Impact of regional 5-HT depletion on the cognitive enhancing effects of a typical 5-ht$_6$ receptor antagonist, Ro 04-6790, in the Novel Object Discrimination task

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Abstract

Rationale Selective 5-ht$_6$ receptor antagonists like Ro 04-6790 prolong memory in many rodent preclinical paradigms, possibly by blocking tonic 5-HT-evoked GABA release and allowing disinhibition of cortico-limbic glutamatergic and cholinergic neurones. If this is the case, behavioural responses to Ro 04-6790 should be abolished by depletion of endogenous 5-HT, and selective lesions of dorsal raphé (DR) or median raphé (MR) 5-HT pathways would allow the neuroanatomical substrates underlying the cognitive effects of 5-ht$_6$ receptor antagonists to be elucidated.

Objectives This study compared the effect of Ro 04-6790 on novel object discrimination (NOD) before and after sham or 5,7-dihydroxytryptamine (5,7-DHT)-induced lesions produced by injection into the lateral ventricles (LV), DR or MR.

Materials and methods NOD tests used a 4 h inter-trial interval (ITI) and Ro 04-6790 (10 mg kg^{-1} i.p.) was administered 20 min before the familiarisation trial. Brain region-specific 5-HT depletion was assessed by high performance liquid chromatography with electrochemical detection (HPLC-ED).

Results Widespread LV or selective MR, but not DR lesions, abolished the ability of Ro 04-6790 to delay natural forgetting. Successful performance of all lesioned rats in subsequent 'drug-free' NOD tests using a 1 h ITI excluded the possibility of any confounding effects on visual acuity or motivation.

Conclusions The ability of Ro 04-6790 to prolong object recognition memory requires blockade of MR 5-HT function. Because DR lesions did not produce the expected depletion of striatal 5-HT an additional contribution of DR inputs to this region cannot be completely excluded.

Keywords Serotonin receptor · Memory · Cognition · Object recognition · Lesion

Introduction

The 5-ht$_6$ receptor is one of the most recent additions to the 5-HT receptor family, and its activation stimulates cAMP production via G$_s$-proteins. Expression is largely confined to the central nervous system, with greatest abundance in the striatum, olfactory tubercle, nucleus accumbens, cortex and hippocampus (Monsma et al. 1993; Ruat et al. 1993; Kohen et al. 1996; Gérard et al. 1996, 1997). Accumulating evidence suggests a role in the regulation of feeding behaviour (Fisas et al. 2006; Halford et al. 2007; Heal et al. 2008), seizure threshold, anxiety (Wesołowska and Nikiforuk 2007; Wesołowska 2008), affective state (Svenningsson et al. 2007) and the sleep–wake cycle (Morairty et al. 2008), although links to cognitive processing have received the most interest (for reviews see Woolley et al. 2004; Mitchell and Neumaier 2005; Fone 2008).

Selective 5-ht$_6$ receptor antagonists such as Ro 04-6790 (Sleight et al. 1998) and SB-271046 (Bromidge et al. 1999) prolong memory retention in rodent cognitive paradigms, including Morris water maze (Woolley et al. 2001; Rogers

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and Hagan 2001; Stean et al. 2002) and novel object discrimination (NOD) tasks (King et al. 2004a; Schreiber et al. 2007). They also overcome muscarinic receptor antagonist-induced deficits in a wide range of cognitive tests (Sleight et al. 1999; Bös et al. 2001; Meneses 2001; Woolley et al. 2003; Foley et al. 2004; Lieben et al. 2005; Hirst et al. 2006; Mitchell et al. 2006; Schreiber et al. 2007; Mitchell and Neumaier 2008) and reverse age-related memory impairments in Morris water maze (Foley et al. 2004; Hirst et al. 2006), NOD (King et al. 2005) and T-maze tasks (Rogers et al. 1999, 2000). These findings have been attributed, at least in part, to 5-ht_6 receptor antagonist-induced increases in ACh efflux within the hippocampus and frontal cortex (Sleight et al. 1999; Shirazi-Southall et al. 2002; Riemer et al. 2003; Zhang et al. 2007) and may be predictive of therapeutic utility against the cognitive dysfunction present in Alzheimer's disease. Some recent reports indicate reductions in both 5-ht_6 receptor expression and functional activation in Alzheimer's disease (Garcia-Alloza et al. 2004; Lorke et al. 2006; Marcos et al. 2008) and suggest that these changes may correlate with the incidence of psychotic symptoms. Interestingly, 5-ht_6 receptor antagonists also increase glutamate efflux within the cortex and hippocampus (Dawson et al. 2000, 2001), and can reverse NOD deficits produced by the NMDA receptor antagonist phencyclidine (King et al. 2005). This, together with the ability to enhance attentional set shifting (Hatcher et al. 2005), has led to the suggestion that 5-ht_6 receptor antagonists may offer a novel therapeutic adjunct treatment for the cognitive defects seen in schizophrenia.

5,7-Dihydroxytryptamine (5,7-DHT) lesions of the serotonergic system fail to decrease 5-ht_6 receptor mRNA levels in the striatum, nucleus accumbens, hippocampus, or raphé (Gérard et al. 1996), suggesting that the 5-ht_6 receptor is expressed postsynaptic to serotonergic neurones and does not function as a nerve terminal or cell body autoreceptor. Dopaminergic (Roberts et al. 2002) or cholinergic (Marcos et al. 2006) lesions also fail to influence 5-ht_6 receptor expression, and co-localisation of 5-ht_6 receptor-like immunoreactivity (5-ht_6-LI) with choline acetyltransferase-like immunoreactivity is low (Woolley et al. 2004), suggesting that there is limited expression on either dopaminergic or cholinergic neurones. There may be some 5-ht_6 receptor expression on the dendrites of glutamatergic pyramidal and granular cells within the hippocampus (Gérard et al. 1997), but in the striatum 5-ht_6 receptor mRNA co-localises with various neuropeptides that are present in GABAergic projections to the globus pallidus and substantia nigra (Ward and Dorsa 1996). Furthermore, there is extensive (>50%) co-localisation of 5-ht_6-LI with GAD_{67}-like immunoreactivity (GAD_{67}-LI; a marker of GABAergic neurones) in the dentate gyrus, presubiculum, subiculum, caudate putamen, lateral septum, posterior

