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ORIGINAL INVESTIGATION

E-6801, a 5-HT$_6$ receptor agonist, improves recognition memory by combined modulation of cholinergic and glutamatergic neurotransmission in the rat

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Abstract
Rationale and objectives In rats, 5-hydroxytryptamine$_6$ (5-HT$_6$) receptor antagonists improve learning and memory, but the effects of agonists are poorly defined. This study investigated the effects of 5-HT$_6$ receptor agonists and antagonists on a rodent model of recognition memory.
Methods Selective 5-HT$_6$ receptor agonists and antagonists were administered either alone, after a scopolamine-induced impairment, or combined with sub-effective doses of the acetylcholinesterase inhibitor, donepezil, or the glutamate NMDA receptor antagonist, memantine, in a novel object discrimination paradigm in adult rats.
Results After a 4-h inter-trial delay to induce natural forgetting, vehicle-treated rats spent an equivalent time exploring novel and familiar objects during the choice trial. The 5-HT$_6$ receptor agonists, E-6801 (1.25–10 mg/kg i.p.) and EMD-386088 (5–10 mg/kg i.p.), and antagonists, SB-271046 and Ro 04–6790 (5 and 10 mg/kg), along with donepezil (0.1–3 mg/kg) and memantine (5–20 mg/kg) all produced significant and mostly dose-dependent increases in novel object exploration, indicative of memory enhancement. Furthermore, sub-effective doses of E-6801 (1 mg/kg) when co-administered with either SB-271046 (3 mg/kg), donepezil (0.1 mg/kg) or memantine (5 mg/kg), and EMD-386088 (2 mg/kg) co-administered with SB-271046 (3 mg/kg) also significantly enhanced object-recognition memory. Additionally, using a 1-min inter-trial delay, E-6801 (2.5 and 5 mg/kg) was as effective as donepezil (0.3 and 1 mg/kg) in reversing a scopolamine-induced (0.5 mg/kg) impairment in object recognition.
Conclusions This is the first study to demonstrate that E-6801, a potent 5-HT$_6$ receptor agonist, improves recognition memory by combined modulation of cholinergic and glutamatergic neurotransmission.

Keywords 5-HT$_6$ receptor · Novel object discrimination · E-6801 · Memantine · Donepezil · Memory enhancement

Introduction

The 5-hydroxytryptamine$_6$ (5-HT$_6$) receptor is one of the 14 mammalian 5-HT receptors which has been cloned in man, rat and mouse (Kohen et al. 1996; Monsma et al. 1993; Ruat et al. 1993). This receptor is almost exclusively expressed in the CNS where increasing evidence suggests that it modulates cognitive function (King et al. 2008; Mitchell and Neumaier 2005; Woolley et al. 2004). The first selective 5-HT$_6$ receptor antagonist developed in 1998 (Sleight et al. 1998) caused a yawning and stretching behaviour that was reversed by pre-treatment with cholinergic muscarinic receptor antagonists (Bentley et al. 1999), suggesting that they enhance acetylcholine release. This led to studies examining the effects of 5HT$_6$ receptor antagonists on learning and memory. Acute administration of selective 5-HT$_6$ receptor antagonists or antisense oligonucleotides directed against the initiation codon of

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the 5-HT$_6$ receptor improved retention of spatial learning in the Morris water maze (Foley et al. 2004; Rogers and Hagan 2001; Woolley et al. 2001) and recognition memory in the novel object discrimination (King et al. 2004a, b) paradigms in rats. Selective 5-HT$_6$ receptor antagonists, such as Ro 04-6790 (5 mg/kg, i.p.), SB-357134 (1–30 mg/kg, p.o.) and SB-399885 (1–30 mg/kg, p.o.), have also been found to improve memory consolidation in an autoshaping Pavlovian/instrumental learning task (Meneses 2001a; Perez-Garcia and Meneses 2005) and reverse a scopolamine-induced impairment in step-through passive avoidance (Foley et al. 2004). A structurally unrelated tryptamine derivative (5-methoxy-2-phenyl-*N*,*N*-dimethyl tryptamine, BGC20-761), which is a high-affinity 5-HT$_6$ receptor antagonist, also reversed a scopolamine-induced deficit in a social recognition paradigm in rats and improved a time delay-induced impairment in novel object recognition in adult rats (Mitchell et al. 2006). Furthermore, Hatcher et al. (2005) showed that 7–8 days treatment of normal adult rats with either of the two 5-HT$_6$ receptor antagonists, SB-399885T and SB-271046-A, significantly improved reversal and extra-dimension set-shift performance in an attentional set shifting paradigm and enhanced acquisition and retention of spatial learning in the water maze (Stean et al. 2002). However, not all research groups have been able to replicate the pro-cognitive effects of 5-HT$_6$ receptor antagonists in the Morris water maze (Lindner et al. 2003; Russell and Dias 2002) or the reversal of scopolamine-induced deficits in a test of contextual fear conditioning or the autoshaping task (Lindner et al. 2003).

The first 5-HT$_6$ receptor agonist reported, 2-ethyl-5-methoxy-*N*,*N*-dimethyltryptamine (EMDT), showed a 16-nM affinity but poor selectivity over 5-HT$_{1A}$, 5-HT$_{1D}$ and 5-HT$_7$ receptors, making it of little use for in vivo studies. More recently, several research groups have identified selective, high-affinity 5-HT$_6$ receptor full and partial agonists (Cole et al. 2005; Glennon et al. 2000; Holenz et al. 2005; Mattsson et al. 2005; Romero et al. 2006, 2007; Schechter et al. 2004, 2007). The few investigations carried out to date suggest that 5-HT$_6$ receptor agonists impair recognition memory in social recognition (Loiseau et al. 2008) and short- and long-term memories in autoshaping tasks (Meneses et al. 2008). In contrast, one recent study reported that the 5-HT$_6$ receptor agonist, WAY181187, selectively enhanced extradimensional set shifting, a prefrontal cortex dependent rule reversal task (Burnham et al. 2010). However, no previous study (except for an abstract, King et al. 2006) has reported the pro-cognitive behavioural effects of 5-HT$_6$ agonists in the novel object recognition task in rats. Recent studies using intracerebral microdialysis have examined the in vivo functional effects of the 5-HT$_6$ receptor agonists, WAY-181187 (Beyer et al. 2005; Schechter et al. 2007) and WAY-466 (Schechter et al. 2004) in rats. Both compounds increased extracellular gamma-aminobutyric acid (GABA) overflow in the frontal cortex and corticolimbic regions, an effect that was blocked by the 5-HT$_6$ receptor antagonist, SB-271046, suggesting that they possess CNS activity.

The current study characterised the in vivo behavioural effects of E-6801 (6-chloro-*N*-(3-(2-(dimethylamino)ethyl)-1*H*-indol-5-yl)imidazo[2,1-b]thiazole-5-sulfonamide, Fig. 1), a potent agonist at rat 5-HT$_6$ receptors. E-6801 was compared with two well-established 5-HT$_6$ receptor antagonists, SB-271046 (5-Chloro-*N*-(4-methoxy-3-(piperazin-1-yl) phenyl)-3-methyl-2-benzothiophenesulfonamide) (Bromidge et al. 1999) and Ro 04-6790 (4-amino-*N*-(2,6 bis-methylamino-pyrimidin-4-yl)-benzene sulphonamide) (Sleight et al. 1998), and the agonist, EMD-386088 (5-Chloro-2-methyl-3-(1,2,3,6-tetrahydro-4-pyridinyl)-1*H*-indole) (Mattsson et al. 2005).

Having established that the 5-HT$_6$ receptor agonist had a pro-cognitive effect similar to the antagonists in the novel object discrimination paradigm, a further set of experiments was performed to elucidate the neuropharmacological mechanism involved. Firstly, the ability of E-6801 and donepezil to reverse a scopolamine-induced impairment in recognition memory was compared to the previously reported effect of 5-HT$_6$ receptor antagonists (Woolley et al. 2003). Secondly, the effect of combined administration of sub-effective doses of the 5-HT$_6$ receptor agonist, E-6801 or EMD-386088, with the 5-HT$_6$ receptor antagonist, SB-271046 was given to examine their interaction. Finally, the study investigated whether E-6801 had any

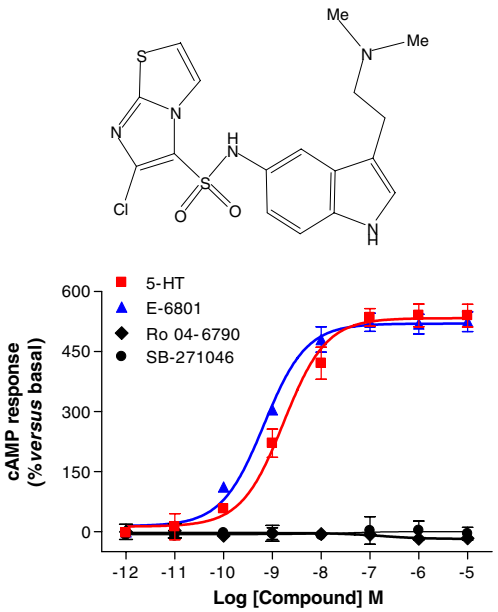

Fig. 1 Chemical structure of E-6801 (*upper diagram*) and comparison of the effect of a range of concentrations of 5-HT, E-6801, Ro 04-6790 or SB 271046 on cAMP accumulation in HEK-293F cells stably expressing the rat 5-HT$_6$ receptors (lower dose-response curve, mean± S.E.M. of basal cAMP, from six to 12 independent experiments performed in duplicate)

synergistic pro-cognitive effect when combined at sub-effective doses with an acetylcholinesterase inhibitor, donepezil, or the low affinity NMDA receptor antagonist, memantine, in the novel object-recognition paradigm to see if both cholinergic and glutamatergic mechanisms could be potentiated by the 5-HT_6 receptor agonist.

Methods

Binding profile and functional assay

Binding profile Binding affinities were determined through commercial radioligand binding assays by CEREP or MDS Pharma, as indicated in Table 1, according to their standard assay protocols (see below for details).

http://www.cerep.fr/Cerep/Users/pages/catalog/binding/catalog.asp http://discovery.mdsps.com/Catalog/Discovery/Profiling/Assays/AssayList.aspx?id=5

The 5-HT_{1A} functional assay was performed at MDS Pharma, according to their standard assay protocol (see below for details).

http://discovery.mdsps.com/Catalog/Services/Profiling/Assays/AssayDetails.aspx?id=3.

