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RESEARCH PAPER

In vivo evidence that 5-HT_{2C} receptors inhibit 5-HT neuronal activity via a GABAergic mechanism

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Background and purpose: Recent evidence suggests that 5-HT_{2C} receptor activation may inhibit midbrain 5-HT neurones by activating neighbouring GABA neurones. This hypothesis was tested using the putative selective 5-HT_{2C} receptor agonist, WAY 161503.
Experimental approach: The effect of WAY 161503 on 5-HT cell firing in the dorsal raphe nucleus (DRN) was investigated in anaesthetised rats using single unit extracellular recordings. The effect of WAY 161503 on DRN GABA neurones was investigated using double label immunohistochemical measurements of Fos, glutamate decarboxylase (GAD) and 5-HT_{2C} receptors. Finally, drug occupancy at 5-HT_{2A} receptors was investigated using rat positron emission tomography and *ex vivo* binding studies with the 5-HT_{2A} receptor radioligand [^{11}C]MDL 100907.
Key results: WAY 161503 caused a dose-related inhibition of 5-HT cell firing which was reversed by the 5-HT_2 receptor antagonist ritanserin and the 5-HT_{2C} receptor antagonist SB 242084 but not by the 5-HT_{1A} receptor antagonist WAY 100635. SB 242084 pretreatment also prevented the response to WAY 161503. The blocking effects of SB 242084 likely involved 5-HT_{2C} receptors because the drug did not demonstrate 5-HT_{2A} receptor occupancy *in vivo* or *ex vivo*. The inhibition of 5-HT cell firing induced by WAY 161503 was partially reversed by the $GABA_A$ receptor antagonist picrotoxin. Also, WAY 161503 increased Fos expression in GAD positive DRN neurones and DRN GAD positive neurones expressed 5-HT_{2C} receptor immunoreactivity.
Conclusions and implications: These findings indicate that WAY 161503 inhibits 5-HT cell firing in the DRN *in vivo,* and support a mechanism involving 5-HT_{2C} receptor-mediated activation of DRN GABA neurones.
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Abbreviations: 5-HT, 5-hydroxytryptamine; DRN, dorsal raphe nucleus; GABA, gamma amino-butyric acid; GAD, glutamate decarboxylase; HIDAC, high-density avalanche chamber; PET, positron emission tomography

Introduction

Feedback regulation is an essential aspect of the physiology of central 5-hydroxytryptamine (5-HT, serotonin) neurones (Aghajanian, 1978), and the roles of presynaptic 5-HT autoreceptors are well established. Thus, somatodendritic 5-HT_{1A} autoreceptors inhibit the firing of 5-HT neurones in the dorsal raphe nucleus (DRN), while terminal 5-HT_{1B} autoreceptors inhibit 5-HT release (Barnes and Sharp, 1999).

In addition to 5-HT autoreceptors, recent evidence suggests that postsynaptic 5-HT receptors located on afferent inputs to 5-HT neurones are also involved in 5-HT feedback control. For example, in rats cortical lesions attenuate the inhibitory effect of 5-HT_{1A} receptor agonists on the firing of DRN 5-HT neurones, suggesting the involvement of post-synaptic 5-HT_{1A} receptors (Ceci *et al*., 1994; Hajós *et al*., 1999). Also, the inhibition of 5-HT cell firing by 5-HT_{1A} receptor agonists persists following the local inactivation of somatodendritic 5-HT_{1A} autoreceptors (Martin-Ruiz and Ugedo, 2001b).

A role for postsynaptic 5-HT_2 receptors in 5-HT feedback control is evident in *in vivo* electrophysiological findings that 5-HT_2 receptor agonists, including (±)-2,5 dimethoxy-4-iodoamphetamine (DOI), inhibit the firing of DRN 5-HT neurones (Aghajanian *et al*., 1970; Garratt *et al*., 1991). A recent pharmacological analysis of this effect of DOI suggested a prominent role for the 5-HT_{2A} receptor subtype, but an involvement of 5-$HT_{2B/C}$ receptors was also indicated (Boothman *et al*., 2003). Accordingly, *in vitro* electrophysiological findings also emphasize the importance of the 5-HT_{2A} over the 5-HT_{2C} receptor subtype in the induction of inhibitory post-synaptic potentials (IPSPs) in DRN 5-HT neurones by 5-HT and DOI (Liu *et al*., 2000). However, these

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studies may under-estimate the importance of the 5-HT$_{2C}$ receptor subtype due to the limited selectivity of the drugs used, and in particular the lack of a selective 5-HT$_{2C}$ receptor agonist.

The neuroanatomical substrates which mediate 5-HT$_2$ receptor agonist-induced inhibition of 5-HT cell firing are unknown but 5-HT neuronal afferents are implicated because 5-HT$_2$ receptors are not expressed by 5-HT neurones (Clemett *et al*., 2000; Cornea-Hebert *et al*., 1999). One candidate neuronal substrate is DRN gamma amino-butyric acid (GABA) neurones which synapse onto 5-HT neurones and exert a powerful inhibitory effect (Wang *et al*., 1992; Varga *et al*., 2001). Moreover, DRN GABA neurones are the target of several important DRN afferents from the forebrain (Varga *et al*., 2001; Jankowski and Sesack, 2004). Interestingly, recent immunohistochemical data indicate that DOI administration increases expression of the immediate early gene *c-fos* in DRN GABA neurones (Boothman and Sharp, 2005a), and the presence of 5-HT$_{2C}$ receptor mRNA in DRN GABA neurones was recently reported (Serrats *et al*., 2005).

This study investigated the role of 5-HT$_{2C}$ receptors in the regulation of 5-HT cell firing, using the putative selective 5-HT$_{2C}$ receptor agonist, WAY 161503 (Cryan and Lucki, 2000; Rosenzweig-Lipson *et al*., 2006) and the 5-HT$_{2C}$ receptor antagonist SB 242084 (Kennett *et al*., 1997). The possible confounding factor of SB 242084 occupancy at 5-HT$_{2A}$ receptors *in vivo* was studied using positron emission tomography (PET) scanning and *ex vivo* binding (Hirani *et al*., 2003). Drug effects on DRN GABA neurones were examined using a combined electrophysiological and Fos immunohistochemical approach. Results indicate that WAY 161503 inhibits 5-HT cell firing in the DRN *in vivo,* and support a mechanism involving 5-HT$_{2C}$ receptor-mediated activation of DRN GABA neurones. A preliminary account of these experiments was presented to the British Pharmacological Society (Boothman *et al*., 2005b; Raley *et al*., 2005).

