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5-HT_7 receptor subtype as a mediator of the serotonergic regulation of luteinizing hormone release in the zona incerta

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Abstract

5-Hydroxytryptamine (5-HT) and the 5-$HT_{1A/7}$ receptor agonist (+)-8-hydroxy-2-(di-*n*-propylamino) tetralinHBr (8-OH-DPAT), injected into the zona incerta (an area in the dorsal hypothalamus) of the female rat, inhibit the release of luteinizing hormone (LH) and the effects of both are blocked by the 5-$HT_{2/7}$ receptor antagonist, ritanserin. As both 8-OH-DPAT and ritanserin have moderate activity at the 5-HT_7 receptor subtype, the possibility that this subtype might mediate their effects in the zona incerta has been investigated. Ovariectomised rats were primed with 5 μg oestradiol benzoate followed at 48 h by 0.5 mg progesterone, which induces an LH surge. 5-Carboxamidotryptamine (5-CT), a potent but non-selective agonist at 5-HT_7 receptors, like 5-HT and 8-OH-DPAT, inhibited the LH surge at 5 and 1.25 nmol injected bilaterally into the zona incerta. The non-selective 5-$HT_{2/7}$ receptor antagonist ritanserin and the selective 5-HT_7 receptor antagonist, (*R*)-3-(2-(2-(4-methyl-piperidin-1-yl)-pyrrolidine-1-sulfonyl)-phenol (SB-269970-A) at 0.5 μg/side blocked all three receptor agonists when injected concurrently into the zona incerta. However, lower (0.2 μg) and higher doses (2 and 5 μg) of SB-269970-A were less effective, indicating a bell-shaped dose–response curve. SB-269970-A was also inhibitory when administered systemically (1 mg/kg intraperitoneally (i.p.)). When LH release was suppressed by 5 μg oestradiol benzoate, SB-269970-A (0.5 and 2 μg) did not elevate levels, indicating it is unlikely that 5-HT_7 receptors mediate a tonic inhibition on release but rather are involved in terminating the pre-ovulatory LH surge. These data demonstrate that 5-HT_7 receptors play a role in the regulation of LH by the zona incerta in rat brain.

Keywords: 5-HT; Zona incerta; 5-HT_7 receptor; 5-Carboxamidotryptamine; Ritanserin; SB-269970-A

1. Introduction

The zona incerta, an area in the dorsal hypothalamus, is an integrative centre in the regulation of a wide variety of functions. A number of reports indicate that neuronal systems either originating from or innervating the zona incerta are involved in the control of gonadotrophin release. These neuronal systems include dopamine, γ-amino-butyric acid (GABA) (Oertel et al., 1982; Kalia et al., 1999), melanin-concentrating hormone (Bittencourt et al., 1992; Gonzalez et al., 1997), orexins (Sakurai et al., 1993; Pu et al., 1998; Small et al., 2003) and 5-hydroxytryptamine (5-HT; Bosler et al., 1984; James et al., 1989). There are 5-HT nerve terminals surrounding the zona incerta (Bosler et al., 1984) and we have shown that there is an inverse relationship between 5-HT activity in the zona incerta and plasma levels of luteinizing hormone (LH) (James et al., 1989). We have also shown that administration of 5-HT into the zona incerta inhibits the LH surge normally observed after priming ovariectomised rats with oestradiol benzoate followed by progesterone. This effect is mimicked by a 5-HT_{1A} receptor agonist ((+)-8-hydroxy-2-(di-*n*-propylamino) tetralinHBr; 8-OH-DPAT) and inhibited by a 5-HT_{1A} receptor antagonist (WAY 100135; *n-tert*-butyl-3-(4-methoxfphenyl) piperazin-lyl indicating that 5-HT_{1A} receptors play a role in mediating the inhibitory effect of 5-HT on LH release. In the same investigation we showed that the non-selective 5-HT_2 receptor agonist (−)-2,5-dimethoxy-4-iodoamphetamine HCL (DOI) had no effect on LH release, but unexpectedly the 5-HT_2 receptor antagonist, ritanserin antagonised the inhibitory effect of both 5-HT and 8-OH-

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DPAT (Siddiqui et al., 2000). A possible explanation might be that a 5-HT receptor subtype, other than the 5-HT_{1A} receptor, is involved in mediating the inhibitory effect of 5-HT and since both 8-OH-DPAT and ritanserin have affinity for the 5-HT_7 receptor subtype (Lovenberg et al., 1993) we have investigated this hypothesis.

The 5-HT_7 receptor was first cloned from mouse, rat, guinea-pig and human (Bard et al., 1993; Plassat et al., 1993; Shen et al., 1993; Tsou et al., 1994; Raut et al., 1993) and has a distinctive pharmacological profile which is consistent across species (Lovenberg et al., 1993; Bard et al., 1993; Plassat et al., 1993; Shen et al., 1993; Tsou et al., 1994; Raut et al., 1993) and between cloned and native tissue 5-HT_7 receptors (To et al., 1995; Boyland et al., 1996). The receptors are defined pharmacologically by their high affinity for 5-CT, 5-HT, 5-methoxytryptamine and methiothepin, moderate affinity for 8-OH-DPAT and ritanserin and low affinity for pindolol, sumatriptan and buspirone (To et al., 1995) 5-HT_7 receptors couple positively to adenylyl cyclase in heterologous expression systems and in native tissue (Hagan et al., 2000; Hoyer et al., 1994; Thomas et al., 1999).

5-HT_7 receptors have a wide distribution within the central nervous system (CNS) and there is reasonable agreement between mRNA in situ hybridisation, semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR), autoradiographic ligand-binding studies and immunocytochemistry (Lovenberg et al., 1993; Shen et al., 1993; Raut et al., 1993; Eglen et al., 1997; Neumaier et al., 2001). The receptor is found predominantly in the thalamus, hippocampus, amygdala and hypothalamus (Raut et al., 1993; To et al., 1995; Gustafson et al., 1996; Kinsey et al., 2001) and the receptors have been reported to be present on both perikerya and proximal fibres (Neumaier et al., 2001) and astrocytes (Hirst et al., 1997). In spite of their presynaptic presence on cell bodies they do not appear to exert an autoregulatory control on 5-HT release (Roberts et al., 2001).