Functional assay cAMP measurements on HEK-293F cells that stably expressed rat 5-HT_6 receptors were performed by using a system based on homogeneous time resolved fluorescence (HTRF) (Gabriel et al. 2003). This technology allows the direct measurement of cAMP in living cells. The principle of this assay is based on competition between cAMP produced by cells and cAMP-XL665 conjugate for the binding with monoclonal anti-cAMP–cryptate conjugate. The HTRF cAMP kit from CisBio was used according to the manufacturer's directions. The experimental procedure was performed as stated below.

After 2-h serum-free incubation, suspended cells (20,000 cells per well) were added to 96-well culture plates in incubation buffer composed of Ham's F12 (Gibco) plus 1-mM 3-isobutyl-1-methyl-xanthine (IBMX (Sigma)) and 20-μM pargyline (Sigma). For agonist and antagonist experiments, 40 μl of cell suspension was added to each well. Two microliters of either compound or vehicle was added at indicated concentrations, and plates were pre-incubated for 10 min at room temperature after this initial compound addition. Then, 10 μl of either vehicle or 5-HT, E-6801 or the antagonists, R0 04-6790 and SB 271046, was added (10^{-12} to 10^{-5}M). After 30 min at 37°C, the reaction was stopped, lysing the cells with a mixture of 25 μl of cryptate and 25 μl of XL-665 prepared in the lysis buffer supplied by the manufacturer. Plates were incubated for an additional hour at room temperature and read at 665 nm/620 nm using a Ruby Star Plate reader (BMG LabTech). The cAMP response was expressed as a percentage of basal release.

Animals and drug treatment

Adult male Lister Hooded rats (n=176, Charles River, UK) weighing 270±50 g at the start of each experiment were housed in groups of four on a 12:12 h light:dark cycle (lights on at 07:00 h). Food (B&K Universal small rodent chow) and water were available ad libitum throughout the study, and the room temperature (21±2°C) and relative humidity (45–65%) were kept constant. Six groups of rats received i.p. injections (2 ml/kg, −20 min prior to the familiarisation trial) of either E-6801 (n=16), at 1.25, 2.5, 5 and 10 mg/kg, EMD-386088 (n=12) at 5 and 10 mg/kg, SB-271046 (n=12) at 5 and 10 mg/kg, Ro 04-6790 (n=12) at 5 and 10 mg/kg, donepezil (n=16) at 0.1, 0.3, 1 and 3 mg/kg or memantine (n=12) at 5, 10, 15 and 20 mg/kg in all cases with vehicle (0.5% methylcellulose in saline, 2 ml/kg) treatment. Thus, all rats in each group received all doses of the selected compound (and vehicle) in a random order with each behavioural test occurring at 7-day intervals, and the familiarisation and choice trials were separated by 4 h.

In the scopolamine-induced impairment experiment (which used a 1-min inter-trial interval), four groups of rats (n=12 each) received i.p. injections of either scopolamine (0.5 mg/kg) or vehicle (−40 min) and (−20 min prior to the familiarisation trial) either E-6801 (2.5 and 5 mg/kg, two groups) or donepezil (0.3 and 1 mg/kg, two other groups) and vehicle (0.5% methylcellulose in saline, 2 ml/kg) in a random order over a period of 4 weeks. As before, each drug dose within each group was examined at 7-day intervals such that each rat had all treatment combinations.

For the synergy studies (using a 4-h inter-trial interval), three groups of rats (n=12 each) received i.p. injections of a sub-effective dose of E-6801 (1 mg/kg) or vehicle (0.5% methylcellulose in saline, 2 ml/kg), administered alone or combined with either SB-271046 (3 mg/kg), memantine (5 mg/kg) or donepezil (0.1 mg/kg). Sub-effective doses were selected from the previous dose-response studies, and again each drug combination was administered to all rats over a period of 4 weeks in a random order using a 7-day behavioural test interval.

Studies were conducted in full compliance with the principles of laboratory animal care, the UK Animals (Scientific Procedures) Act 1986 and approval of the local ethical committee (University of Nottingham) by an observer who was unaware of the treatment given. Additionally, the rats were also examined visually for any abnormal behaviour caused by the weekly repeated i.p. injection procedure.

Table 1 Selectivity binding profile of E-6801

Receptor	K_i (nM)
(*h*)5-HT_{1A}	13[a]–96[b]
(*r*)5-HT_{1B}	280[a]
(*h*)5-HT_{2A}	>1,000[a]
(*h*)5-HT_{2B}	>1,000[b]
(*h*)5-HT_{2C}	>1,000[a]
(*h*)5-HT_3	330[a]
(*gp*)5-HT_4	>1,000[b]
(*h*)5-HT_{5A}	410[a]
(*h*)5-HT_6	0.58[a]
(*h*)5-HT_7	>1,000[a]
(*h*)5-HT transporter	>10,000[a,b]
(*h*)adrenergic α_{2A}	166[b]
(*r*)Ca channel L-type, benzothiazepine	222[b]
(*r*)Ca channel L-type, dyhydropyridine	325[b]
(*h*)M_4	700[a]
Other receptors	n.s.[c]

[a] Cerep

[b] MDS Pharma

[c] In vitro pharmacology screening package including the following assays: Cerep binding assays: (*h*) 5-HT transporter; (*h*) 5-HT_{1A}; (*r*) 5-HT_{1B}; (*h*) 5-HT_{2A}; (*h*) 5-HT_{2B}; (*h*) 5-HT_{2C}; (*h*) 5-HT_3; (*h*) 5-HT_{4e}; (*h*) 5-HT_{5A}; (*h*) 5-HT_6; (*h*) 5-HT_7; (*h*) A_1; (*h*) A_{2A}; (*h*) A_3; (*r*) adrenergic α_1 (non-selective); (*r*) adrenergic α_2 (non-selective); (*r*) AMPA; (*h*) AR; (*h*) AT1; (*h*) AT_2; (*h*) B_1; (*h*) B_2; (*h*) $beta_1$; (*h*) $beta_2$; (*h*) BLT_1 (LTB_4); (*r*) calcium channel L-type, benzothiazepine; (*r*) calcium channel L-type, dihydropyridine; (*r*) calcium channel N-type; (*r*) calcium channel L-type, phenylalkylamines; (*h*) CB_1; (*h*) CB_2; (*h*) CCK_1 (CCKA); (*h*) CCK_2 (CCKB); (*h*) CCR_1; (*h*) CCR_2; (*h*) CCR_3; (*h*) CGRP; (*h*) choline transporter (CHT_1); (*r*) Cl-channel; (*h*) CRF_1; (*h*) $CXCR_2$ (IL-8B); (*h*) $CysLT_1$ (LTD_4); (*h*) D_1; (*h*) D_{2S}; (*h*) D_3; (*h*) $D_{4.4}$; (*h*) D_5; (*h*) opiate δ_2; (*h*) dopamine transporter; (*h*) EP_4; (*h*) ER (non-selective); (*h*) ET_A; (*h*) ET_B; (*r*) GABA (non-selective); (*r*) GABA transporter; (*h*) GAL_1; (*h*) GAL_2; (*r*) glycine (strychnine insensitive); (*h*) GR; (*h*) H_1; (*h*) H_2; (*h*) H_3; (*h*) hERG; (*bv*) I_1; (*r*) I_2; (*h*) IP (PGI_2); (*r*) kainate; (*r*) opiate κ; (*r*) K_{ATP}; (*r*) K_V; (*h*) M_1; (*h*) M_2; (*h*) M_3; (*h*) M_4; (*h*) M_5; (*h*) MC_4; (*h*) MT_1 (ML_{1A}); (*h*) opiate μ; (*r*) N neuronal α-BGTX-insensitive ($\alpha_4\alpha_2$); (*h*) NK_1; (*h*) NK_2; (*h*) NK_3; (*r*) NMDA; (*h*) NMU_2; (*h*) NOP (ORL_1); (*h*) norepinephrine transporter; (*h*) NTS_1 (NT_1); (*r*) P_{2X}; (*r*) P_{2Y}; (*h*) PAC_1 (PACAP); (*h*) PAF; (*r*) PCP; (*m*) PDGF; (*h*) PPARγ; (*h*) PR; (*r*) sigma (non-selective); (*r*) Na channel, site 2; (*r*) SKCa; (*m*) sst (non-selective); (*h*) TNF alpha; (*h*) TP (TXA_2/PGH_2); (*h*) TRH_1; (*h*) UT_1; (*h*) V_{1a}; (*h*) V_2; (*h*) $VPAC_1$ (VIP_1); (*h*) Y_1; (*h*) Y_2.

Cerep enzyme assays: (*h*) COX_1; (*h*) COX_2; (*h*) 12-LO; (*h*) constitutive NOS; (*bv*)PDE1; (*h*) PDE2; (*h*) PDE3; (*h*) PDE4; (*h*) PDE5; (*bv*)PDE6; (*h*) ACE; (*h*) ACE-2; (*h*) ECE-1; (*h*) elastase; (*h*) caspase-3; (*h*) caspase-8; (*h*) cathepsin D; (*h*) cathepsin L; (*h*) neutral endopeptidase; (*h*) MMP-1; (*h*) tryptase; (*h*) phosphatase 1B; (*h*) Abl kinase; (*h*) Akt1/PKBα; (*h*) CaMK2α; (*h*) CaMK4; (*h*) CDC2/CDK1; (*h*) FGFR2 kinase; (*h*) FLT-1 kinase; (*h*) IRK; (*h*) Lyn kinase; (*h*) MEK1/MAP2K1; (*h*) p38αkinase; (*h*) PKA; (*h*) PKCα; (*h*) RAF-1 kinase; (*h*) ZAP70 kinase; (*h*) acetylcholinesterase; (*h*) MAO-A; (*h*) MAO-B; (*pr*)ATPase (Na^+/K^+).