hypothalamus and basal medial amygdala, as well as several areas of the cortex, thalamus and cerebellum (Woolley et al. 2000, 2004). These regions all receive serotonergic innervation from the median raphé (MR) and/or dorsal raphé (DR) and, within the hippocampus at least, serotonergic projections appear to synapse preferentially onto GABAergic neurones (Freund et al. 1990; Freund 1992) which, in turn, make contact with glutamergic granule and pyramidal cells (Freund and Buzaki 1996). Blockade of excitatory 5-ht_6 receptors on GABAergic neurones could therefore lead to disinhibition of glutamate release, with increased ACh release occurring either via a parallel pathway or in response to increased glutamatergic stimulation (Dawson et al. 2001). The ability of Ro 04-6790 to reverse GABA-related impairments in NOD supports the proposal that reduced GABAergic inhibition may underlie the cognitive enhancing effects of 5-ht_6 receptor antagonists (King et al. 2004b). Furthermore, the ability of the recently developed 5-ht_6 receptor agonists WAY-181187 and WAY-208466 to elevate hippocampal, striatal and frontal cortical GABA efflux (Schechter et al. 2008) provides direct evidence for a regulatory effect of the receptor on GABAergic neurotransmission. Interestingly, 5-ht_6 receptor agonists also appear to have procognitive effects in rodent NOD (Fone 2006, 2008; King et al. 2006a), attentional set shifting (Burnham et al. 2007) and autoshaping tasks (Meneses et al. 2008; but maybe not in social recognition; see Loiseau et al. 2008). The mechanism by which 5-ht_6 receptor agonist and antagonist drugs can both produce procognitive effects is currently unclear. Some possibilities are: (1) that the agonists activate a separate population of 5-ht_6 receptors located on glutamatergic neurones which normally receive little tonic 5-HT input and so are not influenced by the antagonists (King et al. 2006a) or (2) that both agonists and antagonists activate the Fyn tyrosine kinase signalling pathway (Yun et al. 2007).

Ascending serotonergic projections from raphé nuclei to the forebrain comprise a dual system of morphologically distinct fibres, with different origins and target regions. Fibres from the DR project to associative and limbic cortical areas within layers III–IV, the ventral hippocampus, lateral septum, dorsal striatum, globus pallidus and amygdala, as well as the suprachiasmatic, arcuate, dorsomedial and periventricular nuclei of the hypothalamus, while fibres from the MR innervate neo- and allo-cortical regions within layers I–II, the dorsal and ventral hippocampus, medial septum, plus suprachiasmatic, paraventricular and periventricular hypothalamic nuclei (Azmitia and Segal 1978; Baumgarten and Grozdanovic 1994). Thus, some areas of the forebrain receive selective inputs from either the DR or MR, while other areas are innervated by both. It is unknown precisely where the 5-ht_6 receptors responsible for the cognitive enhancing effects of selective antagonists are located, and

whether they receive serotonergic innervation from the DR and/or MR.

Initial in vitro characterisation indicated that Ro 04-6790 has a pKi of 7.35 at the rat 5-ht$_6$ receptor and no detectable affinity for the 23 other receptor binding sites studied, including the other 5-HT receptors (Sleight et al. 1998). Furthermore, Ro 04-6790 had no effect on basal cAMP accumulation in either Hela cells stably expressing the human 5-ht$_6$ receptor (Sleight et al. 1998), or HEK293F cells stably expressing the rat 5-ht$_6$ receptor (Romero et al. 2006), suggesting that it is neither an agonist nor inverse agonist. As a result, the behavioural effects of Ro 04-6790 should be abolished by lesion-induced depletion of endogenous 5-HT within relevant brain regions. The current study therefore compared the ability of Ro 04-6790 to delay natural forgetting in a NOD paradigm before and after sham lesions, widespread forebrain 5-HT depletion caused by administration of the serotonergic neurotoxin 5,7-DHT into the lateral ventricles (LV), or more localised serotonergic lesions caused by injection into the DR or MR. The extent of brain region-specific 5-HT depletion was confirmed by HPLC-ED. The NOD task was selected because it is based on the innate preference of rats for novelty. The absence of food rewards should avoid any confounds arising from 5-ht$_6$ receptor antagonist-induced hypophagia (Woolley et al. 2001; Fisas et al. 2006; Halford et al. 2007; Heal et al. 2008), while the lack of any aversive stimuli has obvious ethical advantages and also increases the translational relevance of the paradigm, since arousal and motivational states should be similar to those under which human memory is normally assessed (Ennaceur and Delacour 1988; Dere et al. 2007). Previous experiments showed that Ro 04-6790 (10 mg kg^{-1} i.p) given either before or immediately after the familiarisation trial (but not just prior to the choice trial) improved performance in a 4 h ITI version of the NOD task, justifying the use of this dose to enhance consolidation and perhaps also acquisition in the current study (King et al. 2004a). Portions of the current research have been published in abstract form (King et al. 2006b).

Materials and methods

Animals

For each of the three lesion target sites, 24 young adult male Lister hooded rats (Charles River UK, weighing 134–186 g on the first day of the study) were housed in groups of four on a 12 h light–dark cycle (lights on at 07:00 hours) with food and water available ad libitum and a single cardboard play tube per cage. Following surgical procedures rats were singly housed, and provided with wet mash for the first 4 days and extra bedding material (paper wool: Datesand; Manchester, UK) until completion of the study. Room temperature (21±2°C) and relative humidity (55±10%) were kept constant throughout. Body weights were recorded at 3–4 day intervals prior to surgery, daily for 4 days post-surgery, and then at 3 day intervals thereafter. All procedures were carried out in accordance with the UK Animals (Scientific Procedures) Act (1986), with approval of the University of Nottingham Local Ethical Committee.