Methods

Animals

Experiments were carried out in accordance with the Animals (Scientific Procedures) Act (1986) and a local ethical review process. Male Sprague–Dawley rats (220–320 g; Harlan Olac, Bicester, UK) were group housed (5–6) under conditions of constant temperature ($21 \pm 1^{\circ}$C) and humidity under a 24 h light–dark cycle (lights on 0800–2000) with food and water freely available. Before immunocytochemical experiments, rats were handled daily for 3–5 days and familiarized with the testing room to minimize stress.

Electrophysiological recording of 5-HT neuronal activity

Rats were anaesthetized with chloral hydrate (460 mg kg^{-1} i.p. with additional doses as required), supplemented during surgery with saffan (1.2 mg kg^{-1} i.v.), and maintained at 36°C using a thermoregulated blanket. Extracellular single-unit recordings were made as described previously (Boothman *et al*., 2003). Single barrel glass electrodes (2 M NaCl, 2% pontamine sky blue; 6–20 MΩ) were stereotactically implanted into the DRN (coordinates relative to Bregma and the dural surface of A/P −7.5 mm, L/M 0.0 mm D/V −4.5 to −5.5 mm, Paxinos and Watson, 1986). Single-unit potentials were amplified and filtered (Gain 1 k; 500 Hz–1.5 kHz band pass; Neurolog system, Digitimer Ltd), captured using a 1401plus interface, and analysed offline using Spike2 software.

The firing properties of DRN neurones fulfilled three or more of the following criteria which are characteristic of 5-HT neurones (Hajós *et al*., 1995; Allers and Sharp, 2003): slow firing rate (0.2–2 Hz), regular firing pattern (typical coefficient of variation <0.5), triphasic extracellular waveform with a wide spike width (>1.5 ms) and an inhibitory response to the 5-HT$_{1A}$ receptor agonist 8-OH-DPAT (10 μg kg^{-1} i.v.). Most 5-HT neurones discharged single spikes, but a small number that discharged both single spikes and spikes in short bursts (Hajós *et al*., 1995) were included.

After 5 min baseline recording, drugs were injected via a lateral tail vein. Rats ($n=6$–8/group) received WAY 161503 (0.125, 0.25, 0.5 and 1.0 mg kg^{-1} at 2 min intervals) either alone, or following pre-treatment with SB 242084 (1 mg kg^{-1}). In separate experiments, the following antagonists were administered after WAY 161503: SB 242084 (5-HT$_{2C}$; 0.5 mg kg^{-1}), ritanserin (5-HT$_2$; 1 mg kg^{-1}), WAY 100635 (5-HT$_{1A}$; 0.1 mg kg^{-1}) or picrotoxin (GABA$_A$; 0.5–2.0 mg kg^{-1}). At the end of some experiments, 8-OH-DPAT (10 μg kg^{-1} i.v.) was administered followed by the 5-HT$_{1A}$ receptor antagonist WAY 100635 (0.1 mg kg^{-1}). Finally, dye was expelled by iontophoresis (−3.6 mA pulses, 200 ms duration, 21 ms interpulse interval, 30 min) to allow histological identification of the recording site.

Firing rates were quantified in the final min of each baseline and post-drug period. Regularity of firing was measured by coefficient of variation analysis (COV) of the inter-spike interval (s.d. inter-spike interval/inter-spike interval mean). Neurones discharging spikes in short bursts were analysed using the first spike of each burst.

In vivo *and* ex vivo *binding of [^{11}C]MDL 100907*

Rat PET scanning. Rat PET scanning was carried using the high-resolution quad-HIDAC (high-density avalanche chamber) system (Jeavons *et al*., 1999) as described previously (Hume *et al*., 2001; Hirani *et al*., 2003). In brief, rats were maintained under isoflurane anaesthesia with N_2O/O_2 and positioned in the centre of the field of view of the scanner using a perspex stereotaxic frame. The 5-HT$_{2A}$ receptor radioligand [^{11}C]MDL 100907 ($\sim$10 MBq) was then injected via a tail vein catheter in either drug naïve rats ($n=5$) or rats pre-treated with SB 242084 (1 mg kg^{-1} i.v., $n=4$), or MDL 100907 (0.2 or 0.4 mg kg^{-1} i.v., $n=4$) 5 min before radioligand injection. Each rat was scanned for 60 min with data acquired in list-mode.

For data acquisition, quad-HIDAC sinograms were reconstructed into 0.5 mm cubic voxels with the Hamming filter at a cut-off of 0.6. As the current quad-HIDAC scanner technology does not enable quantitative determination of the full dynamics of delivery and development of specific signal, data acquired during a 40 min time frame (20–60 min after radioligand injection) were chosen to represent a

compromise between time to reach secular equilibrium and increasing noise in data. Data were transferred into ANALYZE-AVW imaging software and a standard volume of interest (VOI) template was projected onto each volume. Eight VOIs were sampled, including frontal with cingulate cortex (452 voxels), striatum (404 voxels), hippocampus (264 voxels) and cerebellum (576 voxels). As the number of 5-HT$_{2A}$ receptors in the cerebellum are negligible (Lopez-Gimenez *et al*., 1997), data were expressed relative to cerebellar VOI counts, to give a measure of total:non-specific binding.