MDS Pharma: (*h*) A_1; (*h*) A_{2A}; (*h*) A_3; (*r*) adrenergic α_{1A}; (*r*) adrenergic α_{1B}; (*h*) adrenergic α_{1D}; (*h*) adrenergic α_{2A}; (*h*) adrenergic β_1; (*h*) adrenergic β_2; (*h*) B_1; (*h*) B_2; (*r*) calcium channel L-type, benzothiazepine; (*r*) calcium channel L-type, dihydropyridine; (*r*) calcium channel N-type; (*h*) D_1; (*h*) D_{2S}; (*h*) D_3; (*h*) $D_{4.2}$; (*h*) ET_A; (*h*) ET_B; (*h*) epidermal growth factor (EGF); (*h*) eostrogen ERα; (*r*) $GABA_A$, agonist site; (*r*) $GABA_A$, flunitrazepam, central; (*r*) $GABA_A$, muscimol, central; (*h*) $GABA_{B1A}$; (*h*) glucocorticoid; (*r*) glutamate, kainate; (*r*) glutamate, NMDA, agonism; (*r*) glutamate, NMDA, glycine; (*r*) glutamate, NMDA, phencyclidine; (*h*) H_1; (*h*) H_2; (*h*) H_3; (*r*) I_2, central; (*m*) interleukin IL-1; (*h*) leukotriene, cysteinyl CysLT1; (*h*) MT_1; (*h*) M_1; (*h*) M_2; (*h*) M_3; (*h*) NPY Y_1; (*h*) NPY Y_2; (*h*) nicotinic acetylcholine; (*h*) nicotinic acetylcholine α1, bungarotoxin; (*h*) opiate δ; (*h*) opiate κ; (*h*) opiate μ; (*m*) phorbol ester; (*h*) platelet activating factor (PAF); (*h*) hERG; (*ht*)K_{ATP}; (*h*) prostanoid EP_4; (*rb*)P_{2X}; (*r*) P_{2Y}; (*r*) rolipram; (*h*) 5-HT_{1A}; (*h*) 5-HT_{2A}; (*h*) 5-HT_3; (*gp*) 5-HT_4; (*h*) σ_1; (*r*) σ_2; (*r*) sodium channel, site 2; (*h*) NK_1; (*r*) testosterone; (*r*) thyroid hormone; (*h*) dopamine transporter; (*r*) GABA transporter; (*h*) norepinephrine transporter; (*h*) serotonin transporter

n.s., not significant (K_i>1 μM or % inhibition at 10^{-6} M lower than 50%); (*bv*), bovine; (*gp*), guinea pig; (*ht*), hamster; (*h*), human; (*m*), mouse; (*pr*), porcine; (*r*), rat; (*rb*), rabbit.

Behavioural assessment

The two trial novel object discrimination paradigm utilised was as described by Ennaceur and Delacour (1988), with minor modifications (King et al. 2004b; Woolley et al. 2003). Experiments were performed between 09:00 and 15:00 h in dim light (180 Lux at the arena floor). The 12 open-field test arenas used for object discrimination were clear perspex boxes (39×23.5 cm with 24.5-cm high walls) to which each rat was habituated for 60 min the day prior to each test day, with the same box being used for each rat. Dose-response studies were performed with all compounds tested giving treatments −20 min prior to the initial acclimatisation on the test day to establish doses to be used in the subsequent combined drug-treatment studies. For the synergy- and scopolamine-impairment experiments, the first drug was administered −40 min on the test day and the second drug −20 min before the familiarisation trial. Therefore, 20 min after the injection, each rat received 3-min acclimatisation to the perspex box in the absence of objects which was then followed by the 3-min familiarisation trial and a second 3-min choice trial following a 4-h inter-trial interval (except in the scopolamine study, see later). This interval was selected from previous studies showing that a 4-h inter-trial delay prevented drug-naive adult rats from discriminating between the familiar and novel objects (King et al. 2004b), thus permitting the pro-cognitive effects of compounds to be evaluated. In contrast, for the scopolamine-induced impairment experiments, an inter-trial delay of 1 min was used as drug-naïve rats discriminate the novel from the familiar object using short-time intervals, so that the impairment produced by scopolamine could be readily observed. During the first familiarisation trial (T_1), rats were exposed to two objects (A1 and A2) of identical size, shape, colour and pattern; in the second choice trial (T_2), one of the objects (selected in a pseudo-random manner) was replaced by an identical object but with a striped (novel) pattern (B1) with a clean original object (A3). The objects were 8-cm high×5-cm diameter plastic bottles filled with water, covered in white masking tape and secured with Blu-Tac® through holes in a removable floor 5 cm from the side and 10 cm from the end wall in opposite corners of the arena. The novel object had three horizontal stripes comprising 1.2-cm wide black insulating tape added to an otherwise identical bottle to those used in trial 1. All objects were cleaned with 20% v/v ethanol prior to each study to remove any olfactory cues and new objects used for each study group.

During both trials, exploration of each object was defined as the time spent (s) sniffing (within 1 cm of it with active vibrissae), licking, chewing or touching the object with the nose. Sitting on the object was not recorded as exploratory activity but rarely occurred for any duration. Exploration times were recorded separately for each object with stopwatches by an observer seated 1 m away. In order to minimise inter-individual variation and allow each animal to act as its own control within every group, each rat was tested with all doses and every animal used was included in the analysis performed.

Compounds

E-6801, Ro 04-6790, SB-271046 and donepezil HCl were synthesised by ESTEVE. EMD-386088 HCl was obtained from Tocris, UK. Memantine HCl and scopolamine HBr were purchased from Sigma, UK. All compounds were dissolved in 0.154-M saline containing 0.5% methyl cellulose and administered in a volume of 2 ml/kg i.p., 20 or 40 min prior to behavioural assessment as described above.

Statistical analysis

Choice trial exploration times were assessed by two-way repeated-measures analysis of variance (ANOVA) with Bonferroni post-test where appropriate. One-way repeated-measures ANOVA was used to analyse the effect of treatment on the discrimination ratio (B1/(B1+A3)) and on cumulative exploratory times in both T_1 (A1+A2) and T_2 (B1+A3). Following ANOVA, post hoc comparison was performed using Bonferroni's multiple comparison test where appropriate. In all cases, data are presented as mean (±S.E.M.), with statistical significance set at $P<0.05$.

Results

Binding profile and functional assay

The binding affinities of E-6801 are summarised in Table 1. E-6801 displayed high affinity for the recombinant human 5-HT_6 receptor, with a high degree of selectivity compared with all the other serotonin receptor subtypes listed in Table 1. Besides this, no significant binding could be detected at 1 μM for a panel of 138 receptors, ion channels and transporters (except for hα_{2A}, rCa channel L-type at benzothiazepine and dyhydropyridine sites, and muscarinic hM_4). The inhibition of 40 enzymes was also evaluated, and E-6801 did not display any significant activity, including at acetylcholinesterase (5% inhibition at 1 μM).

The results on the 5-HT_6 receptor functional assay (Fig. 1) agree with previously reported full agonist activity of E-6801 at the human 5-HT_6 receptor (Romero et al. 2006), showing an EC_{50} of 2.7 nM at the rat 5-HT_6 receptor. E-6801 was evaluated in a GTPγS binding assay for possible functional 5-HT_{1A} receptor agonist/antagonist

activity. Weak 5-HT_{1A} receptor agonist activity was observed (data not shown), showing an estimated EC_{50} value of 320 nM, more than 100-fold less than its activity at the 5-HT_6 receptor.

Dose-response studies with E-6801, EMD-386088, SB-271046, Ro 04-6790, donepezil and memantine

The current study used novel object discrimination to characterise the ability of both 5-HT_6 receptor agonists and antagonists to enhance recognition memory impaired by using either a 4-h inter-trial delay (natural forgetting) or pre-treatment with the muscarinic receptor antagonist, scopolamine. Secondly, synergistic effects were investigated by co-administering sub-effective doses of the 5-HT_6 receptor ligands with drugs that are thought to have pro-cognitive efficacy in clinical studies by the modulation of cholinergic or glutamatergic neuronal function.

In dose-response studies following treatment with either vehicle or any of the 5-HT_6 receptor compounds, donepezil or memantine, rats spent an equivalent time exploring the two identical objects during the familiarisation trial (T_1, data not shown), confirming that rats had no spatial preference for the objects in the task. As expected, with the 4-h interval from the familiarisation trial inducing natural forgetting, vehicle-treated rats from all treatment groups spent an equivalent time exploring the novel and familiar objects in the second (T_2) or choice trial (Fig. 2a–f). Univariate analysis using two-way repeated-measures ANOVA with Bonferroni post hoc tests, where appropriate, to examine any change in profile of object exploration during the choice trial revealed significant interactions between dose and object exploration for all of the compounds tested. E-6801 (A; [$F(4,74)=4.69$, $P=0.002$]), EMD386088 (B; [$F(2,33)=4.43$, $P=0.023$]), SB-271046 (C; [$F(2,33)=4.65$, $P=0.017$]), Ro 04-6790 (D; [$F(2,33)=8.63$, $P<0.001$]), donepezil (E; [$F(4,75)=5.42$, $P=0.0007$]) and memantine (F; [$F_{(4,55)}=11.66$, $P<0.0001$]), all produced significant, and except for EMD386088, dose-related increases in the time spent exploring the novel compared with the familiar object during the second choice trial (Fig. 2a–f).

This restoration of object discrimination resulted from a redistribution of the exploration times towards the novel object during the choice trial, rather than any non-specific change in total exploratory activity, as can be seen from the increases in discrimination ratio (Fig. 3a–f) for each of the compounds tested (E-6801; [$F(4,79)=5.02$, $P<0.01$], EMD-386088 [$F(2,35)=5.25$, $P<0.05$]), SB-271046; [$F(2,22)=5.67$, $P<0.01$]) and Ro 04-6790; [$F(2,22)=6.35$, $P<0.01$], donepezil [$F(4,79)=4.82$, $P<0.01$] and memantine [$F_{(4,59)}=8.92$, $P<0.0001$], repeated-measures ANOVA). Only the highest dose of both antagonists, SB-271046 and Ro 04-6790 (Fig. 3c and d), produced a significant increase in the discrimination ratio, while the effect of both agonists, E-6801 and EMD-386088 (Fig. 3a and b), showed a bell-shaped response, being most effective at 5 mg/kg i.p.

One of the two cognitive enhancers used in this study, the acetylcholinesterase inhibitor, donepezil, restored novel object discrimination at all of the doses used (0.1, 0.3, 1, and 3 mg/kg) with apparently equivalent effectiveness rather than producing a dose-related effect (Figs. 2e and 3e). Of note, the lowest dose of donepezil only just produced a significant increase in the dose ratio (Fig. 3e) so this appeared to be a minimal effective dose. By contrast, only the highest dose of the low affinity NMDA receptor antagonist, memantine (20 mg/kg), restored preferential exploration of the novel over the familiar object (Figs. 2f and 3f). None of these compounds at the doses used evoked any other observable behavioural effect or any motor components of the serotonin syndrome, although this was not formally quantified.

