.*, 1994; Martin & Humphrey, 1994). The 5-HT₇ receptor was recently cloned from several species including rat (Lovenberg *et al.*, 1993; Meyerhof *et al.*, 1993; Ruat *et al.*, 1993; Shen *et al.*, 1993), mouse (Plassat *et al.*, 1993), guinea-pig (Tsou *et al.*, 1994) and human (Bard *et al.*, 1993). 5-HT₇ receptor binding has also been demonstrated in native guinea-pig, rat, mouse, pig and human brain (To *et al.*, 1995; Boyland *et al.*, 1996; Atkinson *et al.*, 2000). The receptor has been shown to be positively coupled to adenylyl cyclase in both human clones (Hoyer *et al.*, 1994) and guinea-pig hippocampal tissue (Tsou *et al.*, 1994; Thomas *et al.*, 1999).

The 5-HT₇ receptor displays a high degree of interspecies homology (>95% between all species; To *et al.*, 1995) but alternative splicing in both human and rat tissues has been identified. The cDNA encoding the receptor contains two

introns: the first located in the gene region corresponding to the second intracellular loop (Bard *et al.*, 1993; Shen *et al.*, 1993; Ruat *et al.*, 1993) and the second in the gene region corresponding to the predicted intracellular carboxyl terminal (Ruat *et al.*, 1993). Alternative splicing of this latter intron has been reported to generate four 5-HT₇ receptor isoforms (5-HT_{7(a)}; 5-HT_{7(b)}; 5-HT_{7(c)}; 5-HT_{7(d)}) which differ in their C-terminal intracellular tails (Heidmann *et al.*, 1997). However, there are no reports on these splice variants differing in pharmacology, signal transduction or tissue distribution (Jasper *et al.*, 1997; Heidmann *et al.*, 1998).

In situ hybridization, immunohistochemistry and autoradiography have all concluded that 5-HT₇ receptors are present on serotonergic neurones throughout the central nervous system (CNS), in both terminal regions such as the cortex and cell body regions such as the raphe. As the biological role of this receptor still remains to be elucidated the distribution of 5-HT₇ receptors has been important in suggesting function.

In the rat, high to moderate levels of mRNA were evident in the cortex (Lovenberg *et al.*, 1993; Ruat *et al.*, 1993; Shen

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et al., 1993; Gustafson *et al.*, 1996; Heidmann *et al.*, 1998) with lower levels in the raphe (Ruat *et al.*, 1993), while in the guinea-pig, moderate levels of mRNA were observed in cortex and low levels in raphe (To *et al.*, 1995). Similarly, moderate levels of 5-HT₇ protein was identified in rat cortex (Waeber *et al.*, 1995; Gustafson *et al.*, 1996; Oliver *et al.*, 1999; Atkinson *et al.*, 2000) with varying levels in the raphe (Gustafson *et al.*, 1996; Oliver *et al.*, 1999), while in the guinea-pig high levels of 5-HT₇ receptor protein were found in the cortex (To *et al.*, 1995; Thomas *et al.*, 2000) and moderate levels in the raphe (To *et al.*, 1995). A review of this data can be found in Lucas & Hen (1995).

The identification of 5-HT₇ receptor mRNA and protein in serotonin cell body and terminal areas raised the possibility that this receptor was another 5-HT autoreceptor which would serve to regulate 5-HT release. Ruat *et al.* (1993) eluded to a possible autoreceptor role for this class of receptor, stating, 'It will be of interest to establish whether 5-HT₇ receptor mRNAs detected in raphe nuclei reflect the local synthesis of another class of autoreceptors.'

With the emergence of selective 5-HT₇ receptor antagonists (Thomas *et al.*, 1998; Hagan *et al.*, 2000) it has now become possible to directly investigate the role of this receptor in the CNS. Therefore, in this study the effect of the 5-HT₇ receptor antagonist, SB-269970 (Hagan *et al.*, 2000), was investigated in guinea-pig and rat cerebral cortex and in rat dorsal raphe nucleus (DRN), using the techniques of *in vitro* [³H]-5-HT release or fast cyclic voltammetry, respectively.