Behavioural testing

Apparatus and basic novel object discrimination procedure

These have been described in detail elsewhere (King et al. 2004a) and were modified from that of Ennaceur and Delacour (1988). All testing was conducted during the light phase of the light–dark cycle, with uplighters providing a light intensity of 140–200 lux at arena level. Each of the 12 identical test chambers comprised a clear Perspex box 39× 23.5 cm with 24.5 cm high walls and a wire lid. The removable arena floor was replaced during trials by one with two circular holes located 5 cm from the side and 10 cm from the end wall in opposite corners. The objects to be discriminated were cylindrical screw–top plastic bottles (8 cm high×5 cm diameter: Bibby Sterilin; Staffordshire, UK) covered in white masking tape alone (familiar objects) or white masking tape with three additional horizontal stripes made of 1.2 cm wide black electrical insulating tape (novel objects). Bottles were filled with water so that they could not be displaced and secured through the holes in the arena floor with blu-tack. The arena and objects were wiped with 20% v/v ethanol before testing to remove any olfactory cues. Each rat received five separate NOD tests always preceded (24 h) by 60 min habituation to the arena in the absence of any objects. On each test day, every rat sequentially received 3 min acclimatisation to the arena, 1 min in the home cage and two consecutive 3 min trials which were separated by an ITI in the home cage (1 or 4 h, as described later). During the first (familiarisation) trial rats were exposed to two objects of identical size, shape and pattern, and in the second (choice) trial one of the objects (selected at random) was replaced by a striped bottle (novel object). The remaining object from the familiarisation trial was left untouched (familiar object), but any faeces were removed from the arena. During the two trials exploration of each object, defined as sniffing, licking, chewing, or having moving vibrissae whilst directing the nose towards and ≤1 cm from the object, was recorded separately using stopwatches by an observer who was unaware of the treatment received. Sitting on an object in the absence of any directed attention was not regarded as exploratory activity, but rarely occurred.

Novel object discrimination tests

Prior to any surgical interventions all rats underwent two NOD tests (on days 2 and 9 of the study), each with a 4 h ITI. They received either methylcellulose vehicle (1 ml kg^{-1} i.p.) or Ro 04-6790 (10 mg kg^{-1} i.p.) 20 min before the familiarisation trial on the first test day, and the opposite treatment at the same time-point on the second test day. This crossover design allowed each rat to serve as its own control for a baseline evaluation of Ro 04-6790 compared to vehicle. On the basis of this data rats were allocated to either sham or 5,7-DHT-induced lesion groups, which were balanced according to the level of discrimination and the order in which the treatments were received. Twenty-one and 28 days post-surgery (on days 32 and 39 of the study) each rat was re-tested using the same crossover design with vehicle and Ro 04-6790. The final NOD test occurred 33–34 days post-surgery (days 44–45 of the study) and employed a short, undemanding 1 h ITI which enables good discrimination in drug-naïve rats (King et al. 2004a). The aim of this test was to determine whether animals were exhibiting either habituation to repeated testing, or gross visual or motivational deficits that could confound interpretation of data from the previous 4 h ITI tests. All rats received methylcellulose vehicle (1 ml kg^{-1} i.p.) 20 min before the familiarisation trial.

Surgical interventions

On day 11 all rats received desipramine hydrochloride (15 mg kg^{-1} i.p., 1 ml kg^{-1}) 30 min prior to central microinjections, either to protect noradrenergic neurones from 5,7-DHT or for control purposes in sham animals (Bjorklund et al. 1975). Rats were anaesthetised with isoflurane (3% in oxygen and nitrous oxide for induction, then 1.75–2% for maintenance) then placed in a stereotaxic frame. The skull surface was exposed and a small hole (bilateral in the case of the LV target groups) drilled to permit insertion of a sterile 31 gauge stainless cannula connected via a polythene tube to a Hamilton syringe. The stereotaxic coordinates (Paxinos and Watson 1997) for each injection site were LV: anterior −0.8 and lateral ±1.5 mm from Bregma, −3.8 mm from the dura according to Cassaday et al. (2003); DR: anterior +1.2, lateral ±0 and +3.5 mm from inter-aural zero according to Body et al. (2002); MR: as for DR except +1.5 mm from inter-aural zero. The incisor bar was set to 3.3 mm below the inter-aural line in each case. Sham rats received either 5 µl of 0.2% w/v ascorbic acid vehicle into each lateral ventricle, or 2 µl of 0.2% w/v ascorbic acid vehicle into either the DR or MR. Lesioned rats received 75 µg of 5,7-DHT creatine sulphate into each lateral ventricle, or 8.4 µg of 5,7-DHT creatine sulphate into the DR or MR. All injections were

made at 5 µl per min, and the injector remained in place for a further 2 min. Injection sites were sealed with bone wax, and wounds closed with surgical sutures were then treated with Xylocaine local anaesthetic, Fuciderm topical antibiotic, and Nobecutane plastic wound dressing. Before completion of surgery all rats received 10 µl kg^{-1} s.c. Rimadyl analgesic, plus 1 ml s.c. sterile saline to aid recovery and prevent dehydration.

Neurochemical analysis

Thirty-five days after surgery (on day 46 of the experiment) rats were euthanised by concussion and immediately decapitated. The brains were rapidly removed and the hypothalamus, frontal cortex, remaining non-frontal regions of the cortex, septum, hippocampus, striatum and brainstem were dissected on a refrigerated dissecting table (4°C), snap frozen in liquid nitrogen, and stored at −80°C for subsequent analysis by HPLC-ED. Samples were thawed, weighed and sonicated (Soniprep 150: MSE Scientific Instruments; Crawley, UK) for 30 s in the following volumes of 0.1 M perchloric acid containing 0.2% w/v sodium metabisulphite: cortex, brainstem and sham hippocampus 1 ml; frontal cortex, striatum and hypothalamus 500 µl; lesion hippocampus 400 µl; and septum 300 µl. Samples were centrifuged (17,500×g, 4°C for 20 min; Harrier 18/80: MSE Scientific Instruments; Crawley, UK) and the supernatant filtered through an Acrodisc syringe tip filter (0.45 µm, Pall: Portsmouth, UK). Monoamines were separated using a PerkinElmer Series 200 autosampler and Nucleosil 5 µm C18 300A column (150 mm×4.6 mm ID), and detected at a potential of +0.75 V by an Antec Intro amperometric detector. Mobile phase was delivered at 0.9 ml min^{-1} by an isocratic pump (Dionex P680) and consisted of 0.15 M sodium dihydrogen phosphate, 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM octane-sulphonic acid and 12% v/v methanol adjusted to pH 3.8 for measurement of 5-HT and dopamine, and 0.05 M potassium dihydrogen phosphate, 0.1 mM EDTA, 2 mM octane-sulphonic acid and 13.5% v/v methanol adjusted to pH 3 for measurement of noradrenaline.