Ex vivo *measurement of tissue [^{11}C]radioactivity*. After PET scanning, rats were administered Euthatal and post-mortem brains were rapidly dissected into regions corresponding to those sampled by the quad-HIDAC (frontal cortex, striatum, hippocampus, cerebellum, olfactory bulbs, hypothalamus/thalamus, superior colliculi and medulla). Extracerebral tissues surrounding the head and within the scanner field of view were also sampled (muscle, skin, submaxillary and lachrymal glands). Carbon-11 radioactivity was measured in whole tissue samples as described previously, using a Wallac gamma-counter, with automatic correction for radioactive decay. Results were normalized to account for radioactivity injected and body weight ('uptake units' = (c.p.m./g tissue)/(injected c.p.m./g body weight)). For brain regions, tissue:cerebellum ratios were calculated to give a measure of specific binding (total:non-specific).

Immunohistochemistry

Pilot experiments ($n = 3$ rats/group) tested the effect of 1, 3 and 10 mg kg^{-1} WAY 161503 compared to saline vehicle. In the main experiments ($n = 6$ rats/group), rats received two i.p. injections 30 min apart as follows: (i) vehicle–vehicle, (ii) vehicle–WAY 161503 (3 mg kg^{-1}), (iii) SB 242084 (1 mg kg^{-1})–vehicle or (iv) SB 242084 (1 mg kg^{-1})–WAY 161503 (3 mg kg^{-1}). At 2 h after the last injection rats were anaesthetized with pentobarbital (300 mg kg^{-1} i.p.), perfused with 200 ml 0.9 % saline followed by 200 ml fixative (4% paraformaldehyde in 0.1 M sodium phosphate buffer with 0.4% picric acid), and brains were post-fixed overnight (4°C). Free-floating sections (40 μm) were cut on a vibratome and stored at 4°C before further use.

Fos/GAD double labelling

Sections were incubated in hydrogen peroxide (0.3%, 10 min), washed in phosphate-buffered saline (PBS: 140 mM NaCl, 30 mM KCl, 80 mM Na_2HPO_4, 15 mM KH_2PO_4, in distilled water), and then treated (30 min) with standard blocking serum (10% normal goat serum, in PBS with 0.3% Triton), prior to overnight incubation (4°C) in rabbit anti-glutamate decarboxylase (GAD)$_{65/67}$ antibody (Chemicon, Hampshire, UK, AB 5992, 1:2000 dilution). Sections were then washed (PBS) and incubated in biotinylated secondary antibody (1:500 dilution, 2 h). GAD$_{65/67}$ immunoreactivity was visualized using a chromagen reaction to give a brown product (Vectastain ABC elite and DAB kit, Vector, Burlinghame, CA, USA). Sections were again washed (PBS), before incubation (72 h at 4°C) with rabbit anti-Fos antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA, sc-253, 1:2000 dilution) followed by biotinylated secondary antibody (goat anti-rabbit, Vector BA-1000 1:500 dilution, 2 h). Fos immunoreactivity was visualized using a chromagen reaction giving a dark-blue product (Vectastain ABC elite and SG kit, Vector).

5-HT$_{2C}$ receptor and GAD$_{65/67}$ or Fos double labelling

Sections were treated with blocking serum before incubation at 4°C in mouse anti-5-HT$_{2C}$ receptor antibody (Pharmigen 556335, 1:200 dilution, overnight, see Bubar *et al*., 2005) together with rabbit anti-GAD$_{65/67}$ antibody (1:2000 dilution, overnight), or in rabbit anti-Fos antibody (1:2000 dilution, 60 h) with the subsequent addition of mouse anti-5-HT$_{2C}$ receptor antibody (1:200 dilution, overnight). Sections were washed (PBS) and then incubated with a green fluorescent goat anti-rabbit antibody (Alexa Fluor-488, InVitrogen, Carlsbad, CA, USA, A-11034, 1:500 dilution, 1 h) before further incubation in a red fluorescent goat anti-mouse antibody (Alexa Fluo–568, InVitrogen A-11004, 1:250 dilution, 2 h).

Cell counting and image collection

Counts of GAD$_{65/67}$/Fos double-labelled cells were made in a defined area of the DRN (250 × 170 μm grid in eyepiece of × 40 objective: Lietz Diaplan light microscope) by an operator blind to treatment. Counts were made bilaterally on six sections per animal. Bright-field images were captured using a colour video camera (Sony) and image software (Scion Image Software, version 1.62c).

5-HT$_{2C}$/Fos and 5-HT$_{2C}$/GAD$_{65/67}$ double-labelled cells were visualized using fluorescence microscopy. Fluorescent images captured using a digital camera (Xillix microimager, Richmond BC, Canada) with the application of false colour (Openlab software, version 3.0.2).

Drugs and materials

The drugs used (with supplier) were as follows: WAY 161503 (8,9-dichloro-2,3,4,4*a*-tetrahydro-1*H*-pyrazino[1,2-*a*]quinoxalin-5(6 H)-one; Tocris, UK), SB 242084 (6-chloro-5-methyl-1-[6-(2-methylpyridin-3-yloxy) pyridin-3-yl carbamoyl] indoline; Eli Lilly & Co, UK), ritanserin (Janssen Phamaceuticals, Belgium), MDL 100907 (R-(+)-(2,3-dimethoxyphenil)-1-[2-(4-flurophenylethyl)]-4-piperidone – methanol; ABX advanced biochemical compounds, Germany), WAY 100635 (*n*-[2-[4-(2-methoxyphenyl)-1-piperazinylethyl]-*n*-(2-pyridinyl) cyclohexane carboxamide trihydrochloride; Wyeth Pharmaceuticals, UK), 8-OH-DPAT (8-hydroxy-2-(di-*n*-propylamino)-tetralin; Sigma-Aldrich, UK), picrotoxin (Sigma-Aldrich, UK). Drugs were dissolved in deionized water except SB 242084 (10% cyclodextrin in 25 mM citric acid) and ritanserin and MDL 100907 (few drops of glacial acetic acid and 5% glucose).

[^{11}C]MDL 100907 was prepared by Hammersmith Imanet radiochemistry according to published methods (Lundkvist *et al*., 1996). The radioligand had a purity of ~99%, specific

activity (at time of injection) of $\sim$45 GBq μmol^{-1} and the dose of MDL 100907 associated with $\sim$10 MBq injection was estimated to be 1.3 nmol kg^{-1}.