5-HT_7 receptors mediate the effect of 5-HT on a number of hypothalamic functions such hypothermia (Meuser et al., 2003; Guscott et al., 2003) and its inhibitory action on spontaneous discharge (Yu et al., 2001) and photic activation of suprachiasmatic nucleus cells (Ying and Rusak, 1997; Pickard and Rea, 1997). 5-HT_7 receptor activation is thought to advance the phase of free-running circadian rhythms (Ehlen et al., 2001), although these observations require confirmation with selective pharmacological tools. The receptors are also involved in the hypothalamic–pituitary–adrenal axis with 5-HT_7 activation directly stimulating the adrenal glomerulosa cells to secrete aldosterone (Lenglet et al., 2002) while in the hippocampus and cortex, adrenal steroids reduce 5-HT_7 receptor expression (Yau et al., 1997; Le Corre et al., 1997; Shimizu et al., 1997).

In order to clarify the role of 5-HT_7 receptors in the control of LH, a study was carried out using the selective 5-HT_7 receptor antagonist SB-269970-A (Hagan et al., 2000; Lovell et al., 2000) and 5-carboxamidotryptamine (5-CT), a non-selective receptor agonist with high affinity and efficacy at the human and guinea-pig 5-HT_7 receptors (Hagan et al., 2000; Lovell et al., 2000; Hirst et al., 1997). Doses of SB-269970-A for systemic injection were selected on the basis of published data (Hagan et al., 2000).

2. Materials and methods

2.1. Animals and steroid priming

Female Wistar rats (200–250 g) bred at St. George's Hospital Medical School were caged in groups of 5 in a lighting system of 12:12 hours (h) light–dark (lights on 7:00 h). The rats were ovariectomised under halothane (Fluothane; Zeneca, Macclesfield, UK) and nitrous oxide anaesthesia at least 2 weeks before the experiment. All rats were then primed with 5 μg/rat oestradiol benzoate subcutaneously (s.c.) 50 h before the experiment. Administration of oestradiol benzoate alone exerts a negative feedback effect on LH release and induces relatively low levels of plasma LH. Some rats also received 0.5 mg/rat s.c. progesterone 48 h after the oestradiol benzoate and 2 h before the experiment; this treatment stimulates the release of an LH surge with a peak concentration about 4 h after the progesterone but often ranging between 3.5 and 5 h after the progesterone injection. The two priming regimes thus produce models, suitable for the investigation of agents with potential stimulatory and inhibitory effects on LH release, respectively. All studies were conducted in compliance with the Home Office Guidance on the operation of the Animals (Scientific Procedures) Act 1986.

2.2. Drugs

In most studies, the compounds were injected bilaterally into the zona incerta in 0.5 μl saline. The serotonergic receptor agonists were 5-hydroxytryptamine creatinine sulphate (5-HT; Sigma, Poole, Dorset, UK), (+)-8-hydroxy-2-(di-*n*-propylamino) tetralin. HBr (8-OH-DPAT; Research Biochemicals International (RBI), Natwick, MA, USA) and 5-carboxamidotryptamine maleate (5-CT; RBI). The serotonergic receptor antagonists were (*R*)-3-(2-(2-(4-methyl-piperidin-1-yl)-pyrrolidine-1-sulfonyl)-phenol (SB-269970-A; GlaxoSmithKline Beecham Pharmaceuticals, Harlow, Essex), *n-tert*-butyl-3-(4-methoxyphenyl) piperazin-l-yl (WAY 100135; Wyeth Research Centre, Taplow, Bucks., UK) and ritanserin (6-[2-[4-[bis-(4-fluorophenyl) methylene]-1-piperidinyl]-ethyl]-7-methyl-5H-thiazole[3,2-1) pyrimidin-5-one; RBI).

When 5-HT and SB-269970-A were given together the final injection volume was maintained at 0.5 μl/side by doubling their concentrations. Groups of rats also received SB-269970-A (1 to 20 mg/kg) intraperitoneally (i.p.) in 1 ml/kg saline, given 30 min prior to the central injections of

5-HT into the zona incerta. The other two antagonists WAY 100135 (2 mg/kg) and ritanserin (0.25 mg/kg), were also given i.p. in 1 ml/kg saline, 1 h before the central injection of the agonist.

2.3. Experimental procedures

Fifty hours after the oestradiol benzoate injection (2 h after progesterone injection), the rats were anaesthetised with Saffan at 3 ml/kg i.p. (Alphaxalone 0.9% + Alphadolone acetate 0.3%; Glaxo Intervet) and a blood sample (0.1 ml) was collected from the tail vein. The animals were then placed in a stereotaxic apparatus and either saline, 5-HT 8-OH-DPAT or 5-CT was injected bilaterally into the zona incerta using co-ordinates derived from the rat brain atlas (Paxinos and Watson, 1998) 2.3 mm behind Bregma, 0.5 mm lateral from the midline and 7.5 mm vertically below the surface of the cortex. The drugs were injected in 0.5 μl saline via a 10 μl Hamilton syringe and the controls received 0.5 μl saline only. One or two saline treated animals were included on each experimental day to make up the final control group. The time of injection was taken as 0 min and blood samples were collected at 10- or 20-min intervals for 2 h from the oestradiol benzoate-alone primed rats and at 30-min intervals for 3 h from the oestradiol benzoate plus progesterone treated animals. During this period, the anaesthetised rats were placed on an electric heating pad in order to maintain body temperature (at 35.5 ± 0.3 °C: see Siddiqui et al., 2000) and induce a degree of vasodilatation. At the end of the sampling period, the rats were autopsied and their brains removed and fixed in 10% formal saline. The blood samples were centrifuged at 2000 rpm for 10 min and the plasma stored at − 20 °C until assayed for LH.