Reversal of scopolamine-induced cognitive impairment by E-6801

To evaluate whether the pro-cognitive effect seen with E-6801 involved modulation of cholinergic mechanisms, a study was performed to see if it could reverse a cognitive impairment induced by the muscarinic receptor antagonist scopolamine, analogous to that previously reported with 5-HT_6 receptor antagonists (Woolley et al. 2003).

Using a 1-min inter-trial period, vehicle-treated animals readily distinguished between the familiar and novel objects during the choice trial an effect which was significantly impaired by scopolamine (Fig. 4a–d). Two-way repeated-measures ANOVA revealed significant interaction between dose and object, with Bonferroni post hoc showing that the scopolamine-induced impairment was significantly reversed by pre-treatment with E-6801 at a dose of 2.5 mg/kg (Fig. 4a; [$F(3,44)=16.58$, $P<0.0001$]) or 5 mg/kg (Fig. 4b; [$F(3,44)=7.33$, $P=0.0004$]). The acetylcholinesterase inhibitor, donepezil, also significantly reversed scopolamine impairment at both doses, 0.3 mg/kg (Fig. 4c; [$F(3,44)=9.32$, $P<0.0001$]) and 1 mg/kg (Fig. 4d; [$F(3,44)=10.34$, $P<0.0001$]).

These findings were reinforced by repeated-measures ANOVA analysis of the discrimination ratio (Fig. 5a–d); which again demonstrated that both doses of E-6801 (Fig. 5a–b), 2.5 mg/kg [$F(3,47)=12.80$, $P<0.001$] and 5 mg/kg [$F(3,47)=9.59$, $P<0.001$]), or donepezil (Fig. 4c–d), 0.3 mg/kg [$F(3,47)=7.56$, $P<0.001$] and 1 mg/kg [$F_{(3,47)}=9.69$, $P<0.001$], significantly reversed the impairment in object recognition produced by scopolamine, confirming that both drugs restored preferential investigation of the novel object in the choice trial following impairment by muscarinic receptor antagonism.

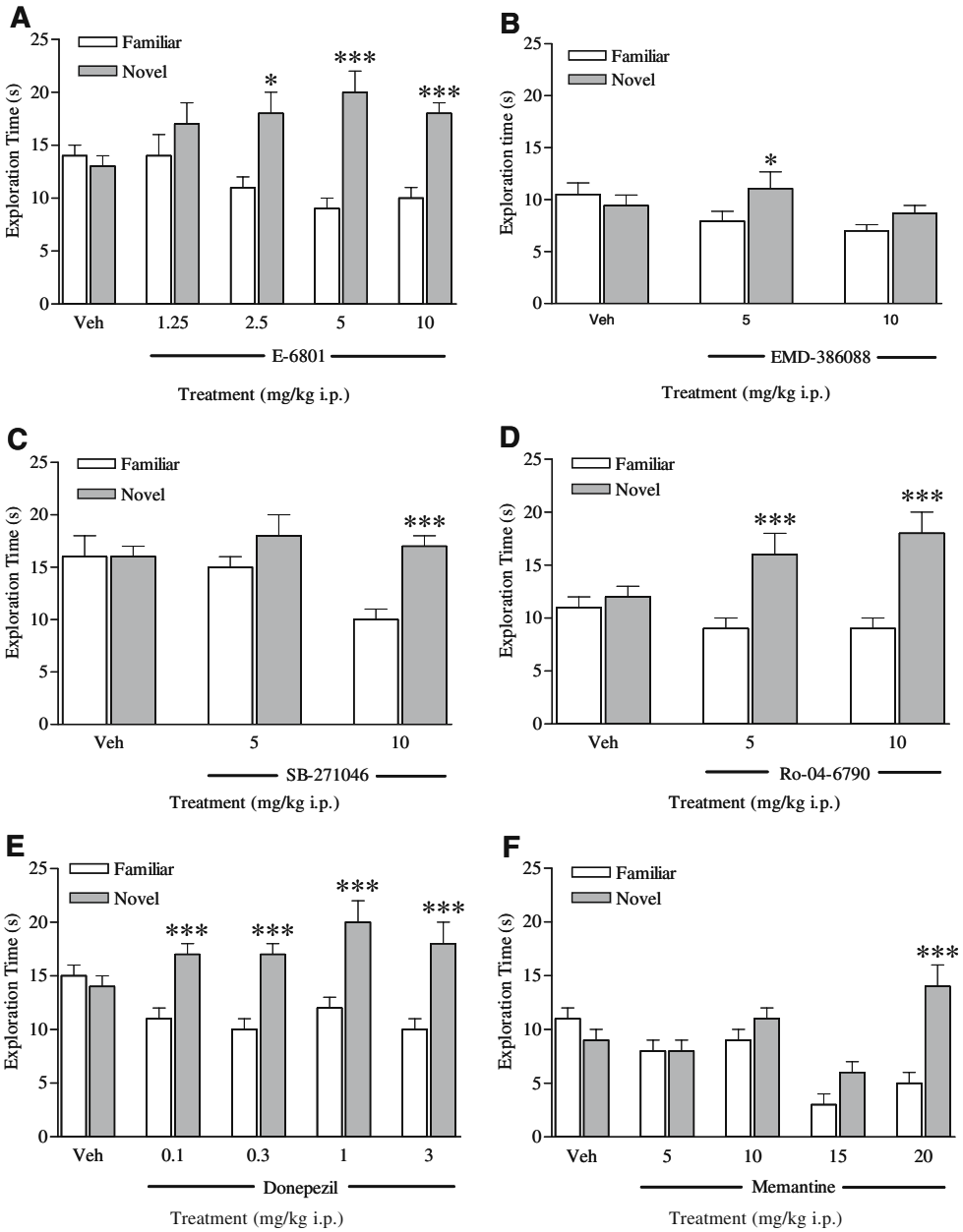

Fig. 2 Dose-response effects of pre-treatment with **a** E-6801, **b** EMD386088, **c** SB-271046, **d** Ro 04-6790, **e** donepezil and **f** memantine on the time spent exploring (s, mean±S.E.M.) the novel versus the familiar object during the choice trial in the novel object discrimination task using a 4-h inter-trial interval. In all groups, each rat (n=12, except for E-6801 and donepezil, n=16) received every dose of the test drug plus vehicle treatment in a random order separated by an interval of 1 week. Two-way repeated-measures ANOVA revealed significant interaction between dose and object exploration for all six compounds tested. *P<0.05 and ***P<0.001 from the familiar object at the same dose within the same treatment group (Bonferroni post-test). Note that with a 4-h inter-trial interval, vehicle-treated rats are unable to discriminate the novel from the familiar object due to natural forgetting but preferential recognition of the novel object was restored by pre-treatment with any of the three 5-HT_6 receptor ligands and both of the selected cognitive enhancers

Interaction between E-6801 with 5-HT_6 receptor antagonists, glutamatergic or cholinergic mechanisms

To further investigate the involvement of cholinergic and/or glutamatergic pathways in the apparent pro-cognitive effect of 5-HT_6 receptor activation, sub-effective doses of E-6801 or EMD386088 were given as a combined pre-treatment with the selective 5-HT_6 receptor antagonist, SB-271046, the acetylcholinesterase inhibitor, donepezil, or the NMDA receptor antagonist, memantine.

Using a 4-h 'natural forgetting' inter-trial interval, vehicle-treated rats showed no preferential exploration of either the novel or familiar objects during the choice trial (Fig. 6a–d). Two-way repeated-measures ANOVA revealed significant interactions between dose and object exploration. Furthermore Bonferroni post hoc tests demonstrated significant preferential exploration of the novel over the familiar object for both agonist/antagonist co-administration studies, E-6801 +SB-271046 (Fig. 6a; [F(3,44)=5.95, P=0.0017]) and EMD386088+SB-271046 (B; [F(3,44)=19.54, P<0.0001]). Co-administration of a sub-effective dose of E-6801 with either memantine (Fig. 6c; [F(3,44)=6.18, P=0.0013]) or donepezil (Fig. 6d; [F(3,44)=5.68, P<0.0022]) also significantly increased novel object exploration, suggesting that a pro-cognitive synergistic interaction occurred between E-6801 and both of these compounds in the object-recognition task. Analyses of the discrimination ratios (Fig. 7a–d) supported the individual object exploration data.