Statistical analysis

Electrophysiological data were analysed using one-way ANOVA with Dunnett's *post hoc* tests (for the effect of agonists alone), two-way ANOVA with Bonferroni *post hoc* tests (for the effect of antagonist pretreatment) and Student's two-tailed paired *t*-test (effect of antagonists alone). [^{11}C]MDL 100907 binding data were analysed on a region-by-region basis using Student's unpaired *t*-test (two-tailed). Cell count data were analysed using one-way ANOVA followed *post hoc* with either Dunnett's *t*-test (multiple group comparison) or Bonferroni's test (between group comparison). *P*-values of $\leqslant$0.05 were considered statistically significant.

Results

Electrophysiological characteristics of DRN neurones

Presumed 5-HT neurones in the DRN ($n=27$) fired broad triphasic spikes (waveform length 2.01$\pm$0.06 ms, range 1.5–2.7 ms), in a slow and regular firing pattern (baseline firing rate 0.91$\pm$0.07 Hz, range 0.32–1.74 Hz; baseline COV 0.26$\pm$0.03, range 0.18–0.4). There was no significant difference in baseline firing rate or COV between treatment groups. The baseline firing rate of these neurones is constant over 20–30 min following an initial 2–3 min stabilization period, and administration of the vehicles used in the current study (deionized water, 10% cyclodextrin in 25 mM citric acid, dilute glacial acetic acid in 5% glucose) have no significant effect firing rate or regularity.

Effect of WAY 161503 on 5-HT cell firing

Systemic administration of the putative 5-HT$_{2C}$ receptor agonist WAY 161503 (0.125–0.5 mg kg^{-1} i.v.) caused a dose-related inhibition of 5-HT cell firing compared to pre-drug values. This effect was apparent at 0.125 mg kg^{-1}, and the highest dose tested reduced firing to 21% of pre-drug values (Figures 1a and 2). WAY 161503 (0.125–0.5 mg kg^{-1} i.v.) had no significant effect on the regularity of 5-HT cell firing (data not shown).

Effect of WAY 161503 on 5-HT cell firing in the presence of 5-HT receptor antagonists

As illustrated in Figure 1, the inhibitory effect of WAY 161503 (0.125–0.5 mg kg^{-1} i.v.) was reversed by administration of the 5-HT$_{2A/C}$ receptor antagonist ritanserin (1.0 mg kg^{-1} i.v.; 15/18 neurones). The effect of WAY 161503 was also reversed by the 5-HT$_{2C}$ receptor antagonist SB 242084 (1.0 mg kg^{-1} i.v.; 3/4 neurones), but not the 5-HT$_{1A}$ receptor antagonist WAY 100635 (0.1 mg kg^{-1} i.v.; 2/2 neurones) (Figure 1). The reversal of the effects of WAY 161503 is not due to rapid clearance of the drug because the reversal was always coincident with administration of the

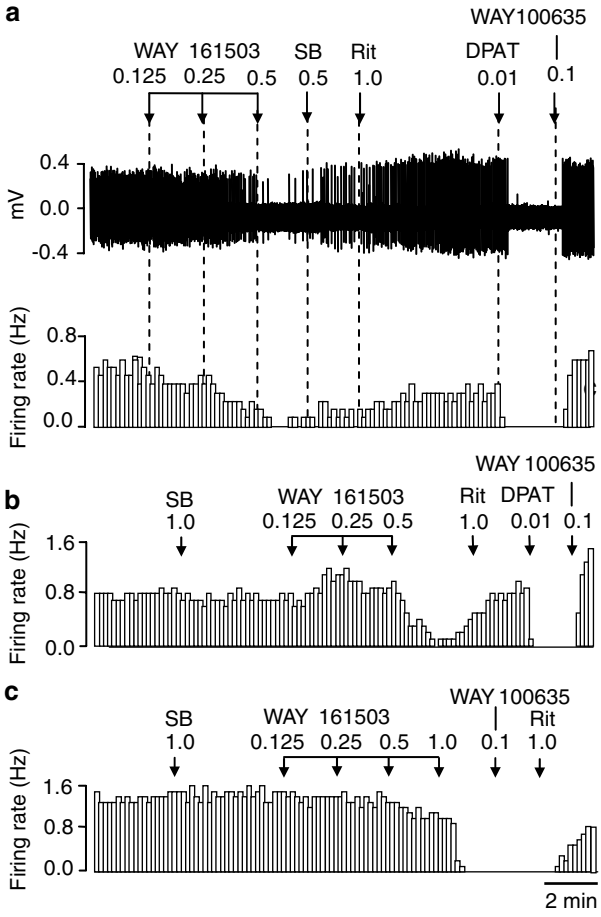

Figure 1 Spike train (upper trace) and rate-meter recordings showing the inhibitory effect of the putative 5-HT$_{2C}$ receptor agonist WAY 161503 on the firing rate of individual DRN 5-HT neurones in the anaesthetized rat. WAY 161503 produced a dose-related inhibition of 5-HT cell firing (**a**), which was attenuated by pretreatment with the 5-HT$_{2C}$ receptor antagonist SB 242084 (**b**, **c**). The effect of WAY 161503 was reversed by both SB 242084 (**a**) and the 5-HT$_2$ receptor antagonist ritanserin (**a**, **b**), but not by the 5-HT$_{1A}$ receptor antagonist WAY 100635 (**c**). Note also the characteristic inhibitory response of 5-HT neurones to the 5-HT$_{1A}$ receptor agonist 8-OH-DPAT, which was reversed by the 5-HT$_{1A}$ receptor antagonist WAY 100635 (**a**, **b**). Drug administration (mg kg^{-1} i.v.) as indicated by arrows. Abbreviations: 8-OH-DPAT (DPAT), SB 242084 (SB), ritanserin (Rit).

5-HT$_{2C}$ receptor antagonist and under the same time-frame (2 min post-agonist), there was no reversal of the effect of WAY 161503 by a 5-HT$_{1A}$ receptor antagonist (Figure 1c). Also recent experiments (Queree *et al.*, unpublished observation) demonstrate that the inhibition of 5-HT cell firing by WAY 161503 has a duration of at least 5–10 min.