Compounds

Ro 04-6790 was provided by F. Hoffmann La-Roche (Basel, Switzerland) and suspended in 0.154 M saline containing 0.5% w/v methylcellulose, using a sonic probe (Soniprep 150: MSE Scientific Instruments; Crawley, UK). Desipramine hydrochloride, 5,7-dihydroxytryptamine creatine sulphate and ascorbic acid were all purchased from Sigma Aldrich (Poole, UK). Desipramine was dissolved in 0.154 M saline, and 5,7-DHT was dissolved in sterile water containing 0.2% w/v ascorbic acid.

Statistical analysis

Data were separated according to lesion target site. Alterations in body weight were assessed using two-way analysis of variance (ANOVA) with factors 'day' and 'lesion type' (sham or 5,7-DHT) followed, where appropriate, by one-way ANOVA and Duncan's new multiple range post hoc test. Object preferences during each NOD trial were detected using two-tailed Student's paired t tests, applied separately to each lesion–treatment combination pre- and post-surgery. For each of these conditions differences in total object exploration from familiarisation to choice trials were assessed in a similar manner. Lesion-related alterations in exploratory activity and/or treatment response were detected using repeated measures ANOVAs, applied separately to sham and 5,7-DHT groups and followed, where appropriate, by Tukey's multiple comparison post hoc test. The repeated measures ANOVAs were performed only on total familiarisation trial, total choice trial and discrimination ratio data, with the latter being defined as 'time at novel object/total choice trial object exploration'. Post-surgery differences in these variables between the sham and lesioned groups were assessed using one-way ANOVAs followed, where appropriate, by Tukey's multiple comparison post hoc test. Lesion-related differences during the 1 h ITI test were detected using two-tailed Student's unpaired t tests (sham versus 5,7-DHT), and regional changes in neurotransmitter levels between sham and 5,7-DHT treated rats were assessed in a similar manner. $P < 0.05$ was regarded as statistically significant in all cases. Body weight and NOD data are expressed as mean ± SEM, and neurochemical data are presented as mean percentage depletion in each 5,7-DHT group compared to the relevant sham control, calculated as ([sham group mean − lesion group mean]/sham group mean)× 100.

Two rats died during surgery and a further seven were excluded from the study because they chewed holes in the objects during NOD testing, which allowed access to the water within and gave unwanted natural significance. These exclusions resulted in $n=9$–12 per group.

Results

Body weight

All three groups of 5,7-DHT-lesioned rats exhibited a transient post-surgery weight loss (equivalent to <7% of total body weight). Body weights of the MR 5,7-DHT group rapidly returned to sham levels (Fig. 1c), but those of LV (Fig. 1a) and DR (Fig. 1b) 5,7-DHT groups remained
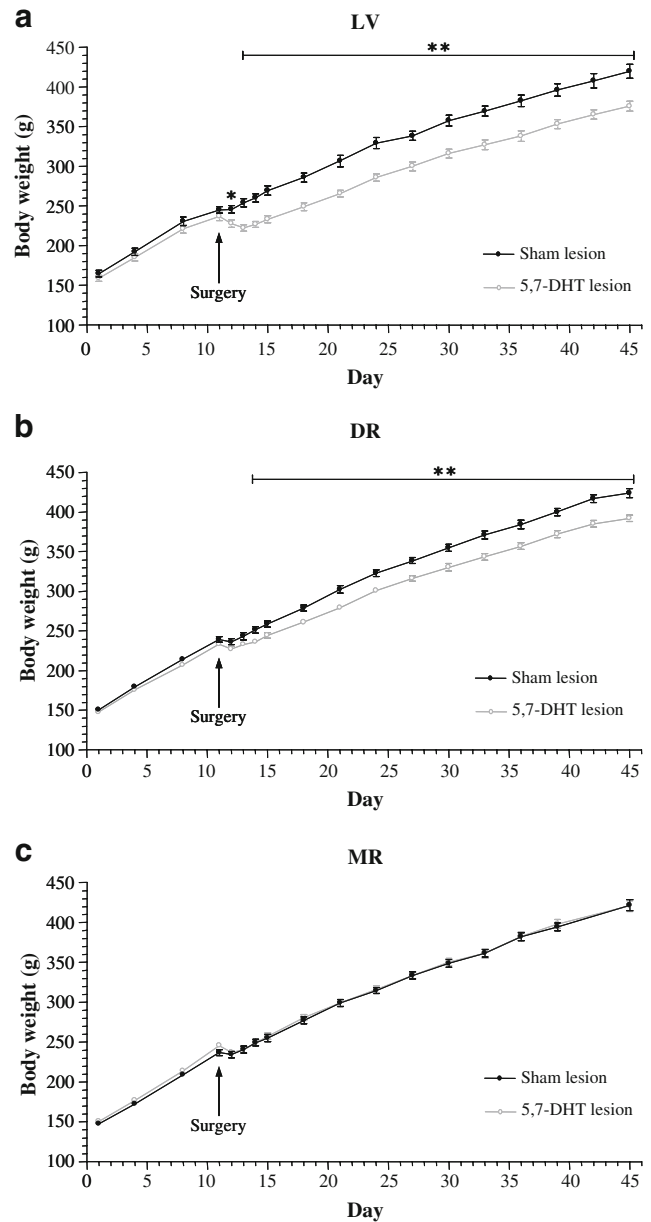

Fig. 1 Effect of sham and 5,7-DHT lesions on body weight. Body weight (g, mean ± SEM) of six separate groups ($n=9$–12) of rats which received sham (*black line*) or 5,7-DHT (*grey line*) lesions produced by injection into either the **a** lateral ventricles (LV); **b** dorsal raphé (DR) or **c** median raphé (MR) on d11. *$P<0.05$; **$P<0.01$ versus the appropriate sham control on the same day, as indicated (Duncan's new multiple range post hoc after one-way ANOVA, preceded by statistically significant lesion × day interaction using two-way ANOVA)

reduced for the rest of the study and differed significantly from sham levels from days 12 and 14, respectively (days 1 and 3 post-surgery, respectively). On the final day LV and DR 5,7-DHT lesioned rats weighed an average of 44 and 32 g less than respective sham controls.