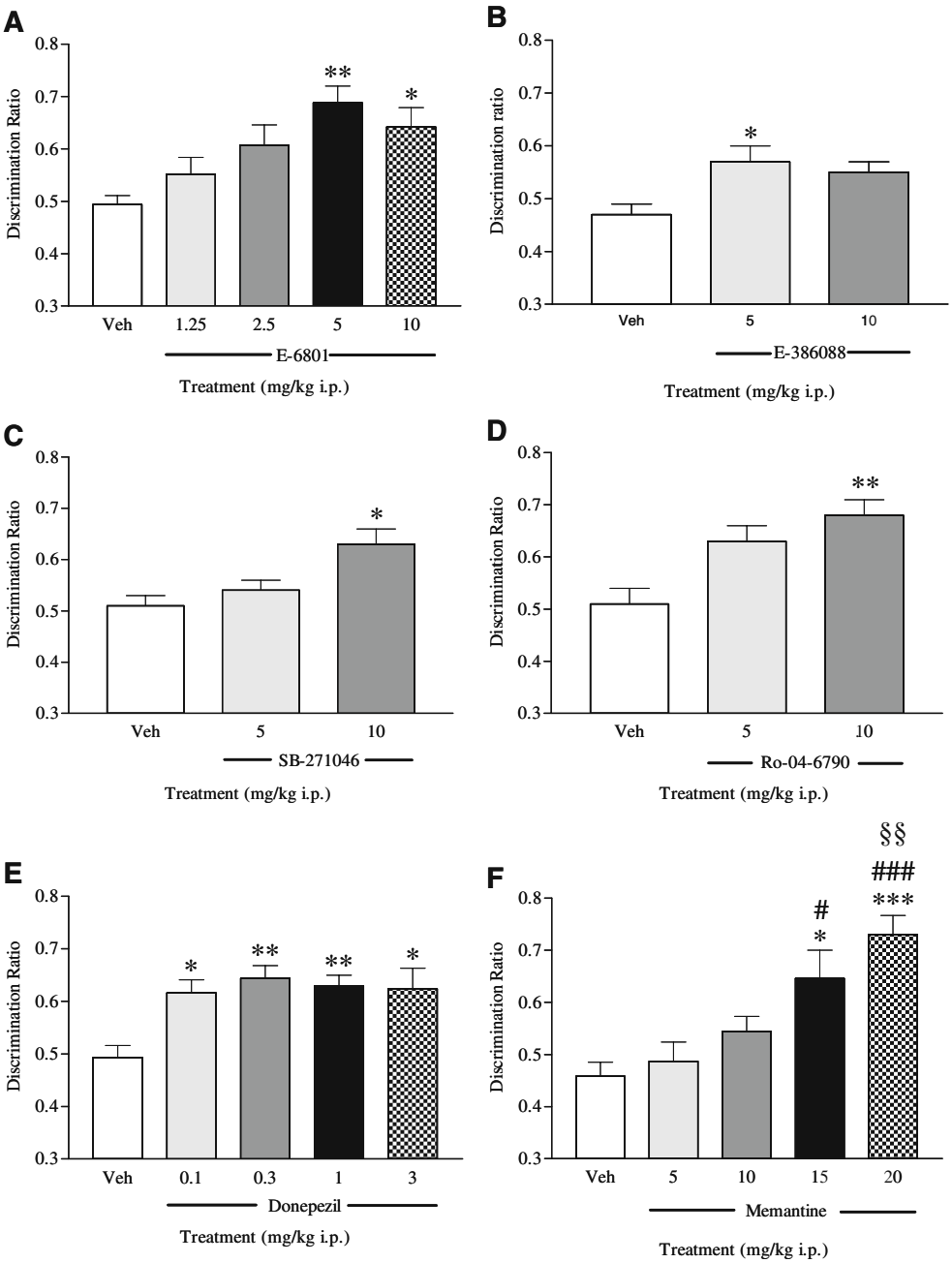

Fig. 3 Effect of test compounds on the discrimination ratio, (calculated from (novel object exploration/(novel+familiar object exploration) in Fig. 2). Values are mean±S.E.M., n=12, except for E-6801 and donepezil, n=16. One-way repeated-measures ANOVA revealed significant increases in discrimination ratio for each of the compounds tested. *P<0.05, **P<0.01, and ***P<0.001 from respective vehicle treatment value (Bonferroni's multiple comparison test). The higher doses of memantine (15 and 20 mg/kg i.p.) also significantly enhanced the discrimination ratio compared with that recorded in lower treatment doses, $^{\#}P$<0.05 and $^{\#\#\#}P$<0.001 from 5 mg/kg, and $^{\S\S}P$<0.01 from 10 mg/kg. Thus, all 5-HT_6 receptor ligands and both cognitive enhancers tested significantly elevated object discrimination compared with respective vehicle treatment

Repeated-measures ANOVA revealed that co-administration of E-6801+SB-271046 (Fig. 7a [F(3,47)=7.74, P<0.0005]), EMD386088+SB-271046 (Fig. 7b [F(3,47)=7.74, P<0.0005]) and E-6801+memantine (Fig. 7c [$F_{(3,47)}$=4.28, P<0.05]) significantly increased the discrimination ratio compared to that with either vehicle treatment or administration of the sub-effective compound alone, indicative of synergy. The effect of E-6801 just failed to reach significance ([$F_{(3,47)}$=2.53, P=0.074] when co-administered with donepezil (Fig. 7d), although a clear trend in improvement was still apparent.

Treatment effects on cumulative object exploration times for both familiarisation (T_1) and choice trials (T_2) are shown in Tables 2, 3 and 4. As expected, T_2 cumulative exploration times were generally lower than those recorded in T_1, irrespective of the drug treatment used probably due to some habituation following a 4-h inter-trial period. As would be expected, the short 1-min inter-trial period adopted with scopolamine-impairment studies produced much closer T_1 and T_2 cumulative times (Table 3).

Of note, only the highest dose of E-6801 produced a small but significant (P<0.05) decrease in the total time spent exploring both objects during the familiarisation trial compared with the vehicle control, and a similar trend was seen with Ro 04-6790 while SB-271046 produced a more pronounced decrease (P<0.001) at 10 mg/kg

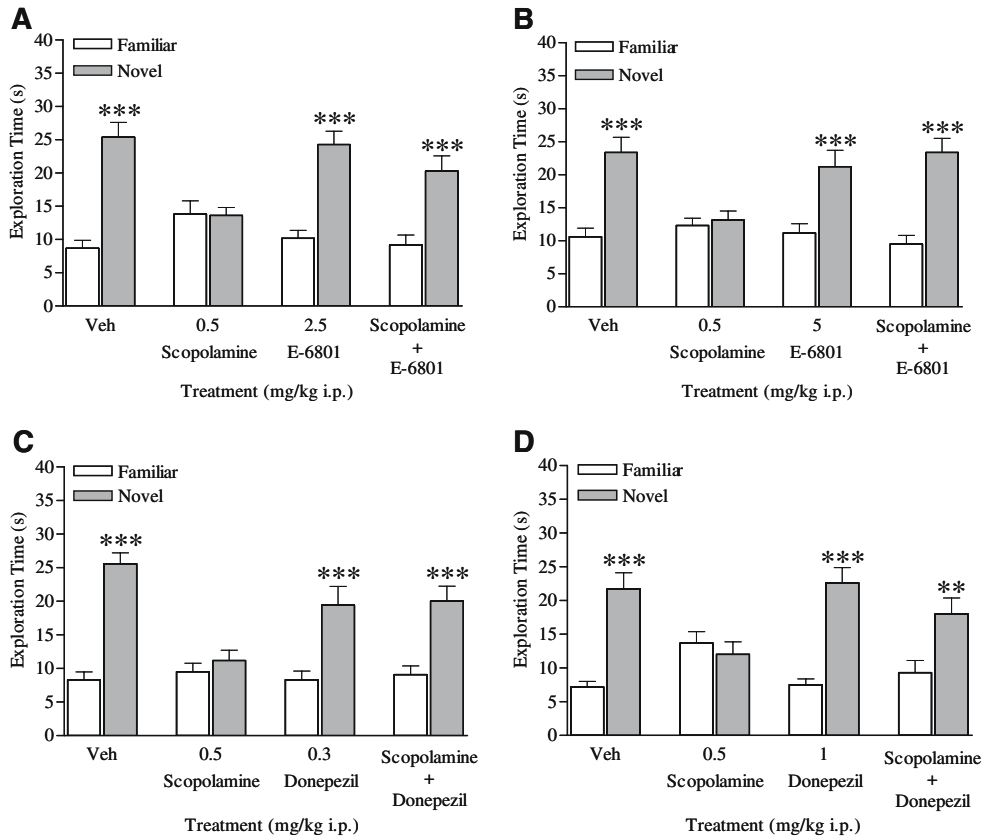

Fig. 4 The effects of E-6801 and donepezil on the time spent exploring the novel versus the familiar objects during the choice trial in the novel object discrimination task following scopolamine-induced impairment using a 1-min inter-trial interval (s, mean±S.E.M.). For each separate experiment, all rats (n=12 per group) received each drug combination plus vehicle in a random order separated by an interval of 1 week. Note that with a 1-min inter-trial interval, vehicle-treated rats readily distinguish the novel from the familiar object, but that administration of scopolamine (0.5 mg/kg i.p.) significantly impairs this recognition process. However, scopolamine impairment is reversed and recognition of the novel object is restored by both E-6801 and donepezil at both doses used. **P<0.01 and ***P<0.001 from the familiar object at that drug combination within the same treatment group (Bonferroni post-test following two-way repeated-measures ANOVA)

(Table 2). Both donepezil and memantine significantly reduced the total T_1 exploration time in a dose-related manner, which reached significance with the two highest doses of both compounds (Table 2). Subjective analysis of behaviours showed that memantine (15 and 20 mg/kg i.p.) induced a mild flat body posture and appeared to reduce locomotor activity during the T_1 trial, but these subjective effects were absent during the second choice trial (T_2) 4 h later. Compared with the total exploratory activity of vehicle treatment in the same group of rats during T_2, none of the compounds, except memantine at 15 mg/kg (Table 2), had any effect on total object exploration times recorded during the choice trial (T_2). Thus, any residual effect of the compounds on exploration was absent during the choice trial for all drugs, except the highest dose of memantine. Tables 3 and 4 show that the cumulative exploration times of both objects in T_1 were not significantly affected by any of the drug combinations used, with the exception that 0.3 mg/kg of donepezil (Table 3) significantly increased and E-6801+memantine (Table 4) significantly decreased the total T_1 exploration time from that of vehicle treatment in the same group. Similarly, none of the compounds used in the drug combination studies (Tables 3 and 4) had any effect on the total T_2 exploration time, except a single dose of scopolamine (0.5 mg/kg i.p., P<0.01) in the donepezil combination study which caused a reduction compared with vehicle treatment in the same group. As expected, in each experiment regardless of compound or dose, there was a small and typically significant reduction in the total time spent exploring the objects in T_2 compared with that observed in T_1 (Tables 2, 3, and 4) due to inter-trial habituation. Taken together, this analysis of change in total trial object exploration supports the proposal that changes seen in object exploration during the choice trial are not due to a non-specific attenuation of exploration but are consistent with a change in recognition memory.