To date, there are no selective 5-HT₇ receptor agonists available. Therefore, in the current studies we have used non-selective 5-HT receptor agonists: 5-CT, which has high affinity for 5-HT₇ receptors but also 5-HT_{1A}, 5-HT_{1B}, 5-HT_{1D} and 5-HT_{1F} receptors and 8-OH-DPAT, which has moderate affinity at 5-HT₇ receptors but high affinity at 5-HT_{1A} receptors (Thomas *et al.*, 1998; 1999). Both 5-CT and 8-OH-DPAT have been demonstrated to act as functional agonists at 5-HT₇ receptors, stimulating adenylyl cyclase activity both in a 5-HT₇ cloned cell line and in guinea-pig hippocampal tissue. However, while 5-CT was a full agonist in both tissues, 8-OH-DPAT was a partial agonist in guinea-pig tissue, with an intrinsic activity of 0.4 (Thomas *et al.*, 1998; 1999).

Methods

In vitro [³H]-5-HT release

Male guinea-pigs (Harlan Porcellus) or rats (Sprague Dawley) were sacrificed, decapitated and the brains removed. The cortex was rapidly dissected and cross-chopped into 300 × 300 μm slices on a McIlwain chopper. The slices were incubated with 100 nM [³H]-5-HT in the presence of pargyline (10 μM) at 37°C for 15 min. Slices were washed, suspended in 2 ml of Krebs buffer (mM: NaCl 118; KCl 4.8; CaCl₂ 1.3; MgSO₄ 1.2; NaHCO₃ 25; NaH₂PO₄ 1.2; glucose 10; L-ascorbate 0.06; Na₂EDTA 0.03) and 100 μl aliquots transferred to a Brandel superfusion 2000 apparatus. The slices were then superfused at 0.5 ml min⁻¹ with oxygenated Krebs in the presence of paroxetine (1 μM).

After 30 min of superfusion (*t*=0) samples were collected every 4 min for a duration of 80 min. [³H]-5-HT release was electrically evoked, using stimulation parameters of 1 Hz, 2 min, 20 mA to investigate effects of antagonists or 3 Hz, 1 min, 20 mA to investigate effects of agonists. Slices were stimulated at *t*=12 (S1) and 56 (S2) min. Compounds were perfused at *t*=24 min and were present until the end of the experiment.

At the end of the experiment the radioactivity in the slices and superfusate samples were determined by scintillation spectrometry. Fractional release (FR) for each sample was calculated and data expressed as either an S2/S1 ratio or as a per cent of control (Roberts *et al.*, 1996).

In vitro fast cyclic voltammetry

Male rats (Sprague Dawley) were killed by terminal anaesthesia (halothane) and decapitated. The brain was rapidly removed and a 350 μm brain slice containing the DRN was taken under ice-cold 'slicing' buffer (mM: KCl 2.5; NaHCO₃ 26; MgCl₂ 5; NaH₂PO₄ 1.2; CaCl₂ 0.1; sucrose 189; glucose 10). The slice was allowed to recover in oxygenated artificial CSF (mM: a.CSF: NaCl 120; KCl 2.5; NaHCO₃ 26; NaH₂PO₄ 1.2; MgCl₂ 1.3; CaCl₂ 2.4; glucose 10) at room temperature for 60 min. The slice was then transferred to a brain slice chamber where it was perfused at 2.5 ml min⁻¹ with oxygenated a.CSF at 32°C. A stimulating (bipolar tungsten, 100 μm tip diameter, 150 μm tip separation) and voltammetric (carbon fibre, 8 μm tip width, 100 μm tip length) electrode was placed in the ventral DRN at a depth of 100 μm. The voltammetric electrode tip was positioned between those of the stimulating electrode, just off linearity.

A triangular voltage waveform (-1.0 to +1.4 V, 1.5 cycles at a rate of 480 V s⁻¹) was applied to the carbon fibre microelectrode (CFE). The voltammetric scan was applied at a frequency of 2 Hz and the current sampled at 525 mV. This signal was fed into a chart recorder and a CED 1401. Data captured with the CED 1401 was analysed using CED 'Signal' software.

5-HT efflux was evoked by electrical stimulation (100 Hz, 10 mA, 0.1 ms, 20 pulses every 5 min, 190 ms duration). 5-HT efflux was taken as the peak signal attained following stimulation. Electrodes were calibrated with standard 5-HT solutions at the end of the experiment.