Only results from animals injected within the zona incerta have been reported. Previous tests, using the dye, Thionine blue, have shown minimal diffusion from the site of injection. Additionally, in contrast to the effect of 5-HT injections inside the site, injections close to, but outside the zona incerta have no effect on LH release (see Siddiqui et al., 2000).

2.4. Histology

Using a freezing microtome 60 μm sections were prepared from the fixed brain and stained with Thionine blue and then examined for the lowest point of the bilateral needle tracts. This was taken as the site of injection (see Siddiqui et al., 2000 for details).

2.5. Radioimmunoassay for LH

Ten microlitre plasma samples were assayed in duplicate employing reagents kindly supplied by the National Hormone and Pituitary Program (Baltimore, MD, USA). The standard was NIADDK-rLH-RP3 and the antibody, NIADDK-anti-rLH S10. The inter- and intra-assay coefficients of variation were 8.0% and 9.5%, respectively and the sensitivity was 10 pg/tube (1 ng/ml).

2.6. Statistical analysis

Data were analysed using one-way analysis of variance (ANOVA) followed by Gabriel's test for unequal groups (Kendall and Stuart, 1968). Student's *t*-test was used for two group comparisons.

3. Results

3.1. The effect of serotonergic agonists on LH release

5-HT, 8-OH-DPAT and 5-CT were injected into the zona incerta of anaesthetised ovariectomised rats primed with 5 μg oestradiol benzoate followed at 48 h by 0.5 mg progesterone. The receptor agonists were given 2 h after the progesterone at 5 and 1.25 nmol (2 and 0.5 μg 5-HT; 1.65 and 0.4 μg 8-OH-DPAT; 1.5 and 0.4 μg 5-CT). Since the actual peak of the LH surge induced by progesterone is variable, the results in Table 1 have been presented as the difference between the basal plasma LH levels measured just before the injection of the compounds and the highest concentration noted in the 3-h interval after the injection. Thus the rise in LH in the saline controls was 7.6 ± 0.7 ng/ml and the receptor agonists at 5 nmol significantly reduced this increase. At the lower dose of 1.25 nmol, 5-HT no

Table 1
The inhibitory effect of serotonergic receptor agonists in the zona incerta on the release of LH

Treatment bilaterally into ZI in 0.5 μl saline	Plasma LH ng/ml ± S.E.M.				
	Dose μg/side	No. rats per group	Basal pre-inj conc	Peak post-inj conc	Difference
Saline	–	18	3.5 ± 0.6	10.9 ± 0.7	7.6 ± 0.7
5-HT	2 μg (5.0 nmol)	16	3.0 ± 0.4	7.8 ± 0.8	4.7 ± 0.7 b
	0.5 μg (1.25 nmol)	6	4.4 ± 0.6	11.0 ± 1.6	6.6 ± 1.0
8-OH-DPAT	1.65 μg (5.0 nmol)	5	3.4 ± 0.8	6.7 ± 0.5	3.9 ± 0.6 b
	0.4 μg (1.25 nmol)	7	2.4 ± 0.5	5.4 ± 0.8	3.1 ± 0.7 c
5-CT	1.5 μg (5.0 nmol)	10	3.4 ± 1.1	8.3 ± 1.0	4.5 ± 0.6 c
	0.4 μg (1.25 nmol)	7	2.4 ± 0.3	6.7 ± 0.8	4.5 ± 0.8 c

The inhibitory effect on LH release of 5-HT, 8-OH-DPAT and 5-CT at 5 and 1.25 nmol given bilaterally into the zona incerta (ZI) in ovariectomised rats primed with 5 μg/rat oestradiol benzoate followed at 48 h by 0.5 mg progesterone. The central injections were administered 1–2 h after the progesterone and blood samples were collected at 30-min intervals for 3 h after the central injection.
ANOVA: $F(6,62) = 4.45$, $P = 0.0008$.
b = $P < 0.005$, c = $P < 0.001$ compared to saline controls (Gabriel's test).

longer had a significant effect, while 8-OH-DPAT and 5-CT still inhibited the release of LH (see Table 1).

3.2. The effect of SB-269970-A, on the action of serotonergic agonists on LH release

Table 2A and Fig. 1 show the effect of 5 nmol 5-HT (2 μg) on the LH surge in oestradiol benzoate plus progesterone primed ovariectomised rats, when given alone or together with increasing doses of SB-269970-A. As before 5-HT reduced the rise in LH when compared to that seen in the saline-treated controls. When SB-269970-A was given with 5-HT, it completely prevented the inhibitory effect of 5-HT at the dose of 0.5 μg/side. It has no effect at the lower dose of 0.2 μg/side; nor at the highest dose tested, which was 5 μg/side. At 2 μg/side SB-269970-A, the overall effect was a slight inhibition of the 5-HT activity, such that the rise in LH was not significantly different from the saline controls, but was also not significant from the value noted after 5-HT alone. Table 2B and C shows that over the dose range of 0.5 to 5 μg/side SB-269970-A had an almost identical antagonistic effect on the action of 8-OH-DPAT and 5-CT in that it significantly prevented their inhibition of LH release

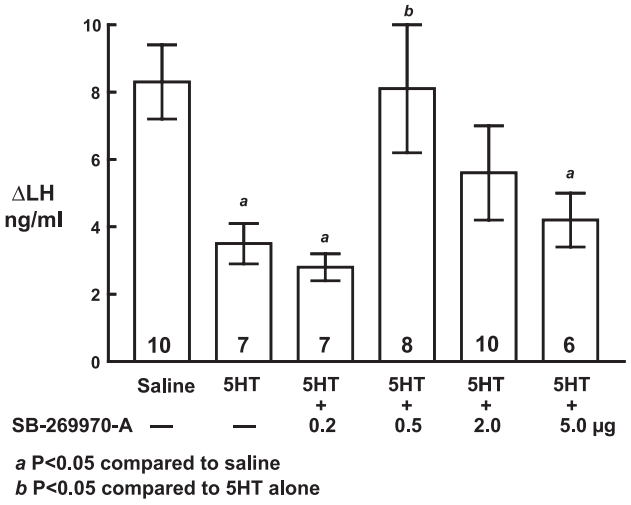

Fig. 1. The antagonistic effect of SB-269970-A on the inhibitory effect of 5 nmol 5-HT given bilaterally into the zona incerta (ZI) on LH release in ovariectomised rats primed with 5 μg oestradiol benzoate and 0.5 mg progesterone. ΔLH ng/ml is the difference of basal pre-injection and peak post-injection concentrations. The vertical lines indicate the standard error of the mean. The numbers in each histogram indicate the number of rats in the group. ANOVA: $F(5,42)=3.5$, $P=0.0099$, [a]$P<0.05$ compared to saline. [b]$P<0.05$ compared to 5-HT alone.