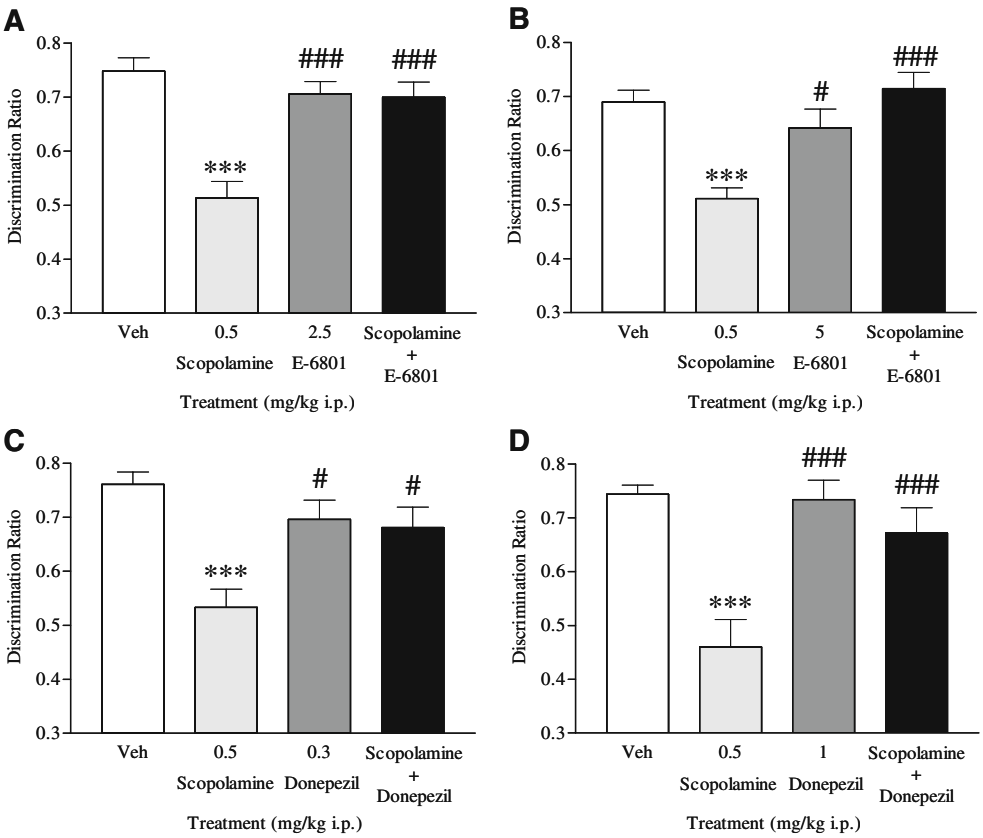

Fig. 5 The effects of E-6801 and donepezil on the discrimination ratio (calculated from novel object exploration/(novel+familiar object exploration) in Fig. 3). Values are mean±S.E.M., $n=12$ per group. Both doses of E-6801 and those of donepezil reversed the impairment in novel object discrimination produced by scopolamine. ***$P<0.001$ from respective vehicle control, and $^{\#}P<0.05$ and $^{\#\#\#}P<0.001$ from scopolamine treatment within the same group (Bonferroni's multiple comparison test)

Discussion

The present study compared the activity of E-6801, a potent and efficacious 5-HT_6 receptor agonist, given alone or in comparison with two 5-HT_6 receptor antagonists SB-271046 and Ro 04-6790 in a novel object discrimination paradigm. Later sub-effective doses of E-6801 were administered in combination with those of SB-271046, and cholinergic and glutamatergic compounds (donepezil and memantine, respectively) used to treat human cognitive dysfunction. To further support the observation that E-6801 had a pro-cognitive effect, it was compared with another 5-HT_6 receptor agonist, EMD386088, both given alone and in sub-effective doses with SB-271046. This is the first dose-response study to report that 5-HT_6 receptor ligands, with agonist properties in functional in vitro studies, enhance cognitive function either when given by themselves or when combined with sub-effective doses of donepezil or memantine using a time-induced deficit in novel object-recognition memory in the adult rat.

The pharmacological validity of the rodent novel object discrimination task to predict compounds with potential clinical pro-cognitive actions has been demonstrated with the acetylcholinesterase inhibitor, donepezil (Aricept®), currently used for the treatment of Alzheimer's disease, which reverses a time-induced deficit in this task (Prickaerts et al. 2005). In addition, other compounds with potential pro-cognitive effects such as PDE4 inhibitors (Rutten et al. 2006), 5-HT_4 partial agonists (Lamirault and Simon 2001; Moser et al. 2002), the NMDA receptor antagonist, memantine (current study) and other cholinesterase inhibitors (Lamirault et al. 2003a; Lieben et al. 2005; Prickaerts et al. 2005; Scali et al. 1997) also reverse natural forgetting in novel object discrimination, while performance is impaired by CB_1 receptor agonists (Kosiorek et al. 2003) and the dopamine D_2 receptor antagonist, raclopride (Woolley et al. 2003), supporting the sensitivity of this pre-clinical task to agents operating through a diverse array of pharmacological mechanisms.

The 5-HT_6 receptor has also been implicated in the regulation of memory processes (for a review see ; Fone et al. 2008) and the acute pro-cognitive activity of several 5-HT_6 receptor antagonists has been described in novel object discrimination (Hirst et al. 2006; King et al. 2004a, b; Lieben et al. 2005; Schreiber et al. 2007; Woolley et al. 2003), social recognition (Mitchell et al. 2006; Schreiber et al. 2007), autoshaping (Meneses 2001b; Perez-Garcia and Meneses 2005; Schreiber et al. 2007), Morris water maze (Hirst et al. 2006; Rogers and Hagan 2001; Stean et al. 2002; Woolley et al. 2001) and passive avoidance tasks (Foley et al. 2004; Schreiber et al. 2007). It is therefore an apparent paradox that E-6801, a full 5-HT_6 receptor agonist in in vitro studies (Holenz et al. 2005; Romero et al. 2006, 2007), caused a dose-related restoration of natural forget-

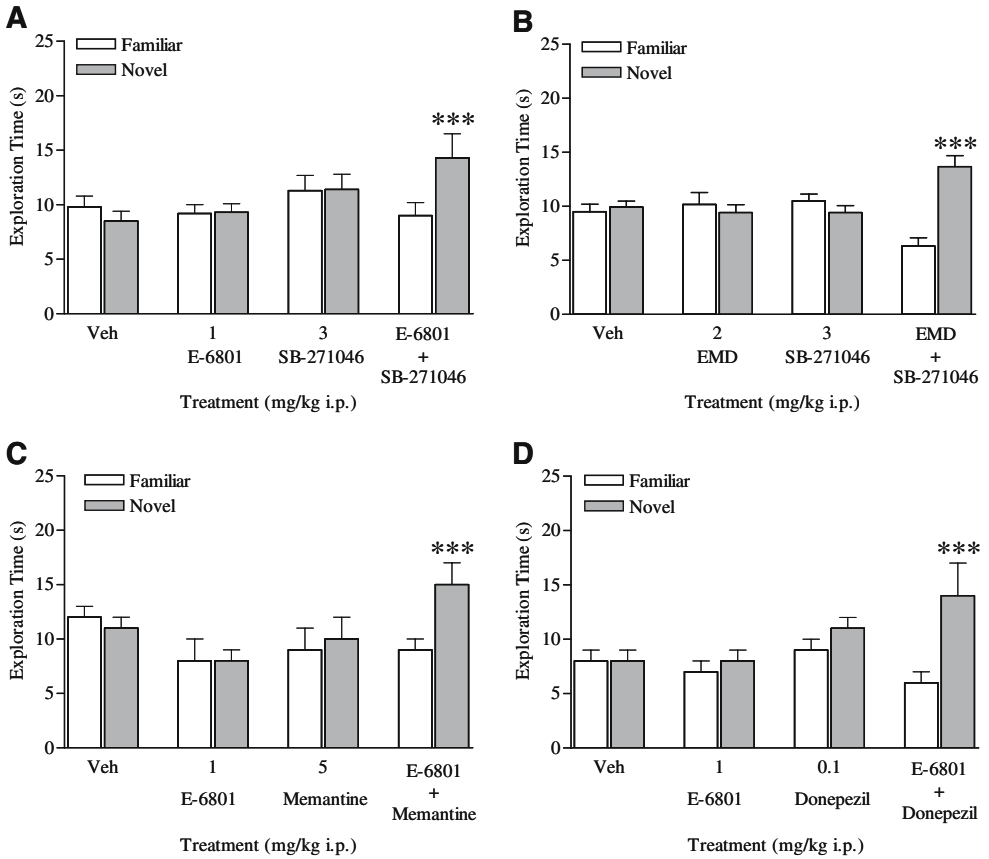

Fig. 6 Effects of sub-effective doses of E-6801 or EMD386088 co-administered with sub-effective doses of either; SB-271046 (**a** and **b**), memantine (**c**) or donepezil (**d**) on the time spent exploring (s, mean± S.E.M.) the novel versus the familiar object during the choice trial in the novel object discrimination task following a 4-h inter-trial interval. All rats in each experiment (n=12) received each compound plus vehicle treatment in a random order separated by an interval of 1 week. Following a 4-h inter-trial, interval rats treated with either vehicle or a sub-effective dose of any test compound were unable to distinguish the familiar from the novel object due to natural forgetting. However, recognition of the novel object is significantly restored by co-administration of E-6801 or EMD386088 with SB-271046 and E-6801 with memantine or donepezil. ***P<0.001 from the familiar object within the same group (Bonferroni post-test following two-way repeated-measures ANOVA)

ting and reversed a scopolamine-induced impairment of novel object discrimination in the current cognitive paradigm. Of note, one very recent study has shown that the 5-HT_6 receptor agonist WAY181187 causes a selective reversal of the extradimensional shift in the set shifting paradigm in rats accompanied by an apparently selective increase in the marker of neuronal activity, c-Fos (Burnham et al. 2010), in the prefrontal cortex although both of these effects were prevented by the antagonist, SB399885. Interestingly, several recent studies investigating models of antidepressant action have also suggested that both 5-HT_6 receptor agonists and antagonists may decrease the immobility time in tail suspension and forced-swim tests (Svenningsson et al. 2007; Wesolowska and Nikiforuk 2007) and increase the expression of genes associated with neuroplasticity in the prefrontal cortex (de Foubert et al. 2007). These studies indicate that both activation and inhibition of this receptor could evoke similar neurochemical and/or behavioural responses. In support of this suggestion, sub-effective doses of E-6801 appeared to act in a synergistic manner (rather than being antagonised) when administered with a selective 5-HT_6 receptor antagonist, SB-271046, in the novel object discrimination paradigm. While this could reflect an action of E-6801 at non-5-HT_6 receptors, it can also be explained by an action of the agonist and antagonist on distinct neuronal populations, as will be discussed in full later. Numerous systems, such as NMDA receptors, have been identified to operate most effectively within an optimal activity range, such that either excessive activation or complete inhibition impairs ultimate performance (Parsons et al. 2007). This may also be the case for the role of the 5-HT_6 receptor in cognitive processes.