The slice was stimulated every 5 min and compounds were added when four consecutive stable 5-HT efflux events were obtained. Subsequent 5-HT efflux was then expressed as a percentage of this pre-compound level. Agonists and antagonists were perfused for at least 20 mins.

Materials

8-OH-DPAT, 5-CT (5-carboxamidotryptamine), 5-HT and WAY 100635 were supplied by RBI. SB-269970 ((R)-1-[(3-Hydroxyphenyl)sulfonyl]-2-[2-(4-methyl-1-piperidinyl)ethyl]-pyrrolidine) and SB-224289 (2,3,6,7-tetrahydro-1'-methyl-5-{2'-methyl-4'-[(5-methyl-1,2,4-oxadiazole-3-yl)biphenyl-4-yl]carbonyl} furo[2,3-f]indole-3-spiro-4'-piperidine oxalate) were synthesized at SmithKline Beecham Pharmaceuticals. All other chemicals were supplied by Sigma.

Results

[³H]-5-HT release from guinea-pig and rat cortex

The 5-HT₇ receptor antagonist, SB-269970 (10 nM–1 μM), had no effect on [³H]-5-HT release in tissue from either species while the 5-HT_{1B} receptor antagonist, SB-224289, significantly potentiated the evoked release of [³H]-5-HT to a maximum of 138 and 168% at 1 μM in rat and guinea-pig respectively ($P < 0.05$; Figure 1). The non-selective 5-HT receptor agonist, 5-CT, inhibited the evoked release of [³H]-5-HT in a concentration-dependent manner, with a maximum inhibition of $60 \pm 4\%$ ($n = 8$) and $61 \pm 4\%$ at 100 nM in rat and guinea-pig respectively (Figures 1 and 2). At a lower stimulation frequency of 1 Hz, to maximize agonist response and minimize antagonist potentiation, SB-269970 was unable to attenuate the 5-CT inhibition of release whereas SB-224289 produced a rightward shift of the 5-CT concentration curve, generating an estimated pK_B of 7.83 ± 0.06 ($n = 3$, Figure 2A) and 7.60 ± 0.06 ($n = 3$, Figure 2B) for guinea-pig and rat tissue respectively, consistent with 5-HT_{1B} receptor blockade.

5-HT efflux from rat DRN

Control stimulations of 20 pulses every 5 min (100 Hz, 10 mA, 0.1 ms width) generated a peak 5-HT efflux of 22 ± 2 nM ($n = 16$). The mixed 5-HT receptor agonist, 8-OH-DPAT (100 nM), inhibited 5-HT efflux to a minimum of 40% of control (Figure 3). Neither the 5-HT_{1A} receptor antagonist, WAY 100635 (100 nM), nor the 5-HT₇ receptor antagonist, SB-269970 (1 μM), had any effect on 5-HT efflux when superfused alone (Figure 3). SB-269970 (1 μM) was unable to attenuate the 8-OH-DPAT effect whereas WAY 100635 (100 nM) fully reversed the 8-OH-DPAT-induced inhibition of stimulated 5-HT efflux (Figure 4).

5-CT (1 nM–100 nM) also inhibited 5-HT efflux in a concentration dependent manner (Figure 5). SB-269970 (1 μM) had no significant effects on the 5-CT inhibition of 5-HT efflux (Figure 5).

Discussion

The 5-HT₇ receptor is the most recently identified member of the family of G-protein-coupled 5-HT receptors. Until recently investigations into the possible role of this receptor has relied on the use of non-selective ligands such as 5-CT, 8-OH-DPAT, risperidone, methiothepin and clozapine but the discovery of the first selective 5-HT₇ receptor antagonist, SB-269970 (Thomas *et al.*, 1998; 1999; Hagan *et al.*, 2000), has been an important first step in determining the biological function of the 5-HT₇ receptor subtype. Hypotheses for a possible role for the 5-HT₇ receptor have been based on the distribution of the receptor mRNA and protein. *In situ* hybridization, immunohistochemistry and autoradiography have all concluded that 5-HT₇ receptors are present on serotonergic neurones in the CNS. Therefore, this raises the possibility for a role of 5-HT₇ receptors as autoreceptors, controlling 5-HT release either through a direct action on serotonergic terminals and somatodendritic regions or through inhibition of serotonergic cell firing. In this present study we investigated these possibilities at both the serotonergic terminal and cell body regions, using the techniques of *in vitro* [³H]-5-HT release and fast cyclic voltammetry respectively.