Table 2
The antagonistic effect of SB-269970-A on the inhibitory actions of serotonergic receptor agonists in the zona incerta on LH release

	Treatment bilaterally in ZI at 5 nmol		Plasma LH ng/ml ± S.E.M.			
		SB-269970-A μg/side	No. rats per group	Basal pre-inj conc	Peak post-inj conc	Difference
A	Saline	–	10	4.5 ± 0.6	12.8 ± 1.4	8.3 ± 1.1
	–	0.5	7	3.5 ± 0.5	11.1 ± 1.1	7.6 ± 0.4
	–	2.0	5	2.5 ± 0.6	9.2 ± 1.1	6.7 ± 1.0
	5-HT	–	7	2.4 ± 0.4	5.9 ± 0.7	3.5 ± 0.6 [a]
	5-HT	0.2	7	2.7 ± 0.6	5.6 ± 0.5	2.8 ± 0.4 [a]
	5-HT	0.5	8	4.2 ± 0.5	12.2 ± 2.1	8.1 ± 1.9 [d]
	5-HT	2.0	10	2.2 ± 0.4	7.9 ± 1.4	5.6 ± 1.4
	5-HT	5.0	6	2.7 ± 0.7	6.9 ± 1.3	4.2 ± 0.8 [a]
B	Saline	–	10	2.6 ± 0.4	9.5 ± 0.5	6.7 ± 0.5
	8-OH-DPAT	–	5	3.4 ± 0.8	6.7 ± 0.5	3.2 ± 0.6 [c]
	8-OH-DPAT	0.5	8	3.2 ± 0.7	10.6 ± 1.4	7.4 ± 1.2 [e]
	8-OH-DPAT	2.0	6	2.2 ± 0.2	4.4 ± 0.6	2.3 ± 0.7 [c]
	8-OH-DPAT	5.0	6	2.3 ± 0.7	5.0 ± 1.0	2.6 ± 0.5 [c]
C	Saline	–	13	2.8 ± 0.3	11.1 ± 1.9	7.0 ± 1.0
	5-CT	–	5	3.4 ± 1.2	7.8 ± 0.9	3.8 ± 0.6 [a]
	5-CT	0.5	6	6.9 ± 2.1	13.2 ± 2.9	6.2 ± 0.9 [d]
	5-CT	2.0	8	4.5 ± 0.5	6.8 ± 0.9	3.4 ± 0.7 [a]
	5-CT	5.0	6	3.8 ± 0.9	6.9 ± 2.2	3.1 ± 1.4 [a]

The antagonistic effect of SB-269970-A on the inhibitory actions of 5 nmol 5-HT (A), 8-OH-DPAT (B) and 5-CT (C) given bilaterally into the zona incerta (ZI), on LH release in ovariectomised rats primed with 5 μg oestradiol benzoate and 0.5 mg progesterone.
ANOVA: 5-HT, $F(5, 42)=3.5$, $P=0.0099$; 8-OH-DPAT, $F(4, 30)=9.75$; $P<0.0001$; 5-CT, $F(4, 33)=3.31$, $P=0.022$.
[a]$=P<0.05$, [c]$=P<0.001$ compared to saline controls.
[d]$=P<0.05$, [e]$=P<0.001$ compared to receptor agonist given alone (Gabriel's test).

at 0.5 μg/side and had no significant effect at 2 or 5 μg/side. SB-269970-A alone had no effect on LH release in the oestradiol benzoate plus progesterone primed model at 0.5 or 2 μg/side (Table 2).

Table 3 shows the effect of systemic administration of SB-269970-A on the inhibition of LH release exerted by 5-HT injected into the zona incerta. The compound was given intraperitoneally to groups of rats over a dose range of 1 to 20 mg/kg, 30 min prior to the central injection of 5-HT. The lowest dose of 1 mg/kg significantly antagonised the effect of 5-HT, while the higher doses (3, 10 and 20 mg/kg) were less effective.

Table 3
The antagonistic effect of SB-269970-A given intraperitonally on the inhibitory effect of 5-HT in the zona incerta on LH release

Treatment bilaterally in ZI. 5-HT at 5 nmol (2 μg)/side	SB-269970-A mg/kg i.p.	No. of rats	Plasma LH ng/ml ± S.E.M.		
			Basal pre-inj conc	Peak post-inj conc	Difference
Saline	–	13	2.8 ± 0.3	11.1 ± 1.9	7.0 ± 1.0
5-HT	–	5	0.9 ± 0.6	4.5 ± 1.1	3.6 ± 0.6 [a]
5-HT	1.0	5	2.5 ± 0.7	8.3 ± 0.8	5.8 ± 0.7 [d]
5-HT	3.0	6	0.3 ± 0.2	5.4 ± 1.2	5.1 ± 1.2
5-HT	10.0	5	2.1 ± 0.6	7.5 ± 2.0	5.4 ± 1.5
5-HT	20.0	5	3.4 ± 0.5	8.6 ± 3.4	5.8 ± 2.5

The antagonistic effect of SB-269970-A given intraperitoneally (i.p.) 30 min prior to central injection of 5-HT (5 nmol bilaterally into the zona incerta (ZI) in ovariectomised rats primed with oestradiol benzoate and 0.5 mg progesterone.
[a]$=P<0.05$ compared to saline controls; [d]$=P<0.05$ compared to 5-HT given alone (Student's *t*-tests).