Novel object discrimination

Pre- and post-surgery 4 h ITI tests with Ro 04-6790

During the familiarisation trial all rats spent an equivalent amount of time at each object, irrespective of treatment or lesion type (data not shown). The total level of object exploration within each trial was also unaffected by treatment or lesion type, with the single exception of the DR 5,7-DHT group which showed an increase in total familiarisation trial object exploration following post-surgery vehicle compared to pre-surgery Ro 04-6790 (Table 1). In most cases a decrease in total object exploration occurred from the familiarisation to the choice trial, and although this did not always reach significance the extent of the change did not appear to be related to either treatment or lesion type (Table 1). During the pre-surgery choice trials all groups spent an equivalent amount of time at the novel and familiar object following vehicle administration, confirming that a 4 h ITI produced a

robust natural forgetting. In the same groups of rats pre-surgery administration of Ro 04-6790 produced a significant exploratory preference for the novel object ($P<0.05$ for LV sham and both MR groups, and $P<0.01$ for LV 5,7-DHT and both DR groups), indicating retention of some memory from the familiarisation trial (Fig. 2a–c). In the post-surgery choice trials discrimination by LV sham animals was similar to that observed before surgery ($P>0.05$ following vehicle, and $P<0.01$ following Ro 04-6790), whereas LV 5,7-DHT rats were unable to discriminate the novel object even in the presence of Ro 04-6790 (Fig. 2a). Similar post-surgery findings were observed for the MR group. Thus, Ro 04-6790-treated rats spent more time exploring the novel than the familiar object after MR sham ($P<0.01$) but not MR 5,7-DHT, although in this case the sham group also unexpectedly spent more time exploring the novel object following vehicle administration ($P<0.05$; Fig. 2c). In contrast, Ro 04-6790 administration was associated with significant discrimination in the DR group regardless of whether sham or 5,

Table 1 Total levels of object exploration during the familiarisation and choice trials of 4 h ITI pre- and post-surgery NOD tests

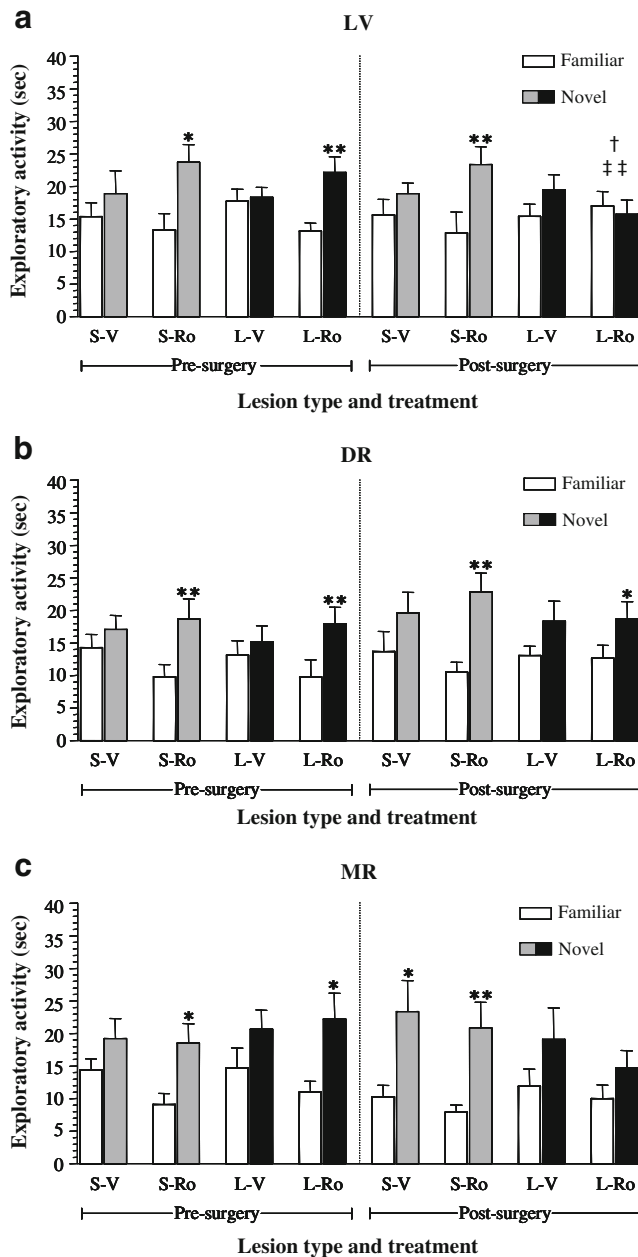| Target site	Timepoint	Lesion type	Treatment	Number of animals	Total (s) in familiarisation trial	Total (s) in choice trial
LV	Pre-surgery	Sham	V	10	49±4	34±4*
			Ro	10	48±4	37±3
		5,7-DHT	V	12	48±4	36±3
			Ro	12	52±5	35±3**
	Post-surgery	Sham	V	10	50±4	35±2**
			Ro	10	52±4	36±5**
		5,7-DHT	V	12	53±3	35±4**
			Ro	12	54±3	33±4***
DR	Pre-surgery	Sham	V	10	44±6	31±3
			Ro	10	31±6	29±5
		5,7-DHT	V	12	37±4	28±4
			Ro	12	29±5	28±5
	Post-surgery	Sham	V	10	36±3	33±5
			Ro	10	40±4	33±4
		5,7-DHT	V	12	51±4††	31±3**
			Ro	12	40±4	31±4
MR	Pre-surgery	Sham	V	10	38±3	34±4
			Ro	10	37±4	28±4
		5,7-DHT	V	9	38±6	35±5
			Ro	9	31±5	33±5*
	Post-surgery	Sham	V	10	42±3	34±5
			Ro	10	48±4	29±4
		5,7-DHT	V	9	53±5	31±7**
			Ro	9	47±8	25±4***

Total duration (s, mean ± SEM) of object exploration by six separate groups of rats which were each tested on four separate occasions following 1 ml kg^{-1} methylcellulose vehicle (V) and 10 mg kg^{-1} Ro 04-6790 (Ro) in a random order before and after their designated surgical intervention. Treatments were administered via the i.p. route 20 min before the familiarisation trial, and surgical interventions comprised sham or 5,7-DHT lesions produced by injection into either the lateral ventricles (LV), dorsal raphé (DR) or median raphé (MR).