The technique of *in vitro* [³H]-5-HT release studies the effects at serotonergic terminal autoreceptors. Using this method, the 5-HT agonist, 5-CT, decreased the evoked release of [³H]-5-HT from cortical slices through activation of a 5-HT autoreceptor (Roberts *et al.*, 1996). The magnitude of this effect was stimulation frequency dependent: 5-CT

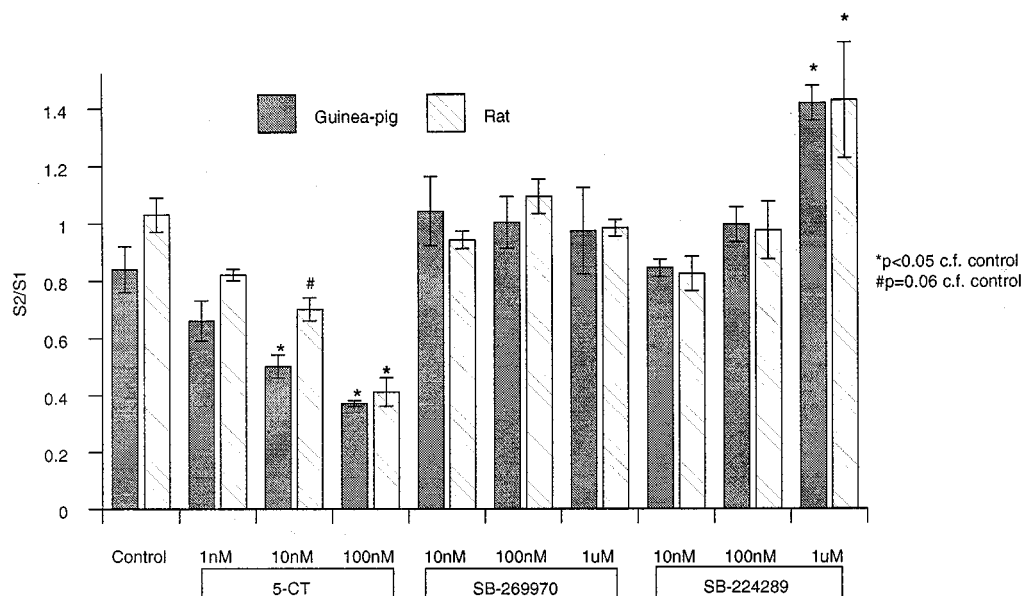


Figure 1 Effect of 5-CT, SB-269970 and SB-224289 on electrically evoked [³H]-5-HT release from guinea-pig and rat cortical slices. Data are expressed as an S2/S1 ratio \pm s.e. mean and are the mean of at least three determinations. Statistical analysis was by one-way ANOVA, followed by a *post-hoc* *t*-test (least squares difference).