Table 4
The antagonistic effect of ritanserin and WAY 100135 given intraperitonally on the inhibitory effect of 5-CT in the zone incerta on LH release

Treatment bilaterally in ZI at 5 nmol	Antagonist treatment	No. rats per group	LH ng/ml ± S.E.M.		
			Basal pre-inj conc	Peak post-inj conc	Difference
Saline	–	10	2.1 ± 0.3	7.9 ± 0.4	5.6 ± 0.4
5-CT	–	6	2.2 ± 0.3	5.5 ± 0.2	3.2 ± 0.4 [a]
5-CT	Ritanserin	9	3.0 ± 0.8	10.6 ± 2.8	5.8 ± 1.1 [d]
5-CT	WAY100135	9	1.7 ± 0.4	8.1 ± 0.6	6.4 ± 0.3 [d]

The antagonistic effect of ritanserin (0.25 mg/kg i.p.) and WAY 100135 (2 mg/kg i.p.) given 1 h prior to central injection of 5-CT (5 nmol bilaterally into the zona incerta (ZI)).
ANOVA: $F(3,30) = 3.53$, $P = 0.027$.
[a] = $P < 0.05$ compared to saline controls; [d] = $P < 0.05$ compared to 5-CT given alone (Gabriel's test).

3.3. The effect of 5-HT$_{1A}$ and 5-HT$_2$ antagonists on the action of 5-carboxamidotryptamine (5-CT) on LH release

Previous experiments show that the inhibitory effect of 5-HT and 8-OH-DPAT in the zona incerta on LH release can be prevented by the selective 5-HT$_{1A}$ receptor antagonist, WAY 100135, and the 5-HT$_2$ receptor antagonist, ritanserin. When these agents were given alone they had no effect on LH release (Siddiqui et al., 2000). Table 4 shows that both antagonists also block the inhibitory effect of 5-CT in the zona incerta when given intraperitoneally, 1 h before the central administration of the agonist.

3.4. Investigation into a putative stimulatory effect of SB-269970-A on LH release

Since SB-269970-A appeared to prevent the inhibitory effect of exogenous 5-HT release, an experiment was carried out to see if it might antagonise a possible tonic inhibitory action of endogenous 5-HT. SB-269970-A was injected at 0.5 and 2 μg/side into the zona incerta of ovariectomised rats primed with oestradiol benzoate alone so that basal levels of LH were low. Table 5 shows that LH levels after SB-269970-A at both doses were not significantly different from those of the saline controls.

4. Discussion

Previous results obtained in this laboratory, indicate that serotonergic neurons in the zona incerta exert an inhibitory effect on LH release and this is mediated via 5-HT$_{1A}$ receptors, since the 5-HT$_{1A}$ receptor agonist, 8-OH-DPAT mimics the inhibitory effect of 5-HT and the effect of both receptor agonists is prevented by a selective 5-HT$_{1A}$ receptor antagonist, WAY 100135 (Siddiqui et al., 2000). However, the same studies indicated that another receptor subtype may be involved in mediating the inhibitory serotonergic effect. The 5-HT$_7$ receptor was a candidate for this role because 5-HT$_7$ receptors are present in the zona incerta (Neumaier et al., 2001) and the inhibitory effect of 8-OH-DPAT (as well as 5-HT) could be reversed by ritanserin. Although 8-OH-DPAT has a high affinity and potency at the 5-HT$_{1A}$ receptor and ritanserin has a high affinity for the 5-HT$_2$ receptor (Hoyer et al., 1994), they also share the common property of moderate affinity for the 5-HT$_7$ receptor (Lovenberg et al., 1993; To et al., 1995) and the experiments described in this report investigated the possibility that 5-HT$_7$ receptors might mediate serotonergic regulation of LH release in the zona incerta.

The first approach was to show that the serotonergic receptor agonist 5-CT, which has highest affinity and potency for 5-HT$_7$ receptors, although it is still non-selective, had a similar inhibitory effect on LH release to that of 5-HT and 8-OH-DPAT. In vitro functional and binding studies indicate that the rank order of 5-HT$_7$ potency of the three compounds is 5-CT>5-HT>8-OH-DPAT (Thomas et al., 1998; Inoue et al., 2003). However, this was not demonstrated in vivo, with 5-HT the natural ligand, being the least potent; perhaps because it is more rapidly degraded than the synthetic compounds. In addition, none of the

Table 5
The effect of SB-269970-A in the zone incerta on the tonic release of LH

LH ng/ml ± S.E.M.										
Bilateral treatment in ZI	0	10	20	30	40	60	80	100	120 min after injection	A.U.C. ± S.E.M.
Saline 0.5 μl/side (8)	3.2 ± 0.6	4.1 ± 1.0	3.4 ± 0.5	3.7 ± 0.7	4.3 ± 1.0	6.8 ± 1.9	7.8 ± 1.7	6.6 ± 1.3	6.9 ± 5.6	684 ± 103
SB-269970-A 2 μg/side (8)	4.6 ± 0.7	5.3 ± 1.1	3.3 ± 0.5	5.5 ± 0.9	5.6 ± 1.3	8.7 ± 1.4	7.9 ± 1.3	5.2 ± 0.5	4.8 ± 1.2	732 ± 110
SB-269970-A 0.5 μg/side (8)	3.1 ± 0.7	3.9 ± 0.6	5.0 ± 0.9	3.5 ± 0.8	3.6 ± 0.6	3.3 ± 1.1	6.0 ± 1.6	4.7 ± 1.4	5.1 ± 1.0	563 ± 84

The effect of SB-269970-A given bilaterally into the zona incerta (ZI) on plasma LH concentrations (ng/ml ± S.E.M.) in ovariectomised rats primed with 5 μg oestradiol benzoate alone.
Blood samples were collected starting 48 h after oestradiol benzoate and at 10- or 20-min intervals for 2 h, post-central injection.
A.U.C. ± S.E.M. = Mean area-under-the-curve ± standard error of the mean. Figures in parenthesis indicate number of samples in the group.

agents are selective and their additional effect on the 5-HT_{1A} receptors may have affected the final result.