*$P<0.05$
**$P<0.01$
***$P<0.001$ versus the opposite trial following the same treatment at the same time-point (Student's paired t test)
†† $P<0.01$ versus the same trial on the pre-surgery Ro 04-6790 test day (Tukey's multiple comparison post hoc test following repeated measures ANOVA)

a LV

b DR

c MR

Fig. 2 Effect of sham and 5,7-DHT lesions on the ability of Ro 04-6790 to restore choice trial object discrimination following a 4 h ITI. Time spent (s, mean ± SEM) at the novel and familiar object by six separate groups ($n=9$–12) of rats, which were each tested on four separate occasions following 1 ml kg^{-1} methylcellulose vehicle (V) and 10 mg kg^{-1} Ro 04-6790 (Ro) in a random order before and after their designated surgical intervention. Treatments were administered via the i.p. route 20 min before the familiarisation trial, and surgical interventions comprised sham (*grey*) or 5,7-DHT (*black*) lesions (S or L, respectively) produced by injection into either the **a** lateral ventricles (LV), **b** dorsal raphé (DR) or **c** median raphé (MR). *$P<$ 0.05; **$P<0.01$ versus the familiar object following the same treatment at the same time-point (Student's paired t test). †$P<0.05$ versus the discrimination ratio of the same group of rats following the same treatment prior to surgery (repeated measures ANOVA followed by Tukey's multiple comparison post hoc test), and ‡‡ $P<0.01$ versus the comparable post-surgery discrimination ratio in sham lesioned rats (one-way ANOVA followed by Tukey's multiple comparison post-hoc test), where discrimination ratio is defined as 'time at novel object/ total choice trial object exploration'

0.09), but not the MR sham group (pre-surgery Ro 04-6790 0.66±0.07, post-surgery Ro 04-6790 0.69±0.05), although this failed to reach statistical significance using repeated measures ANOVA. In contrast, in the DR sham and lesion groups there was no main effect of surgery on the discrimination ratio (sham $F_{3,43}=1.908$, $P=0.1495$; lesion $F_{3,43}=1.953$, $P=0.1424$). One-way ANOVAs on the post-surgery discrimination ratio data revealed a comparable pattern of results, with a significant difference ($P<0.01$) between LV sham and lesioned groups for Ro 04-6790 but not vehicle (ANOVA $F_{3,43}=4.159$, $P=0.0118$), and a similar trend between MR sham and lesion, but not DR sham and lesion groups. Thus, overall, the discrimination ratio data support the proposal made from the actual novel and familiar object exploration times, that Ro 04-6790 could not improve NOD following either LV or MR lesions of the serotonergic neuronal system.

Post-surgery 1 h ITI test

Irrespective of either lesion type or target site, all rats spent significantly more time exploring the novel than the familiar object during the choice trial (Fig. 3). There was a small but significant ($P<0.05$) difference in discrimination ratio between MR sham and 5,7-DHT groups (0.787± 0.039 and 0.659±0.047, respectively) which may be a reflection of the unexpected cognitive improvement noted in the MR sham group during post-surgery 4 h ITI tests, rather than any indication of a lesion-related deficit in the 5,7-DHT group (values for comparison: LV sham 0.730± 0.031; LV 5,7-DHT 0.674±0.037; DR sham 0.737±0.030; DR 5,7-DHT 0.704±0.039, $P>0.05$ in each case). As with the 4 h ITI tests most groups showed a decrease in total object exploration across the two trials, although this did not always reach significance (Table 2). Findings from the
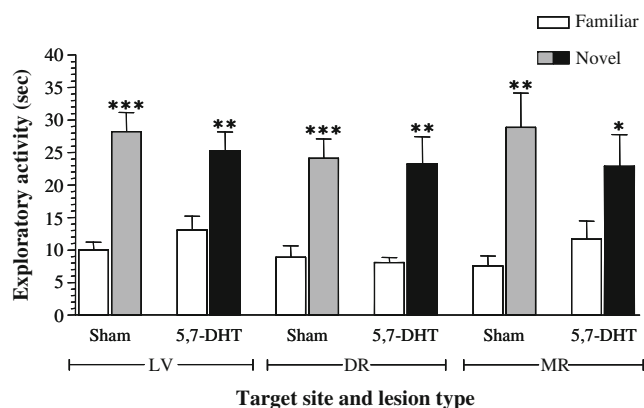
7-DHT lesions were received ($P<0.01$ and $P<0.05$, respectively; Fig. 2b). In summary, administration of 5,7-DHT into either the LV (Fig. 2a) or MR (Fig. 2c) abolished the ability of Ro 04-6790 to prevent natural forgetting, but administration of 5,7-DHT into the DR did not (Fig. 2b). Repeated measures ANOVA performed on the discrimination ratio data from the LV lesion group showed a significant main effect ($F_{3,47}=3.098$, $P=0.0401$), due to a significant decrease ($P<0.05$) in the activity of Ro 04-6790 from pre-surgery to post-surgery (discrimination ratios being 0.62±0.04 versus 0.48±0.04). This was not observed for vehicle treatment, or in the LV sham group (ANOVA $F_{3,39}=2.400$, $P=0.0898$). A similar trend occurred in the MR lesion group (pre-surgery Ro 04-6790 0.68±0.05, and post-surgery Ro 04-6790 0.57±

Fig. 3 Effect of sham and 5,7-DHT lesions on choice trial object discrimination following a 1 h ITI. Time spent (s, mean ± SEM) by six separate groups (*n*=9–12) of rats at the novel and familiar object, following sham (*grey*) or 5,7-DHT (*black*) lesions produced by injection into either the lateral ventricles (LV), dorsal raphé (DR) or median raphé (MR). All rats received 1 ml kg^{-1} i.p. methylcellulose vehicle 20 min before the familiarisation trial. *$P<0.05$; **$P<0.01$; ***$P<0.001$ versus the familiar object in the same group of rats (Student's paired *t* test). There were no significant differences between sham and 5,7-DHT for any of the target sites

1 h ITI test indicate that the inability of Ro 04-6790 to restore object discrimination after surgery in the LV and MR 5,7-DHT groups was not due to any gross visual or motivational deficit, and can therefore be attributed to abolition of the pro-cognitive effect of the 5-ht$_6$ receptor antagonist by the serotonergic lesion.