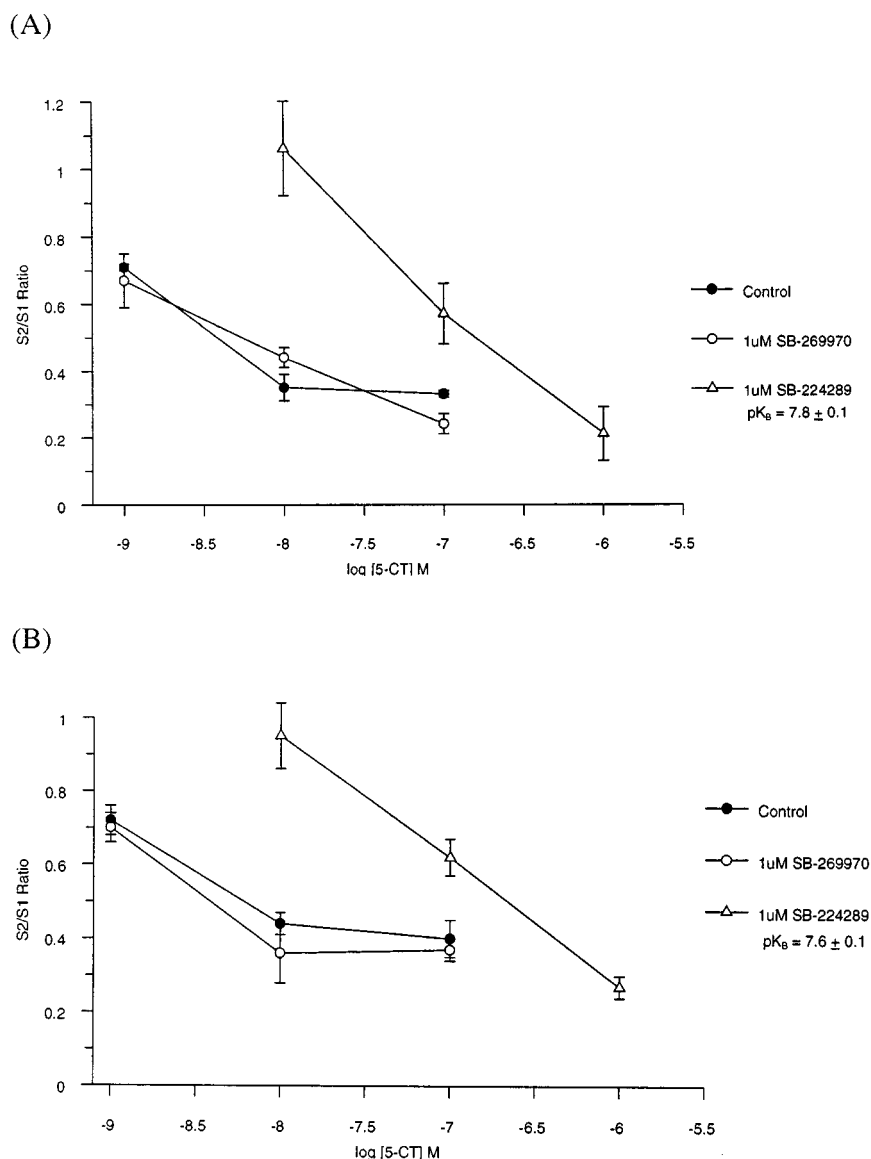


Figure 2 Inhibition of electrically evoked [³H]-5-HT release from (A) guinea-pig or (B) rat cortex with 5-CT, or 5-CT in the presence of 1 μM SB-269970 or 1 μM SB-224289. Data are expressed as an S2/S1 ratio ± s.e.mean and are the mean of at least three determinations.

inhibition of release was greater at 1 Hz than 3 Hz. This was probably a reflection of the biophase concentration of endogenous 5-HT; higher frequencies of stimulation results in a greater endogenous 5-HT tone on the system at hence a smaller inhibition from exogenously applied 5-HT receptor agonists.

In the present study, the selective 5-HT_{1B} receptor antagonist, SB-224289, increased the evoked release of 5-HT through antagonism of endogenous 5-HT at the autoreceptor (see also Selkirk *et al.*, 1998) whereas the 5-HT₇ receptor antagonist, SB-269970, had no effect. SB-224289 compound also attenuated the 5-CT response, shifting the concentration response curve to the right. The calculated pK_B values of 7.8 and 7.6, for guinea-pig and rat respectively, were consistent with competitive antagonism of 5-HT_{1B} receptors (Selkirk *et al.*, 1998). SB-269970 had no effect on the 5-CT-induced inhibition of release. Therefore, although

the 5-HT₇ receptor is present in the cortex, these data indicate that the serotonergic terminal autoreceptor is of the 5-HT_{1B} receptor subtype and there is no evidence for an autoreceptor function for the 5-HT₇ receptor. Thus cortical 5-HT₇ receptors are unlikely to be present on the terminals of serotonergic projections and are presumably located postsynaptically on cortical neurones.

This study was then extended to investigate effects of 5-HT receptor agonists and antagonists at serotonergic autoreceptors located in the cell body region, the DRN, the brain region responsible for the serotonergic innervation of the frontal cortex (Kosofsky & Molliver, 1987), using the technique of fast cyclic voltammetry. The 5-HT agonists, 5-CT and 8-OH-DPAT, both inhibited 5-HT efflux through activation of the cell body autoreceptors.