Pre-treatment with SB 242084 (1.0 mg kg^{-1} i.v.) caused a rightward shift in the dose response to WAY 161503 (Figures 1 and 2). When administered alone, SB 242084 (0.5 mg kg^{-1} i.v.) had no significant effect on the rate or regularity of 5-HT cell firing (Figures 1b and c).

In some animals, the 5-HT$_{1A}$ agonist 8-OH-DPAT (10 μg kg^{-1} i.v.) was administered following the reversal of the effect of WAY 161503 by a 5-HT$_2$ receptor antagonist. In all cases (15/15 neurones) 8-OH-DPAT inhibited cell firing

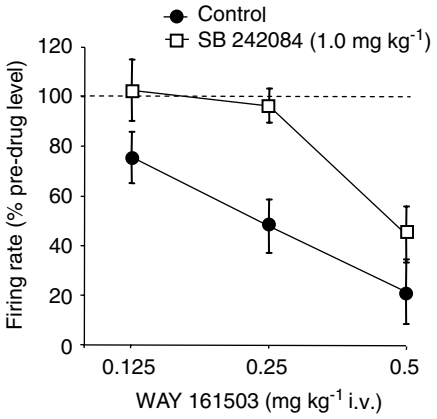

Figure 2 Inhibition of 5-HT cell firing by WAY 161503 and its blockade by the 5-HT$_{2C}$ receptor antagonist SB 242084 (1.0 mg kg^{-1}). Control animals received WAY 161503 alone and showed a dose-related inhibition of cell firing. Note that pre-treatment with SB 242084 caused a significant shift to the right of the dose response to WAY 161503. Data are mean ± s.e.m. of *n* observations at agonist doses of 0.125, 0.25, 0.5 mg kg^{-1}, respectively: control *n* = 8,8,7; SB 242084 *n* = 6,6,5. Effect of WAY 161503 alone, $P<0.0001$, one-way ANOVA; $P<0.01$ at 0.25 and 0.5 mg kg^{-1}, Dunnett's *post hoc* test. Effect of SB 242084 pre-treatment, $P<0.01$, two-way ANOVA; $P<0.05$ at 0.25 mg kg^{-1} WAY 161503, Bonferroni *post hoc* test.

and this effect was typically reversed by subsequent administration of WAY 100635 (11/15 neurones) (Figure 1).

Occupancy of SB 242084 at 5-HT$_{2A}$ receptors

As 5-HT$_{2A}$ receptors modulate 5-HT cell firing, the occupancy of SB 242084 at these receptors was investigated. In rat PET studies, [^{11}C]MDL 100907 demonstrated high levels of binding (VOI:cerebellum ratios) in the frontal cortex and striatum with lower levels in the olfactory bulbs, hypothalamus, hippocampus, superior colliculi and medulla (Figure 3a). Pre-treatment with SB 242084 (1 mg kg^{-1} i.v.) had no statistically significant on [^{11}C]MDL 100907 binding in any region tested. In comparison, pre-treatment with unlabelled MDL 100907 (0.2 or 0.4 mg/kg i.v.) reduced [^{11}C]MDL 100907 specific binding to unity in all regions sampled.

Ex vivo binding of [^{11}C]MDL 100907 (tissue:cerebellum [^{11}C]radioactivity ratios) confirmed the PET data (Figure 3b). As with the PET data, pre-treatment with SB 242084 (1 mg kg^{-1} i.v.) had no statistically significant effect on [^{11}C]MDL 100907 binding whereas unlabelled MDL 100907 reduced specific binding to unity.

Effect of WAY 161503 in presence of GABA$_A$ receptor antagonist

Additional experiments tested whether the inhibitory effect of WAY 161503 on 5-HT cell firing involved GABA. WAY 161503 (0.125–1.0 mg kg^{-1} i.v.) inhibited 5-HT cell firing, and this effect was partially restored (4/7 neurones) by administration of the GABA$_A$ receptor antagonist picrotoxin (⩽2 mg kg^{-1}) (Figure 4). Picrotoxin (0.5–1.0 mg kg^{-1}) alone caused a slight (+25%) increase in the firing rate of 5-HT cells (3/3 neurones).

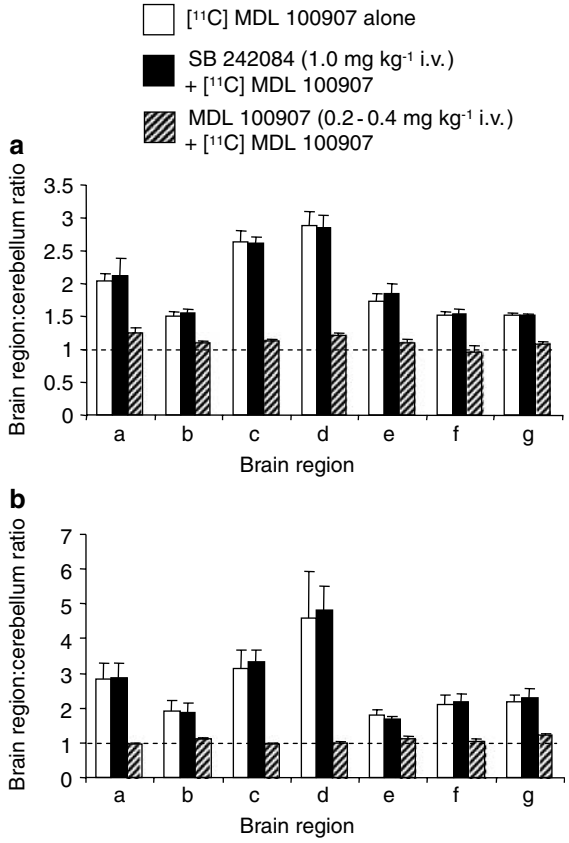

Figure 3 Binding of the radioligand [^{11}C]MDL 100907 in rat brain determined by (**a**) PET and (**b**) *ex vivo* methods. Rats received [^{11}C]MDL 100907 alone (open columns, *n* = 5), 5 min after SB 242084 1 mg kg^{-1} i.v. (filled columns, *n* = 4), or 5 min after non-radioactive MDL 100907 (0.2–0.4 mg kg^{-1} i.v.) (striped columns, *n* = 4). Brain regions: a, olfactory bulbs; b, hypothamus; c, striatum; d, frontal with cingulate cortex; e, hippocampus, f, superior colliculi; g, medulla. Data are mean ± s.d. values. Note that SB 242084 did not affect [^{11}C]MDL 100907 binding in any region whereas unlabelled MDL 100907 reduced [^{11}C]MDL 100907 binding to unity ($P<0.05$: Student's *t*-test).