In order to further characterise the pharmacological mechanism of action of E-6801, it was also given in sub-effective doses with both the non-competitive NMDA

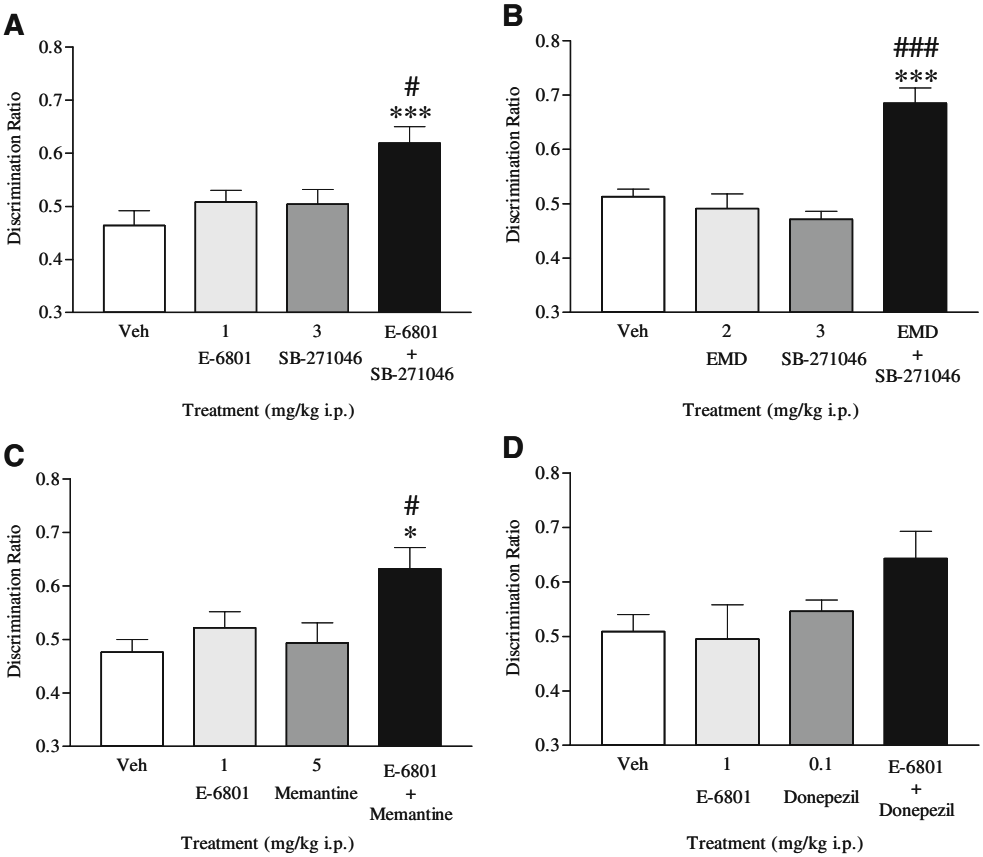

Fig. 7 By using sub-effective doses of all compounds, the synergistic effects of E-6801 or EMD386088 with either SB-271046 (**a**), memantine (**c**) or donepezil (**d**) were examined on the discrimination ratio (calculated from novel object exploration/(novel+familiar object exploration) reported in Fig. 6). Values are mean±S.E.M., $n=12$ per group. One-way repeated-measures ANOVA revealed significant effects of E-6801+SB-271046, EMD386088+SB-271046 and E-6801+memantine $^{*}P<0.05$ and $^{***}P<0.001$ from vehicle treatment, $^{\#}P<0.05$ from memantine alone and $^{\#\#\#}P<0.001$ from both EMD386088 and SB-271046 alone (Bonferroni's multiple comparison test). The discrimination ratio for E–6801 combined with donepezil just failed to reach significance ([$F_{(3,47)}=2.59$, $P=0.074$])

receptor antagonist, memantine, and the acetylcholinesterase inhibitor, donepezil, at doses which alone had no ability to reverse natural forgetting. Analogous to the response seen with a silent 5-HT_6 receptor antagonist SB-271046 (herein), E-6801 also acted in an apparently synergistic manner with both memantine and donepezil, restoring a time delay-induced deficit in object discrimination. It is noteworthy that a similar synergistic interaction has been reported to occur between sub-effective doses of 5-HT_4 partial agonist, RS-67333, and the acetylcholinesterase inhibitor, galanthaminium, restoring a time delay-induced novel object discrimination impairment (Lamirault et al. 2003b).

In vivo microdialysis studies imply that the 5-HT_6 receptor tonically inhibits acetylcholine release in the prefrontal cortex which is elevated by pre-treatment with a 5-HT_6 receptor antagonist (Hirst et al. 2006; Riemer et al. 2003; Shirazi-Southall et al. 2002). Yet, dual-labelled immunohistochemistry suggests that the 5-HT_6 receptor is not extensively co-localised with choline acetyltransferase, and immunotoxin-induced lesion of the cholinergic input to the frontal cortex with 192-IgG-saporin into the nucleus basalis magnocellularis failed to reduce 5-HT_6 receptor mRNA or protein levels, suggesting that there is little 5-HT_6 receptor expression on cortical cholinergic neurones (Marcos et al. 2006). Instead, immunohistochemical evidence suggests that the 5-HT_6 receptor may be extensively co-expressed on cortical and hippocampal GABAergic neurones (Ward and Dorsa 1996; Woolley et al. 2004). Thus, 5-HT_6 receptor effects on acetylcholine release may be mediated indirectly in these two brain areas by disinhibition of GABAergic release onto cholinergic neurones. Of note, both cholinergic and GABAergic neurones in the medial septum receive serotonergic input from the midbrain raphé nuclei (Farr et al. 1999; King et al. 2009; Leranth and Vertes 1999). A critical issue is whether the 5-HT_6 receptor populations on either or both cholinergic and GABAergic neurones are tonically activated by 5-HT, as a silent antagonist would be ineffective on a system with no tone while a full agonist would be active. Thus, a potential explanation for the comparable pro-cognitive effect of 5-HT_6 receptor agonist and antagonist compounds, reported herein, is that the receptors on cholinergic (and glutamatergic) neurones receive no serotonergic tone under normal conditions but may be directly activated by 5-HT_6 receptor agonist administration. In contrast, 5-HT_6 receptor antagonists will also enhance acetylcholine and glutamate release but do this indirectly by blockade of the tonic serotonergic input to upstream GABAergic interneurones. Destruction of medial raphé serotonergic neurones with the neurotoxin 5,7 dihydroxytryptamine prevents the pro-cognitive effects of the 5-HT_6 receptor antagonist Ro

Table 2 Total (mean±S.E.M.) exploration time (s) spent exploring both objects in the familiarisation (T_1) and choice (T_2) trials for the dose-response studies with each compound tested. For each compound, all rats received all treatments in a random order separated by an interval of 1 week. In T_1, ANOVA revealed significant main effects of E-6801 [$F(4,79)=3.87$, $P<0.0073$], SB-271046 [$F(2,22)=15.95$, $P<0.0001$], donepezil [$F(4,79)=12.58$, $P<0.0001$], and memantine [$F(4,47)=14.17$, $P<0.0001$]. $^{*}P<0.05$ and $^{***}P<0.001$ from vehicle treatment in the same group in T_1, $^{\dagger}P<0.05$ from 5 mg/kg same group in T_1, $^{\$\$}P<0.01$ from 1 mg/kg and $^{@@@}P<0.001$ from 0.1 and 0.3 mg/kg same group in T_1, $^{\ddagger\ddagger}P<0.01$ and $^{\ddagger\ddagger\ddagger}P<0.001$ from 5 mg/kg and $^{\int\int}P<0.01$ from 10 mg/kg same group in T_1 (Bonferroni's multiple comparison test). In T_2, repeated-measures ANOVA revealed a significant decrease in exploration time only in the memantine group, $^{*}P<0.05$ from vehicle, $^{\ddagger\ddagger}P<0.01$ from 5 mg/kg and $^{\int\int\int}P<0.001$ from 10 mg/kg in the same treatment group (Bonferroni's multiple comparison test). Comparing the total exploratory time in T_2 with that in T_1 in the same group of rats $^{\#}P<0.05$, $^{\#\#}P<0.01$ and $^{\#\#\#}P<0.001$ from T_1 (Student's paired *t* tests)

Compound	Dose (i.p.)	T_1 (s)	T_2 (s)
E-6801	Vehicle	41±2	27±2$^{\#}$
	1.25 mg/kg	40±2	31±2$^{\#\#}$
	2.5 mg/kg	35±2	29±2$^{\#}$
	5 mg/kg	35±2	28±2$^{\#}$
	10 mg/kg	33±2*	29±3
SB-271046	Vehicle	38±2	32±3$^{\#}$
	5 mg/kg	34±3	33±3
	10 mg/kg	22±3***,†	27±2
Ro 04-6790	Vehicle	33±3	23±2$^{\#\#\#}$
	5 mg/kg	27±3	25±3
	10 mg/kg	24±3	26±2
Donepezil	Vehicle	42±2	29±2$^{\#\#\#}$
	0.1 mg/kg	37±3	28±2$^{\#\#}$
	0.3 mg/kg	33±2	27±2$^{\#}$
	1 mg/kg	32±2*	31±3
	3 mg/kg	19±3***,$^{\$\$}$,$^{@@@}$	28±3$^{\#\#}$
Memantine	Vehicle	25±4	20±2
	5 mg/kg	21±2	15±2
	10 mg/kg	18±2	21±1
	15 mg/kg	5±1***,‡‡‡,$^{\int\int}$	9±2*,‡‡,$^{\int\int\int}$
	20 mg/kg	9±2***,‡‡	19±3$^{\#}$

04-6790, consistent with the idea that the effect of these antagonists depends on tonic 5-HT release (King et al. 2008). Consistent with this theory, two selective 5-HT_6 receptor agonists, WAY-466 (Schechter et al. 2004) and WAY-181187 (Beyer et al. 2005; Schechter et al. 2007), elevate GABA levels in corticolimbic microdialysates in rats, and the effect of the latter agonist is blocked by SB-271046. The decrease in monoamine levels produced by WAY-181187 is also blocked by local infusion of bicuculline, a $GABA_A$ receptor antagonist into the microdialysate, supporting the hypothesis that 5-HT_6 receptor stimulation may evoke GABA release which indirectly affects downstream neurotransmitter systems.