In common with 5-HT and 8-OH-DPAT the inhibitory effect of 5-CT was prevented by both the 5-HT_{1A} receptor antagonist, WAY 100135, and the 5-HT_2 receptor antagonist, ritanserin. This indicates that all three serotonergic receptor agonists exert their action via the 5-HT_{1A} receptor and additionally by another receptor that is blocked by ritanserin. It is unlikely that this is the 5-HT_2 receptor since the 5-HT_2 receptor agonist DOI does not inhibit LH release (Siddiqui et al., 2000), nor do 8-OH-DPAT or 5-CT bind to the 5-HT_2 receptor (Hoyer et al., 1994). Instead, as hypothesised above, the second receptor may be the 5-HT_7 subtype.

SB-269970-A is a highly potent 5-HT_7 receptor antagonist with selectivity of 100-fold or greater across a wide range of receptors and enzymes, except for the 5-HT_{5A} receptor for which selectivity is 50-fold (Lovell et al., 2000; Hagan et al., 2000). On rat cortical membranes, it has a K_D of 0.9 ± 0.1 nM and B_{max} of 30 ± 20.1 fmol/mg protein (Thomas et al., 2002). When injected into the zona incerta together with 5-HT, 8-OH-DPAT or 5-CT, SB-269970-A at 0.5 μg/side prevented the inhibitory effects on LH release of the three agonists. The lower dose of 0.2 μg/side and the higher doses of 2 and 5 μg/side had no significant effect. Thus in this model, SB-269970-A had an inverse U-shaped dose–response curve and is only effective over a relatively narrow dose range. Similar results were obtained when SB-269970-A was given systemically, in that it was more effective at 1 mg/kg i.p. than at higher doses (3–20 mg/kg). It is difficult to explain the lack of effect of the higher concentrations and the appearance of the bell-shaped response-curve. This has not been reported in other pharmacological studies. It is possible that the effect on the 5-HT_5 receptor comes into play and masks the action on the 5-HT_7 subtype when tissue concentration is high. Alternatively, perhaps the 5-HT_7 receptor mediates the regulation of other neuroendocrine functions and at the higher doses SB-269970-A affects endogenous 5-HT activity on these other systems which then interact and/or mask its effect on LH release.

If exogenous 5-HT exerts an inhibitory effect on LH release via 5-HT_7 receptors, it is possible that endogenous 5-HT exerts a similar tonic effect. This would be revealed by administering the 5-HT_7 antagonist alone to see if it removes the putative inhibitory effect and induces a rise in circulating LH. SB-269970-A was therefore injected alone into the zona incerta of ovariectomised rats treated with oestradiol benzoate, which suppresses LH release allowing observation of any stimulatory effect. No rise in basal levels of LH was noted after either 0.5 or 2 μg/side of the receptor antagonist. Previous findings showed that the selective 5-HT_{1A} receptor antagonist Way 100135 was also ineffective in this model (Siddiqui et al., 2000), indicating that neither 5-HT_{1A} and 5-HT_7 receptors are involved in mediating any endogenous inhibitory control of LH release at this site and in these conditions. This is in contrast to the effect of naloxone (a μ-opioid receptor antagonist), which induces a rise in LH plasma levels indicating it has disinhibited the LH system from a tonic inhibitory effect of endogenous β-endorphin (Van Vught et al., 1982). The physiological significance of the involvement of 5-HT receptors in the zona incerta on the control of LH release is unclear. It is important that the pre-ovulatory LH surge (mimicked in the ovariectomised-steroid-primed model) is transient, thus mechanisms are required for initially stimulating LH release followed rapidly by an inhibition. Perhaps the 5-HT_7 receptor-mediated activity is involved in this rhythmic change in a similar manner to its proposed involvement in circadian rhythms (Lovenberg et al., 1993; Pickard and Rea, 1997).

In conclusion, it is suggested that the inhibitory effect of 5-HT in the zona incerta may be mediated, in part, by 5-HT_7 receptors. This is based on the pharmacological evidence that the inhibitory effects of 5-HT and two other serotonergic agents with affinity for the 5-HT_7 receptor (5-CT and 8-OH-DPAT) are blocked by a non-selective 5-HT_7 receptor antagonist (ritanserin) and more importantly by a highly selective 5-HT_7 receptor antagonist (SB-269970-A).

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References

Bard, J.A., Zgombick, J., Adham, N., Vaysse, P., Branchek, T.A., Weinshank, R.L., 1993. Cloning of a novel human serotonin receptor (5-HT_7) positively linked to adenylate cyclase. J. Biol. Chem. 268, 23422–23426.

Bittencourt, J.C., Presse, F., Aouas, C., Peto, C., Vaughan, J., Nahon, J.-L., Vale, W., Sawchenko, P.E., 1992. The melanin-concentrating hormone system of the rat brain: an immuno- and hybridization histochemical characterisation. J. Comp. Neurol. 319, 218–245.

Bosler, O., Joh, T.D., Beaudet, A., 1984. Ultrastructural relationships between serotonin and dopamine neurons in the rat arcuate nucleus and medial zona incerta: a combined radioautographic and immunocytochemical study. Neurosci. Lett. 4, 279–285.

Boyland, P.S., Eastwood, S., Ellis, C., Bergsma, D., Jones, B.J., Gloger, I.S., Upton, N., Middlemass, D.N., 1996. High specific activity [^{3}H]-5-CT binding: correlation of guinea-pig cortex with human cloned 5-HT_7 receptors. Br. J. Pharmacol. 117, 132P.

Eglen, R.M., Jasper, J.R., Chang, D.J., Martin, G.R., 1997. The 5-HT_7 receptor: orphan found. Trends Pharmacol. Sci. 18, 104–107.