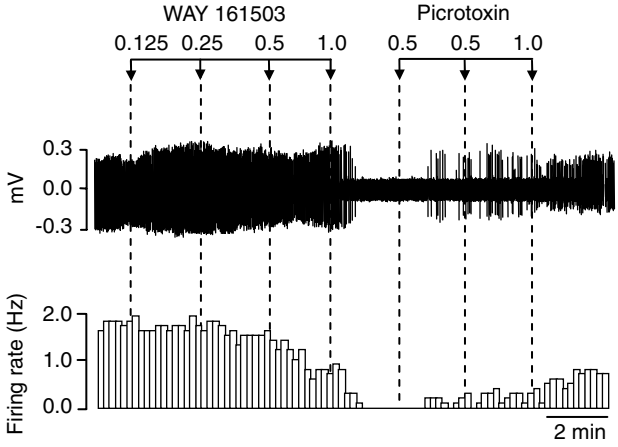

Figure 4 Spike train (upper trace) and rate-meter recording illustrating a partial reversal of the inhibitory effect of WAY 161503 on 5-HT cell firing by administration of the GABA$_A$ receptor antagonist picrotoxin. Drug administration (mg kg^{-1} i.v.) as indicated by arrows.

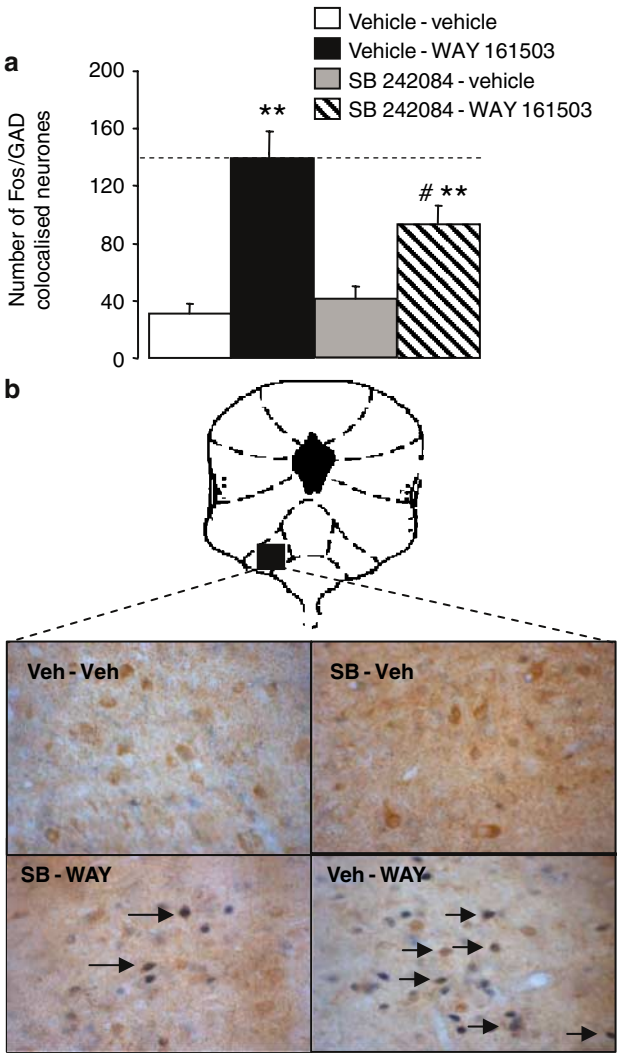

Figure 5 Effect of WAY 161503 on the expression of Fos in GAD-positive DRN neurones. (**a**) Drug treatments were vehicle-vehicle, vehicle-WAY 161503 (3 mg kg^{-1}), SB 242084 (1.0 mg kg^{-1})-vehicle and SB 242084 (1.0 mg kg^{-1})-WAY 161503 (3 mg kg^{-1}). Data ($n=6$) are mean$\pm$s.e.m. Note that WAY 161503 increased the number of Fos/GAD double-labelled cells and that this effect was reduced by pre-treatment with SB 242084. $^{**}P<0.001$ versus vehicle–vehicle (one way ANOVA with Dunnett's *post hoc* test). $^{\#}P<0.05$ versus vehicle–WAY 161503 (one-way ANOVA with Bonferroni's *post hoc* test). (**b**) Photomicrographs showing cells double labelled (arrows) with Fos and GAD immunoreactivity in the DRN of rats administered one the following drug treatments: vehicle–vehicle, SB 242084 (1.0 mg kg^{-1})–vehicle, SB 242084 (1.0 mg kg^{-1})–WAY 161503 (3 mg kg^{-1}), vehicle–WAY 161503 (3 mg kg^{-1}). Images at $\times$40 magnification.

Effect of WAY 161503 on Fos expression in GAD positive DRN neurones

In sections from vehicle-treated animals, GAD immunoreactivity was abundant in the lateral wings of the DRN with few GAD positive cells present in the midline. Moreover, in vehicle-treated animals the number of Fos positive cells was low, and there were few cells in which Fos and GAD immunoreactivity was colocalized (Figure 5).

Pilot experiments established that WAY 161503 (1, 3 and 10 mg kg^{-1} i.p.) caused a dose-related increase in Fos positive cells in the rat DRN (data not shown). A subsequent more detailed analysis confirmed this effect and revealed that, in comparison to vehicle controls, WAY 161503 (3 mg kg^{-1} i.p.) increased the number of Fos/GAD double-labelled cells in the lateral wings around three-fold and this effect was significantly reduced by pre-treatment with SB 242084 (1 mg kg^{-1} i.p.) (Figure 5). SB 242084 alone did not alter the number of Fos/GAD double-labelled cells (Figure 5).