Neurochemistry

Administration of 5,7-DHT into the LV resulted in an extensive, highly significant depletion of 5-HT in the cortex, frontal cortex, hippocampus, striatum, septum and hypothalamus ($P<0.001$ versus sham control, mean deple-

tion 62–88%), without affecting levels in the brainstem region containing serotonergic cell bodies. The DR 5,7-DHT group exhibited a much more restricted pattern of 5-HT depletion which only reached statistical significance in the cortex ($P<0.001$, mean depletion 38%) and hypothalamus ($P<0.05$, 24%). Although hippocampal and septal 5-HT levels showed a similar percentage decrease to the aforementioned regions (mean depletions 33% and 19%, respectively) this failed to reach significance when compared to sham levels. The MR 5,7-DHT group showed extensive 5-HT depletion similar to that observed with LV 5,7-DHT, including highly significant reductions in the cortex, striatum and hypothalamus ($P<0.001$, 43–72%) as well as the hippocampus ($P<0.01$, 70%). There were more modest effects in the frontal cortex and septum ($P<0.05$, 42–48%) and a small but significant reduction in the brainstem ($P<0.05$, 22%), the latter region having been unaffected by the other two sites of injection. Irrespective of the 5,7-DHT injection site there was no depletion of noradrenaline in the frontal cortex. When 5,7-DHT was administered into the LV the 5-HT depletion was accompanied by a smaller reduction in striatal dopamine levels ($P<0.01$, 38%) but this did not occur with either the DR or MR lesions (Fig. 4).

Discussion

The aim of the current study was to examine the impact of regional 5-HT depletion on the cognitive enhancing effect of a typical 5-ht$_6$ receptor antagonist in the NOD task, in order to elucidate the neuroanatomical substrates via which such actions are mediated. As expected, the pre-surgery NOD tests (and post-surgery tests in sham lesioned controls) verified that Ro 04-6790 prolongs memory retention in rats that have an intact serotonergic neuronal

Table 2 Total levels of object exploration during the familiarisation and choice trials of the 1 h ITI post-surgery NOD test

Target site	Timepoint	Lesion type	Treatment	Number of animals	Total (s) in familiarisation trial	Total (s) in choice trial
LV	Post-surgery	Sham	V	10	50±5	38±3
		5,7-DHT	V	12	46±3	38±4
DR	Post-surgery	Sham	V	10	56±3	33±4**
		5,7-DHT	V	12	52±4	31±4**
MR	Post-surgery	Sham	V	10	48±5	37±6*
		5,7-DHT	V	9	59±5	35±6*

Total duration (s, mean ± SEM) of object exploration by six separate groups of rats which received sham or 5,7-DHT lesions produced by injection into either the lateral ventricles (LV), dorsal raphé (DR) or median raphé (MR). After being tested in a 4 h ITI version of the NOD task on four previous occasions they received a single 1 h ITI NOD test, following i.p. administration of 1 ml kg^{-1} methylcellulose vehicle 20 min before the familiarisation trial.

*$P<0.05$

**$P<0.01$

***$P<0.001$ versus the opposite trial following the same lesion type (Student's paired *t* test). Object exploration within each trial was unaffected by target site or lesion type
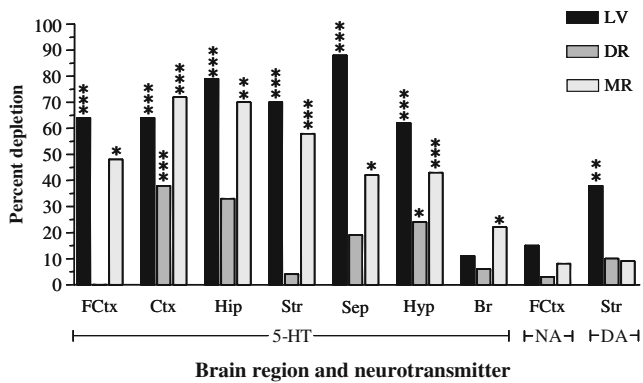
Fig. 4 Percentage depletion of 5-HT, noradrenaline and dopamine following 5,7-DHT lesions. Mean percentage neurotransmitter depletion (calculated as ([sham group mean − lesion group mean]/sham group mean) ×100) produced by injection of 5,7-DHT into either the lateral ventricles (LV), dorsal raphé (DR) or median raphé (MR) ($n=9$–12 per group). $*P<0.05$; $**P<0.01$; $**P<0.001$ compared to absolute neurotransmitter concentrations (pmol mg^{-1} wet weight) in the same brain region following appropriate sham control (Student's unpaired t test), but note that data are presented as percentage depletion rather than absolute levels to aid clarity. *FCtx* frontal cortex, *Ctx* cortex, *Hip* hippocampus, *Str* striatum, *Sep* septum, *Hyp* hypothalamus, *Br* brain stem, *NA* noradrenaline, *DA* dopamine

system. Administration of 5,7-DHT into the LV produced the predicted (Choi et al. 2001; Cassaday et al. 2003) widespread depletion of forebrain 5-HT levels whilst sparing brainstem serotonergic cell bodies, and also completely abolished the response to Ro 04-6790. The ability of such extensive 5-HT depletion to prevent the effect of Ro 04-6790 without altering basal NOD performance under vehicle conditions demonstrates the suitability of the current lesion-based approach to study 5-ht$_6$ receptor antagonists' site of action. The study also confirms that the pro-cognitive effects of Ro 04-6790 are due to blockade of tonic serotonergic neuronal activity. If basal activation of the 5-ht$_6$ receptor by endogenous 5-HT normally inhibits cognitive processes, then the extensive 5-HT depletion obtained herein might be expected to enhance NOD. However, the lack of any such improvement can be attributed to complex interacting effects of 5-HT on multiple receptor subtypes, some of which exert opposing effects on cognition (Buhot et al. 2000; Meneses 2003). Indeed, previous reports have shown improvements (Ward et al. 1999), deficits (Cassaday et al. 2003; Lieben et al. 2004, 2006) or unaltered performance (Lehmann et al. 2000, 2002; Wirth et al. 2000) in either NOD or alternative cognitive paradigms following lesion- or tryptophan depletion-induced decreases in 5-HT levels, supporting this proposal.