In contrast, the 5-HT_6 receptor antagonist, SB-271046, increases acetylcholine and glutamate release in cortical or hippocampal microdialysates (Dawson et al. 2000, 2001) which may also explain the synergistic interaction of E-6801 with donepezil and memantine which operate on these respective neurotransmitter systems. Interestingly, cholinesterase inhibitors appear to preferentially improve the process of acquisition, only enhancing object recognition when administered prior to and not after the first trial (Prickaerts et al. 2005), while the 5-HT_6 receptor antagonists still improve object recognition when administered after the first trial (King et al. 2004b), suggesting that their pro-cognitive effect cannot be mediated by increasing cholinergic activity alone. Consistent with the finding that SB-271046 increases glutamate release (Dawson et al. 2000, 2001), pre-treatment with a non-competitive NMDA receptor antagonist, MK-801 (0.05 mg/kg dizocilpine), impairs cognitive function (Maurice et al. 1994; Willmore et al. 2001) and prevents the 5-HT_6 receptor antagonist, Ro 04-6790, restoring time delay-induced deficits in novel object discrimination (King et al. 2004a). Taken together, these data suggest that 5-HT_6 receptor antagonists may enhance consolidation by increasing central glutamatergic neurotransmission. Supporting this proposal, it has recently been shown that the NMDA receptor antagonist MK-801

Table 3 Total (mean±S.E.M.) time (s) spent exploring both objects in the familiarisation (T_1) and choice (T_2) trials for the scopolamine-impairment reversal studies. For each compound tested, all rats received all treatments in a random order separated by an interval of 1 week. In T_1, repeated-measures ANOVA revealed a significant main effect of donepezil (0.3-mg/kg dosage group) [$F(3,47)=10.21$, $P<0.0001$]. $^{\$\$\$}P<0.001$ from scopolamine and $^{\dagger\dagger}P<0.01$ from scopolamine+donepezil (0.3 mg/kg), Bonferroni's multiple comparison test. In T_2, ANOVA revealed a significant main effect of scopolamine treatment (0.3-mg/kg dosage group) [$F(3,47)=5.97$, $P<0.0023$], $^{**}P<0.01$ from vehicle, Bonferroni's multiple comparison test. Furthermore, $^{\#}P<0.05$, $^{\#\#}P<0.01$ and $^{\#\#\#}P<0.001$ from T_1 for the same dose and treatment group (Student's paired *t* tests)

Study	Injection(s) (i.p.)	T_1 (s)	T_2 (s)
Scopolamine+E-6801	Vehicle	25±2	34±3
	Scopolamine (0.5 mg/kg)	30±2	27±3
	E-6801 (2.5 mg/kg)	39±2	34±3
	Scopolamine+E-6801	28±3	29±4
Scopolamine+E-6801	Vehicle	39±3	34±2$^{\#}$
	Scopolamine (0.5 mg/kg)	32±3	25±2$^{\#\#}$
	E-6801 (5 mg/kg)	42±3	32±3$^{\#\#}$
	Scopolamine+E-6801	33±3	33±3
Scopolamine+donepezil	Vehicle	38±3	34±2
	Scopolamine (0.5 mg/kg)	28±2	21±3$^{\#}$**
	Donepezil (0.3 mg/kg)	47±3$^{\$\$\$\ \dagger\dagger}$	28±3$^{\#\#\#}$
	Scopolamine+donepezil	33±2	29±2$^{\#}$
Scopolamine + Donepezil	Vehicle	39±2	29±3$^{\#\#}$
	Scopolamine (0.5 mg/kg)	33±4	26±3$^{\#}$
	Donepezil (1 mg/kg)	45±3	31±2$^{\#\#}$
	Scopolamine+donepezil	34±4	27±4$^{\#}$

impairs retention of a novel recognition task when given either before or immediately after training, implicating NMDA receptors in this process (de Lima et al. 2005). However, these results contrast to the finding reported herein showing that low doses of memantine, a non-competitive NMDA receptor antagonist licensed for the treatment of Alzheimer's disease under the trade names Axura®, Akatinol®, Namenda® and Ebixa® can reverse natural forgetting in the novel object discrimination paradigm. Previous studies in rodents have reported that memantine improves spatial learning in the Morris water maze task (Minkeviciene et al. 2004; Zoladz et al. 2006),

Table 4 Total (mean±S.E.M.) time (s) spent exploring both objects in the familiarisation (T_1) and choice (T_2) trials for the synergy interaction studies. For each compound tested, all rats received all treatments in a random order separated by an interval of 1 week. In T_1, ANOVA revealed significant main effects of E-6801+memantine [$F(3,47)=3.54$, $P<0.0251$], $^{*}P<0.05$ from vehicle, Bonferroni's multiple comparison test. For T_2 exploration $^{\#}P<0.05$, $^{\#\#}P<0.01$ and $^{\#\#\#}P<0.001$ from T_1 at the same dose and in the same treatment group (Student's paired *t* tests)

Study	Injection(s) (i.p.)	T_1 (s)	T_2 (s)
E-6801+SB-271046	Vehicle	44±3	19±2$^{\#\#\#}$
	E-6801 (1 mg/kg)	32±4	25±5
	SB-271046 (5 mg/kg)	33±5	23±3
	E-6801+SB-271046	39±4	22±3$^{\#\#\#}$
E-6801+memantine	Vehicle	38±2	32±3
	E-6801 (1 mg/kg)	34±3	33±3$^{\#}$
	Memantine (5 mg/kg)	22±3	27±2$^{\#}$
	E-6801+memantine	18±3*	24±3
E-6801+donepezil	Vehicle	33±3	17±1$^{\#\#}$
	E-6801 (1 mg/kg)	24±3	16±2$^{\#\#}$
	Donepezil (0.1 mg/kg)	31±4	20±2$^{\#\#}$
	E-6801+donepezil	29±3	20±3$^{\#}$

consistent with the current findings. While the reason for the disparity in the cognitive effects of the two NMDA receptor antagonists, MK801 and memantine, is unclear, it may reflect structural differences or kinetics in NMDA receptor channel blockade but the additional pharmacological interaction of memantine with 5-HT_3 and/or nicotinic acetylcholine receptors (nAChR) cannot be excluded (Johnson and Kotermanski 2006). Nonetheless, it further validates the predictive utility of the novel object discrimination task to detect the pro-cognitive effects of compounds with widely divergent pharmacological mechanisms.

E-6801 displays sub-nanomolar affinity for 5-HT_6 receptors and has no relevant affinity for a total of 138 receptors, transporters, ion channels assayed, and no activity on acetylcholinesterase or another 39 enzymes. However, E-6801 displays affinity for the 5-HT_{1A} receptor (K_i ranging from 13 to 96 nM) and acts as a weak agonist at the 5-HT_{1A} receptor, with an EC_{50} of 320 nM (current GTPγS functional assay). Interestingly, 5-HT_{1A} receptor antagonists, such as Lecozotan, appear to improve memory and enhance hippocampal acetylcholine and glutamate release in preclinical studies (Schechter et al. 2005). Paradoxically, low doses of the 5-HT_{1A} receptor agonist, 8-OH-DPAT, also reverses a cannabinoid-induced impairment of spatial memory and its reduction in hippocampal acetylcholine release (Inui et al. 2004). Thus, analogous to the present study of 5-HT_6 receptor function, both 5-HT_{1A} receptor agonist and antagonists can improve cognitive function, including recognition memory (Pitsikas et al. 2005). However, the affinity of E-6801 is 20- to 165-fold higher for 5-HT_6 (K_i=0.58 nM) than for 5-HT_{1A}, and its potency as an agonist is over 100-fold higher at the 5-HT_6 (EC_{50}=0.3 nM, cAMP formation (Romero et al. 2006)) than at the 5-HT_{1A} receptor. Therefore, one possibility is that the 5-HT_{1A} receptor activity of E-6801 could contribute to the restoration of memory seen in the current paradigm. In a rodent behavioural test battery scoring components of the serotonin syndrome known to be mediated by 5-HT_{1A} receptor activation, E-6801 did not produce hypothermia, lower lip retraction, flat body posture or reciprocal forepaw treading, up to a dose of 80 mg/kg i.p. (data not shown), suggesting that this is not a likely explanation for the current cognitive effects. The recent report of 5-HT_6 receptor interaction with Fyn tyrosine kinase, its co-localisation within the cell and co-expression in the hippocampus, cortex and hypothalamus (Yun et al. 2007), along with previous studies suggesting a role of Fyn in fearfulness and spatial memory (Grant et al. 1992; Miyakawa et al. 1996) and modulation of the GABAergic (Boehm et al. 2004) and glutamatergic systems (Grant 1996; Nada et al. 2003) may also be a mechanism by which 5-HT_6 receptors could be linked to cognitive function. Studies are clearly required to see if both agonists and antagonists modify 5-HT_6 receptor interaction with Fyn tyrosine kinase.

In conclusion, this study reports for the first time that 5-HT_6 receptor ligands, with agonist properties in vitro, enhance recognition memory, both when administered alone and in low dose combinations with either SB-271046, donepezil or memantine in a novel object discrimination task in rats. Furthermore, these pro-cognitive effects appear analogous with those previously reported with 5-HT_6 receptor antagonists. The mechanism for this apparently paradoxical effect of 5-HT_6 receptor agonists and antagonists could be that the agonist activates 5-HT_6 receptors located directly on glutamatergic and/or cholinergic neurones which receive a low tonic serotonergic input under normal conditions. In contrast, 5-HT_6 receptor antagonists may predominantly attenuate active serotonergic input to upstream inhibitory GABAergic neurones which disinhibit glutamate and/or acetylcholine release. Thus, both 5-HT_6 agonist and antagonist compounds may elevate glutamate and/or acetylcholine release resulting in pro-cognitive effects. The involvement of other receptors, such as the interaction of the 5-HT_6 receptor agonist with 5-HT_{1A} receptors cannot however be ruled out. Further investigation such as microdialysis measurement of acetylcholine or glutamate release and examining the impact of serotonergic lesions (which should only attenuate the action of 5-HT_6 receptor antagonists if these alone depend on serotonergic tone) on the pro-cognitive effects of 5-HT_6 receptor agonist and antagonists would be worthwhile.

The increasing evidence of the pro-cognitive actions of 5-HT_6 receptor compounds in a variety of pre-clinical models warrants evaluation of the potential synergistic effects of these compounds with existing therapies used to treat cognitive deficits seen in Alzheimer's disease, depression and schizophrenia. In particular, since many atypical antipsychotic drugs induce weight gain, the recent reports that 5-HT_6 receptor compounds (both agonists and antagonists by an unknown mechanism) reduce body weight in diet-induced obesity (Fisas et al. 2006; Holenz et al. 2006; Woolley et al. 2004) make this a particularly exciting area for clinical studies. Future preclinical studies in animal models of the cognitive dysfunction seen in schizophrenia, such as rats reared in social isolation from weaning (Fone and Porkess 2008), would be beneficial to establish if 5-HT_6 receptor agonists or antagonists are most effective at reversing these deficits.

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