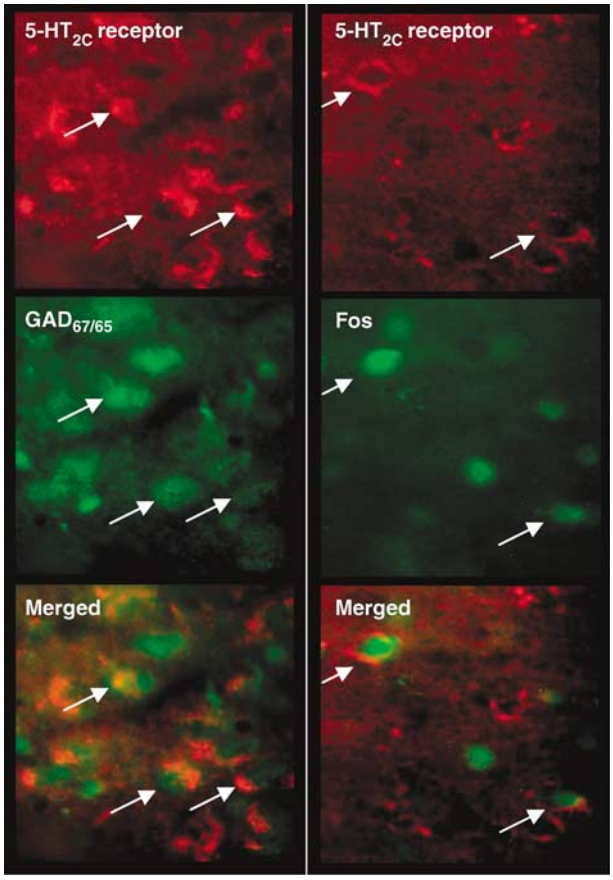

Figure 6 Photomicrographs of the rat DRN showing fluorescent images of 5-HT$_{2C}$ receptor immunoreactivity with either GAD$_{65/67}$ immunoreactivity (left) or Fos immunoreactivity induced by WAY 161503 (3 mg kg^{-1} i.p.) (right). Arrows indicate co-localized neurones as seen in the merged images. Note the presence of GAD positive cells double-labelled with 5-HT$_{2C}$ receptor immunoreactivity. Also Fos positive cells were double-labelled with 5-HT$_{2C}$ receptor immunoreactivity after WAY 161503 administration.

In sections from drug-naive rats, cells with 5-HT$_{2C}$ receptor immunoreactivity were sparsely distributed in the ventrolateral DRN with low levels in other DRN regions. In the ventrolateral DRN many GAD positive cells were double-labelled with 5-HT$_{2C}$ receptor immunoreactivity (Figure 6). In DRN sections from rats treated with WAY 161503 (3 mg kg^{-1} i.p.), numerous Fos positive cells were double-labelled with 5-HT$_{2C}$ receptor immunoreactivity (Figure 6).

Discussion and conclusions

Recent evidence suggests that postsynaptic 5-HT$_2$ receptors are involved in the feedback control of 5-HT neurones in the

midbrain raphe nuclei, and that neighbouring GABA neurones play a role (see Introduction). In particular, a combination of electrophysiological and Fos immunohistochemical studies show that 5-HT$_2$ receptor agonists both inhibit 5-HT neurones and activate GABA neurones in the DRN (Liu *et al*., 2000; Boothman *et al*., 2003). While these studies emphasize the contribution of the 5-HT$_{2A}$ receptor subtype, the importance of the 5-HT$_{2C}$ receptor subtype may be underestimated due to a lack of sufficiently selective drugs. The present study addressed this issue using the putative selective 5-HT$_{2C}$ receptor agonist WAY 161503. In addition, the possible involvement of DRN GABA neurones in 5-HT$_{2C}$ receptor-mediated feedback was investigated.

An important finding was that WAY 161503 caused a dose-dependent inhibition of the firing of DRN 5-HT neurones in anaesthetized rats. WAY 161503 is a recently developed 5-HT$_{2C}$ receptor agonist, with reported $\sim$6-fold selectivity over 5-HT$_{2A}$ receptors and 20-fold selectivity over 5-HT$_{2B}$ receptors in radioligand binding studies, and weak or negligible affinity for other sites reported to date (5-HT$_{1A,1B,1D,1F}$ receptors, 5-HT transporter) (Rosenzweig-Lipson *et al*., 2000, 2006). These data are supported by findings in functional *in vitro* assays that indicate WAY 161503 has 9- and 12-fold higher potency at 5-HT$_{2C}$ than 5-HT$_{2A}$ receptors (calcium mobilization) and 5-HT$_{2B}$ receptors ([^{3}H]-inositol phosphate formation), respectively (Rosenzweig-Lipson *et al*., 2006). Moreover, at doses comparable to those used in the present study (0.1–3.0 mg kg^{-1}) WAY 161503 is active in behavioural models of 5-HT$_{2C}$ receptor function in rats, and sensitive to 5-HT$_{2C}$ receptor antagonist blockade (Cryan and Lucki, 2000; Rosenzweig-Lipson *et al*., 2006).

The present data show that the inhibitory effect of WAY 161503 on 5-HT cell firing was reversed by both the 5-HT$_2$ receptor antagonist ritanserin (Leysen *et al*., 1985) and the 5-HT$_{2C}$ receptor antagonist, SB 242084 but not the 5-HT$_{1A}$ antagonist WAY 100635. In addition, pretreatment with SB 242084 shifted the WAY 161503 dose response curve to the right. The blocking effects of SB 242084 in particular, suggest a role for 5-HT$_{2C}$ receptors in the inhibition of 5-HT cell firing by WAY 161503. However, since activation of 5-HT$_{2A}$ receptors causes inhibition of 5-HT cell firing (Boothman *et al*., 2003) experiments were carried out to exclude the possible *in vivo* occupancy of SB 242084 at 5-HT$_{2A}$ receptors.