The 8-OH-DPAT-induced inhibition of 5-HT efflux was fully reversed by WAY 100635, a selective 5-HT_{1A} receptor

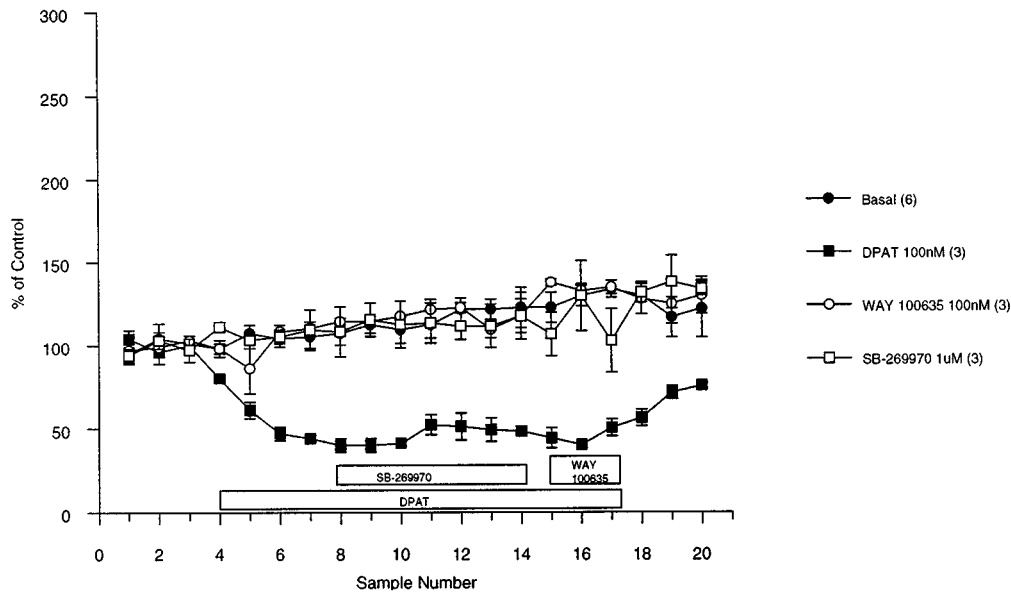


Figure 3 Effect of 100 nM DPAT, 100 nM WAY 100635 and 1 μ M SB-269970 on electrically evoked 5-HT efflux from rat DRN as measured by fast cyclic voltammetry. Data are expressed as a per cent of control \pm s.e. mean and are the mean of at least three determinations.

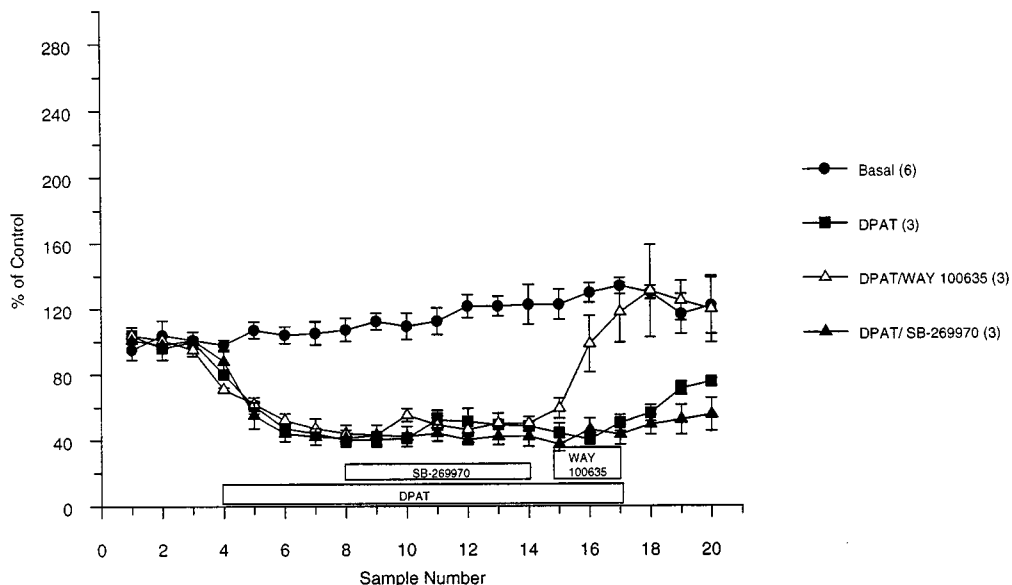


Figure 4 Effect of 1 μ M SB-269970 and 100 nM WAY 100635 on the 8-OH-DPAT-induced inhibition of 5-HT efflux from rat DRN as measured by fast cyclic voltammetry. Data are expressed as a per cent of control \pm s.e. mean and are the mean of at least three determinations.