Ehlen, J.C., Grossman, G.H., Glass, J.D., 2001. In vivo resetting of the hamster circadian clock by 5-HT_7 receptors in the suprachiasmatic nucleus. J. Neurosci. 21 (14), 5351–5357.

Gonzalez, M.I., Baker, B.I., Wilson, C.A., 1997. Stimulatory effect of melanin-concentrating hormone on LH release. Neuroendocrinology 66, 254–262.

Guscott, M.R., Egan, E., Cook, G.P., Stanton, J.A., Beer, M.S., Rosahl, T.W., Hartmann, S., Kulagowski, J., McAllister, G., Fone, K.C.,

Hutson, P.H., 2003. The hypothermic effect of 5-CT in mice is mediated through the 5-HT_7 receptor. Neuropharmacology 44 (8), 1031–1037.

Gustafson, E.L., Durkin, M.M., Bard, J.A., Zgombick, J., Branchek, T.A., 1996. A receptor autoradiographic and in situ hydridization analysis of the distribution of the $5HT_7$ receptor in rat brain. Br. J. Pharmacol. 117, 657–666.

Hagan, J.J., Price, G.W., Jeffrey, P.J., Deeks, N.J., Stean, T., Piper, D., Smith, M.I., Upton, N., Medhurst, A.D., Middlemiss, D.N., Riley, G.J., Lovell, P.J., Bromidge, S.M., Thomas, D.R., 2000. Characterisation of SB-269970-A, a selective 5-HT_7 receptor antagonist. Br. J. Pharmacol. 130, 539–548.

Hirst, W.D., Price, G.W., Rattray, M., Wilkin, G.P., 1997. Identification of 5-hydroxytryptamine receptors positively coupled to adenylyl cyclase in rat cultures astrocytes. Br. J. Pharmacol. 120, 509–515.

Hoyer, D., Clarke, D.E., Fozard, J.R., Hartig, R., Martin, G.R., Mylecharane, E.J., Saxena, P.R., Humphrey, P.P.A., 1994. International union of pharmacology classification of receptors for 5-hydroxytryptamine (serotonin). Pharmacol. Rev. 46, 157–204.

Inoue, M., Kitazawa, T., Cao, J., Taneike, T., 2003. 5-HT_7 receptor-mediated relaxation of the oviduct in nonpregnant pigs. Eur. J. Pharmacol. 461 (2–3), 207–218.

James, M.D., Hole, D.R., Wilson, C.A., 1989. Differential involvement of 5-hydroxytryptamine (5-HT) in specific hypothalamic areas in the mediation of steroid-induced changes in gonadotrophin release and sexual behaviour in female rats. Neuroendocrinology 49, 561–569.

Kalia, V., Fenske, C., Hole, D.R., Wilson, C.A., 1999. Effect of gonadal steroids and gamma-aminobutyric acid on LH release and dopamine expression and activity in the zona incerta in rats. J. Reprod. Fertil. 117, 180–197.

Kendall, M.G., Stuart, J.A., 1968. The Advanced Theory of Statistics. Griffin, London.

Kinsey, A.M., Wainwright, A., Heavens, R., Sirinathsinghji, D.J., Oliver, K.R., 2001. Distribution of 5-ht (5A), 5-ht (5B), 5-ht (6) and 5-HT (7) receptor mRNAs in the rat brain. Brain Res. Mol. Brain Res. 88 (1–2), 194–198.

Le Corre, S., Sharp, T., Young, A.H., Harrison, P.J., 1997. Increase of $5HT_7$ (serotonin-7) and 5HT1A (serotonin-1A) receptor mRNA expression in rat hippocampus after adrenalectomy. Psychopharmacology (Berlin) 130, 368–374.

Lenglet, S., Louiset, E., Delarue, C., Vaudry, H., Contesse, V., 2002. Involvement of T-type calcium channels in the mechanism of action of 5-HT in rat glomerulosa cells: a novel signalling pathway for the 5-HT_7 receptor. Endocr. Res. 28 (4), 651–655.

Lovell, P.J., Bromidge, S.M., Dabbs, S., Duckworth, D.M., Forbes, I.T., Jennings, A.J., King, F.D., Middlemiss, D.N., Rahman, S.K., Saunders, D.V., Collin, L.L., Hagan, J.J., Riley, G.J., Thomas, D.R., 2000. A novel, potent and selective 5-HT_7 antagonist: (*R*)-3-(2-(2-(4-Methylpiperidin-l-yl)ethyl)-pyrrolidine-1-sulfonyl)-phenol (SB-269970). J. Med. Chem. 43, 342–345.

Lovenberg, T.W., Baron, B.M., de Lecea, L., Miller, J.D., Prosser, R.A., Rea, M.A., Foye, P.E., Racke, M., Slone, A.L., Siegel, B.W., Danielson, P.E., Sutcliffe, J.G., Erlander, M.G., 1993. A novel adenylyl cyclase-activating serotonin receptor (5-HT_7) implicated in the regulation of mammalian circadian rhythms. Neuron 11, 449–458.

Meuser, T., Pietruck, C., Gabriel, A., Xie, G.X., Lim, K.J., Peirce Palmer, P., 2003. 5-HT_7 receptors are involved in mediating 5-HT-induced activation of rat primary afferent neurons. Life Sci. 71 (19), 2279–2289.

Neumaier, J.F., Sexton, T.J., Yracheta, J., Diaz, A.N., Brownfield, M., 2001. Localization of $5HT_7$ receptors in rat brain by immunocytochemistry in situ hybridization and agonist stimulated cfos expression. J. Chem. Neuroanat. 21, 63–73.

Oertel, W.H., Tappaz, M.L., Berod, A., Mugnaina, E., 1982. Two-colour immunochemistry for dopamine and GABA neurones in rat substantia nigra and zona incerta. Brain Res. Bull. 9, 463–474.

Paxinos, G., Watson, C., 1998. The Brain in Stereotaxic Co-ordinates, 4th edition. Academic Press, New York.