The findings of several of early studies suggest that 5-ht$_6$ receptor antagonists are unlikely to modulate cognition by either direct or indirect modulation of dopamine release

(Dawson et al. 2000, 2001; Roberts et al. 2002; Woolley et al. 2003), but a number of subsequent investigations have provided evidence to the contrary (Frantz et al. 2002; Dawson et al. 2003; Lacroix et al. 2004; Minabe et al. 2004; Pullagurla et al. 2004; Li et al. 2007). It is therefore possible that decreased striatal dopamine levels following LV 5,7-DHT (which may reflect reduced tonic serotonergic input to dopaminergic neurones rather than a direct effect of the neurotoxin; Iyer and Bradberry 1996) were partly responsible for the impact of this particular lesion type on the activity of Ro 04-6790.

Injections of 5,7-DHT into the DR or MR produced selective 5-HT depletions that were less widespread than those associated with the administration into the LV. The pattern of depletion following MR 5,7-DHT was entirely consistent with the reported anatomical projections of this nucleus. Overall the depletion induced by DR 5,7-DHT also agreed with the expected targets, although a greater decrease in striatal 5-HT levels would have been predicted given the anatomical (Azmitia and Segal 1978) and functional (McQuade and Sharp 1997) evidence for a strong DR projection to this region. The DR is a more diffuse structure than the MR, which could explain the lack of striatal 5-HT depletion following a single neurotoxin injection into the former. It is also possible that increased 5-HT release from MR pathways partially compensates for damage to DR terminals in regions receiving a dual innervation, although it must be noted that the converse does not appear to occur following MR lesions (Thomas et al. 2000). Other compensatory mechanisms such as increased tryptophan hydroxylase activity (Stachowiak et al. 1986; Bendotti et al. 1990) or collateral re-sprouting (Zhou and Azmitia 1984, 1986) could also contribute, since these have been reported within the current post-surgery timescales (which were selected on the basis that a period of 21 days is required in order to obtain maximal decreases in basal levels of extracellular 5-HT; Di Cara et al. 2001).

DR 5,7-DHT caused a significant reduction in post-surgery body weight relative to sham controls, whereas MR 5,7-DHT did not. This is probably due to differential raphé innervation of hypothalamic regions involved in the control of feeding behaviour (for reviews see Leibowitz and Alexander 1998; Meguid et al. 2000). Interestingly, Woolley et al. (2004) attributed 5-ht$_6$ receptor antagonist-induced hypophagia to an inhibitory effect on α-MSH release from POMC-containing neurones in the arcuate nucleus of the hypothalamus, which receives its serotonergic innervation from the DR (Azmitia and Segal 1978). Thus, reduced weight gain following DR 5,7-DHT could theoretically be mediated via a decrease in tonic serotonergic activation of hypothalamic 5-ht$_6$ receptors.

In the NOD test MR 5,7-DHT, like LV 5,7-DHT, prevented the restoration of object discrimination that was

seen with Ro 04-6790 in the same rats prior to surgery, whereas restoration of discrimination in the DR 5,7-DHT group was unaffected. Although the MR 5,7-DHT group subsequently exhibited a lower discrimination ratio than sham controls during the 1 h ITI test they were still able to differentiate the novel from the familiar object. Thus, the cognitive enhancing effects of Ro 04-6790 in the NOD task appear to be mediated via brain regions whose serotonergic innervation is lost following LV and MR, but not DR 5,7-DHT. The brain regions involved in the pro-cognitive effect of Ro 04-6790 are therefore likely to receive prominent serotonergic innervation from the MR, which innervates forebrain regions such as the frontal, cingulate, entorhinal and perirhinal cortices, plus the dorsal hippocampus and medial septum (Azmitia and Segal 1978; Baumgarten and Grozdanovic 1994; Vertes et al. 1999). Of these regions the dorsal hippocampus and frontal cortex are plausible candidates given that serotonergic inputs regulate GABAergic inhibition (Yan 2002), 5-ht$_6$ receptor stimulation enhances GABA efflux (Schechter et al. 2008), and 5-ht$_6$ receptor blockade elevates glutamate efflux therein (Dawson et al. 2001). A key role of the frontal cortex is further supported by the recent finding that SB-271046 enhances social recognition memory when administered directly into this region, but not the nucleus basalis magnocellularis or striatum (Loiseau et al. 2008). The striatum does, however, express some of the highest levels of 5-ht$_6$ receptors (Monsma et al. 1993; Ruat et al. 1993; Kohen et al. 1996; Gérard et al. 1996, 1997), and the combined failure of DR 5,7-DHT to deplete striatal 5-HT and to alter the cognitive response to Ro 04-6790 suggests this region may be important. This proposal is supported by the recent finding that increased striatal 5-ht$_6$ receptor expression impairs cognition (Mitchell et al. 2007), presumably by enhancing GABAergic inhibition (Schechter et al. 2008). Within the striatum 5-ht$_6$ receptors are expressed on GABAergic projections to the globus pallidus (Ward and Dorsa 1996), and this region has been implicated in NOD task performance (Ennaceur 1998). The contribution of the entorhinal and perirhinal cortices to NOD task performance also makes these a strong possibility (Dere et al. 2007) and in addition both the septum and cingulate cortex contain 5-ht$_6$ receptors, apparently expressed on GABAergic interneurones (Woolley et al. 2000), and so could also underlie to the cognitive enhancing effects of 5-ht$_6$ receptor antagonists.

In summary, the current study shows that the cognitive enhancing effects of the 5-ht$_6$ receptor antagonist, Ro 04-6790, in the NOD task stem from blockade of serotonergic neurotransmission from cells originating in the MR. An additional contribution of DR inputs to the striatum cannot be ruled out given the possible incomplete nature of DR lesions, but projections of this nucleus to the cortex and hypothalamus appear not to be involved in the cognitive

effects of Ro 04-6790. Likewise the involvement of DR innervated hippocampal and septal areas also appears questionable since the percentage 5-HT depletion in these areas, while not statistically significant, matched that in the cortex and hypothalamus and would therefore be expected to have at least some effect on the response to Ro 04-6790 if these regions were crucial mediators of its actions. While the impact of 5-HT depletion on the cognitive enhancing activity of Ro 04-6790 clearly has implications for the potential use of 5-ht$_6$ receptor antagonists to treat conditions like Alzheimer's disease, which feature disrupted serotonergic neurotransmission, it should be noted that the depletions employed for localisation purposes herein were much more extensive than those typically observed in patient populations (Adolfsson et al. 1979; Reinikainen et al. 1988).

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