antagonist, confirming that the 5-HT_{1A} receptor regulates 5-HT release at the cell body (Davidson & Stamford, 1995; Starkey & Skingle, 1995). WAY 100635 had no effect *per se*, which may indicate either a low level of endogenous 5-HT or that the effect of 5-HT_{1A} receptor antagonism may be limited by the presence of additional autoreceptors e.g. 5-HT_{1B} or 5-HT_{1D} (Roberts *et al.*, 1998). That is, endogenous 5-HT may act as an agonist at 5-HT_{1B} or 5-HT_{1D} receptors, to negatively feedback and reduce the 5-HT level.

SB-269970 had no effect on basal 5-HT efflux and, in contrast to WAY 100635, was unable to significantly reverse either the 5-CT or 8-OH-DPAT inhibition of 5-HT efflux. These data imply that the 5-HT₇ receptor does not function to regulate 5-HT release in this cell body region.

To conclude, we were unable to provide evidence for the existence of 5-HT₇ serotonergic autoreceptors in either the terminal or cell body regions. However, the location of 5-HT₇ receptors in regions known to contain serotonergic autoreceptors raises the question of whether they serve to

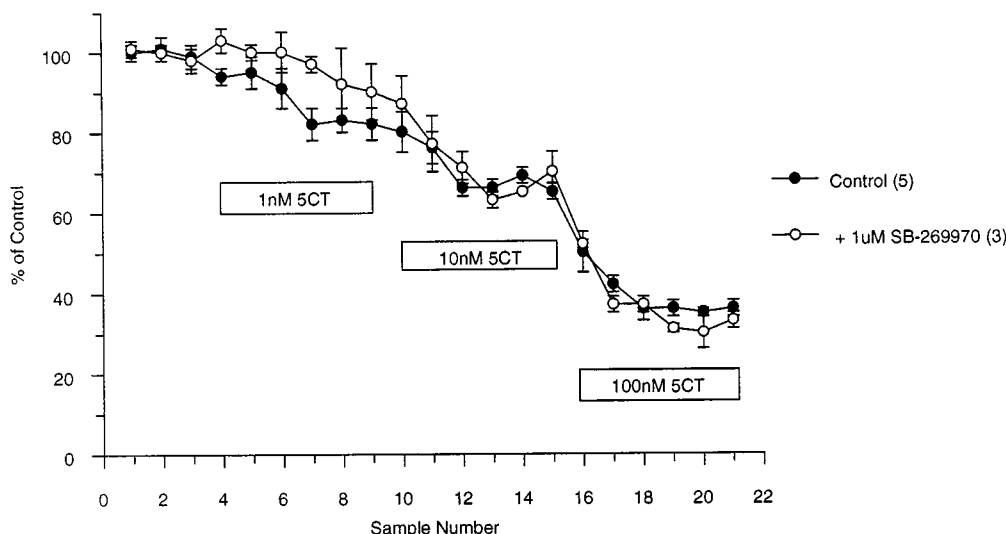


Figure 5 Effect of 5-CT on 5-HT efflux from the rat DRN in the presence or absence of SB-269970 (1 μ M), as measured by fast cyclic voltammetry. Data are expressed as a per cent of control \pm s.e.mean and are the mean of at least three determinations.

modulate the action of 5-HT_{1A}, 5-HT_{1B} or 5-HT_{1D} autoreceptors. Recently Thomas *et al.* (1999) demonstrated an interaction of 5-HT_{1A} and 5-HT₇ receptors in the hippocampus. Both receptors stimulate adenylyl cyclase in the hippocampus but the data suggested that the 5-HT_{1A} effect was indirect, that is, 5-HT_{1A} receptors do not directly

stimulate adenylyl cyclase but serve to augment the 5-HT₇-mediated stimulation. In serotonergic neurones the converse may be true, 5-HT₇ receptors may play an indirect role through other 5-HT autoreceptors. However, such studies are not possible with the currently available, non-selective 5-HT receptor agonists.

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