Pickard, G.E., Rea, M.A., 1997. Serotonergic innervation of the hypothalamic suprachiasmatic nucleus and photic regulation of circadian rhythms. Biol. Cell 89, 513–523.

Plassat, J.L., Amlaiky, N., Hen, R., 1993. Molecular cloning of a mammalian serotonin receptor that activates adenylate cyclase. Mol. Pharmacol. 44, 229–236.

Pu, S., Jain, M.R., Kalra, P.S., Kalra, S.P., 1998. Orexins, a novel family of hypothalamic neuropeptides, modulate pituitary luteinizing hormone secretion in an ovarian steroid-dependent manner. Regul. Pept. 78, 133–136.

Raut, M., Truiffort, E., Lewis, R., Tardivel-Lacombe, J., Diaz, J., Arrang, J.-M., Schwartz, J.-C., 1993. Molecular cloning characterisation and localisation of a high-affinity serotonin receptor (5-HT_7) activating cAMP formation. Proc. Natl. Acad. Sci. 90, 8547–8551.

Roberts, C., Allen, L., Langmead, C.J., Hagan, J.J., Middlemiss, D.N., Price, G.W., 2001. The effect of SB-269970, a 5-HT(7) receptor antagonist, on 5-HT release from serotonergic terminals and cell bodies. Br. J. Pharmacol. 132 (7), 1574–1580.

Sakurai, T., Amemiya, A., Ishii, M., Matsuzaki, I., Chemelli, R.M., Tanaka, H., Williams, S.C., Richardson, J.A., Kozlowski, G.P., Wilson, S., Arch, J.R.S., Buckingham, R.E., Haynes, A.C., Carr, S.A., Annan, R.S., McNulty, D.E., Liu, W.S., Terralt, J.A., Elshourbagy, N.A., Bergsma, D.J., Shen, Y., Monsma, F.J., Metcalf, M.A., Jose, P.A., Hamblin, M.W., Sibley, D.R., 1993. Molecular cloning and expression of 5-hydroxytryptamine$_7$ serotonin receptor subtype. J. Biol. Chem. 268, 18200–18204.

Shen, Y., Monsma Jr., F.J., Metcalf, M.A., Jose, P.A., Hamblin, M.W., Sibley, S.R., 1993. Molecular cloning and expression of a 5-hydroxytryptamine$_7$ serotonin receptor subtype. J. Biol. Chem. 268, 18200–18204.

Shimizu, M., Nishida, A., Zensho, H., Miyata, M., Yamawaki, S., 1997. Down-regulation of 5-hydroxytryptamine$_7$ receptors by dexamethasone in rat frontocortical astrocytes. J. Neurochem. 68 (6), 2604–2609.

Siddiqui, A., Kotecha, K., Salicioni, A.M., Kalia, V., Murray, J.F., Wilson, C.A., 2000. 5-HT in the zona incerta inhibits LH release via 5-HT1A receptors. Neuroendocrinology 72, 272–283.

Small, C.J., Goubillon, M.-L., Murray, J.F., Siddiqui, A., Grimshaw, S.E., Young, H., Sivanesan, V., Kalamatianos, T., Kennedy, A.R., Coen, C.W., Bloom, S.R., Wilson, C.A., 2003. Central orexin A has site-specific effects on luteinizing hormone release in female rats. Endocrinology 144, 3225–3236.

Thomas, D.R., Gittins, S.A., Collin, L.L., Middlemiss, D.N., Riley, G., Hagan, J., Gloger, I., Ellis, C.E., Forbes, I.T., Brown, A.M., 1998. Functional characterisation of the human cloned 5-HT_7 receptor (long form); antagonist profile of SB-258719. Br. J. Pharmacol. 124, 1300–1306.

Thomas, D.R., Middlemiss, D.N., Taylor, S.G., Nelson, P., Brown, A.M., 1999. 5-CT stimulation of adenyl cylase activity in guinea-pig hippocampus evidence for involvement of 5-HT_7 and 5-HT_{1A} receptors. Br. J. Pharmacol. 128, 158–164.

Thomas, D.R., Atkinson, P.J., Hastie, P.G., Roberts, J.C., Middlemiss, D.N., Price, G.W., 2002. [3H]-SB-269970 radiolabels 5-HT_7 receptors in rodent, pig and primate brain tissues. Neuropharmacology 42 (1), 74–81.

To, Z.P., Bonhaus, D.W., Eglen, R.M., Jakeman, L.B., 1995. Characterisation and distribution of putative 5-ht receptors in guinea pig brain. Br. J. Pharmacol. 115, 107–116.

Tsou, A.-P., Kosake, A., Bach, C., Zuppan, P., Yee, C., Tom, K., Alvarez, R., Ramsey, S., Bonhaus, D.W., Stefanich, E., Jakeman, L., Eglen, R.M., Chan, H.W., 1994. Cloning and expression of a 5-hydroxytryptamine7 receptor positively coupled to adenylyl cyclase. J. Neurochem. 63, 456–464.

Van Vught, D.A., Sylvester, P.W., Aylsworth, E., Meites, J., 1982. Counteraction of gonadal steroid inhibition of LH release by naloxone. Neuroendocrinology 34, 274–278.

Yau, J.L., Noble, J., Widdowson, J., Seckl, J.R., 1997. Impact of adrenalectomy on 5-HT_6 and 5-HT_7 receptor gene expression in the rat hippocampus. Brain Res. Mol. Brain Res. 45 (1), 182–186.

Ying, S.W., Rusak, B., 1997. 5-HT_7 receptors mediate serotonergic effects on light-sensitive suprachiasmatic nucleus neurons. Brain Res. 755 (2), 246–254.

Yu, G.D., Liu, Y.L., Jiang, X.H., Guo, S.Y., Zhang, H.Q., Yin, Q.Z., Hisamitsu, T., 2001. The inhibitory effect of serotonin on the spontaneous discharge of suprachiasmatic neurons in hypothalamic slice is mediated by 5-HT(7) receptor. Brain Res. Bull. 54 (4), 395–398.