SB 242084, at a dose (1 mg kg^{-1} i.v.) that blocked the effect of WAY 161503 on 5-HT cell firing, showed no displacement of the 5-HT$_{2A}$ receptor radioligand [^{11}C]MDL 100907 in either rat PET or *ex vivo* binding studies. In comparison unlabelled MDL 100907 (0.2 or 0.4 mg kg^{-1} i.v.) fully displaced the [^{11}C]MDL 100907 signal in both models. Radioactivity levels in extracerebral tissues were unaffected by SB 242084 pre-treatment, indicating that the antagonist did not affect [^{11}C]MDL 100907 delivery. Thus, these data indicate that SB 242084 (1 mg kg^{-1} i.v.) has no significant occupancy of rat brain 5-HT$_{2A}$ receptors *in vivo*. These results are consistent with radioligand binding studies showing that SB 242084 has over 100-fold selectivity for 5-HT$_{2C}$ versus 5-HT$_{2A}$ and 5-HT$_{2B}$ receptors (Kennett *et al*., 1997) and exerts a 5-HT$_{2C}$ receptor antagonist action *in vivo* (Kennett *et al*., 1997).

Taken together, the above data indicate that 5-HT$_{2C}$ receptor activation contributes to the inhibition of 5-HT cell firing by WAY 161503. This evidence of a role for 5-HT$_{2C}$ receptors in 5-HT neurone control accords with observations that the inhibition of 5-HT cell firing by the 5-HT$_2$ receptor agonist DOI was attenuated by pre-treatment with the 5-HT$_{2B/C}$ receptor antagonist SB 206553 *in vivo* (Boothman *et al*., 2003). Also, in a raphe slice preparation the increase of IPSPs in 5-HT neurones induced by 5-HT and DOI was attenuated by SB 242084 (Liu *et al*., 2000).

It is noteworthy that the inhibition of 5-HT cell firing by WAY 161503 was not completely reversed by SB 242084, and that full reversal was only achieved by subsequent administration of ritanserin. As activation of 5-HT$_{2A}$ receptors inhibits 5-HT cell firing (Boothman *et al*., 2003), these results suggest that the inhibitory effect of WAY 161503 may be partially 5-HT$_{2A}$ receptor-mediated. This would be consistent with radioligand and functional assay data showing that even though WAY 161503 demonstrates preference for 5-HT$_{2C}$ versus 5-HT$_{2A}$ receptors, the selectivity is only of the order of 10-fold (see above).

In the present study, administration of the GABA$_A$ receptor antagonist picrotoxin restored the inhibition of 5-HT cell firing induced by WAY 161503. In agreement with previous studies (Gallager and Aghajanian, 1976), picrotoxin by itself had little effect on the firing of 5-HT neurones (+25% increase). These data are consistent with an earlier report in that the inhibition of 5-HT cell firing induced by DOI was restored by picrotoxin (Martin-Ruiz *et al*., 2001a). Moreover, the data suggest that an activation of GABA neurones may be involved in the inhibitory action of WAY 161503.

A possible action of WAY 161503 on GABA neurones within the DRN was investigated using Fos immunohistochemistry. It was found that WAY 161503 caused a marked increase in Fos expression in DRN cells that were immunoreactive for the GABA neurone marker, GAD. As this effect of WAY 161503 was attenuated by pre-treatment with SB 242084 the involvement of 5-HT$_{2C}$ receptors is implicated. Recently, we reported that DOI administration increased the number of Fos/GAD double-labelled cells in the DRN and it is possible that 5-HT$_{2C}$ (as well as 5-HT$_{2A}$) receptors contribute to this effect (Raley *et al*., 2004).

The 5-HT$_{2C}$ receptor-mediated action of WAY161503 on DRN GABA cells may be a direct effect as these cells were found to express 5-HT$_{2C}$ immunoreactivity, a finding which agrees with a recent report describing the presence of 5-HT$_{2C}$ receptor mRNA in DRN GABA neurones (Serrats *et al*., 2005). Also, WAY161503 increased Fos expression in DRN cells immunoreactive for 5-HT$_{2C}$ receptors.

Taking together the above findings, it is plausible that WAY 161503 inhibits 5-HT cell-firing in the DRN by activating local GABA neurons. This idea is consistent with earlier data demonstrating extensive synaptic interactions between GABA and 5-HT neurons in the DRN (Wang *et al*., 1992), and that local application of GABA agonists into this nucleus inhibits 5-HT cell firing (Gallager and Aghajanian, 1976). Thus, current data support a model of 5-HT feedback control in which 5-HT$_{2C}$ receptors activate DRN GABA neurones to inhibit 5-HT neuronal activity. However, 5-HT$_{2C}$ receptors are abundant in other brain regions and

the present data do not exclude the additional possibility that 5-HT$_{2C}$ receptors also play a role in the modulation of DRN afferents from more distant regions such as the lateral habenula or prefrontal cortex.

Interestingly, we observed many instances of DRN 5-HT neurones that were inhibited by both WAY 161503 as well as 8-OH-DPAT. This finding is evidence of 5-HT neurones that are sensitive to feedback control by both 5-HT$_{2C}$ and 5-HT$_{1A}$ receptors, the latter probably involving both pre- and postsynaptic mechanisms (Hajós *et al*., 1999). Our observations suggest that individual 5-HT neurones are subject to 5-HT feedback control at several levels.

In summary, this study demonstrates that WAY 161503 inhibits 5-HT cell firing in the DRN *in vivo,* and support a mechanism involving 5-HT$_{2C}$ receptor-mediated activation of DRN GABA neurones. This mechanism may be one means by which postsynaptic 5-HT receptors located on afferent inputs to 5-HT neurones contribute to 5-HT feedback control. In this context, evidence that 5-HT$_{2C}$ receptor antagonists augment the neurochemical effects of some antidepressants is of particular interest (Cremers *et al*., 2004; Boothman *et al*., 2006). Thus, by analogy to 5-HT autoreceptor-mediated feedback mechanisms, it is possible that 5-HT$_{2C}$ feedback may provide a source of targets for drug therapies addressing neuropsychiatric disorders.

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Conflict of interest

The authors state no conflict of interest.

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