GSK189254, a Novel $H_3$ Receptor Antagonist That Binds to Histamine $H_3$ Receptors in Alzheimer’s Disease Brain and Improves Cognitive Performance in Preclinical Models


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ABSTRACT

6-[(3-Cyclobutyl-2,3,4,5-tetrahydro-1H-3-benzazepin-7-yl)oxy]-N-methyl-3-pyridinecarboxamide hydrochloride (GSK189254) is a novel histamine $H_3$ receptor antagonist with high affinity for human (P$_K_i$ = 9.59–9.90) and rat (P$_K_i$ = 8.51–9.17) $H_3$ receptors. GSK189254 is >10,000-fold selective for human $H_3$ receptors versus other targets tested, and it exhibited potent functional antagonism (P$_A_2$ = 9.06 versus agonist-induced changes in cAMP) and inverse agonism (log P$_C_{50}$ = 8.20 versus basal guanosine 5′-O-[3-35S]thiotriphosphate binding) at the human recombinant $H_3$ receptor. In vitro autoradiography demonstrated specific [3H]GSK189254 binding in rat and human brain areas, including cortex and hippocampus. In addition, dense $H_3$ binding was detected in medial temporal cortex samples from severe cases of Alzheimer’s disease, suggesting for the first time that $H_3$ receptors are preserved in late-stage disease. After oral administration, GSK189254 inhibited cortical ex vivo R-(-)-α-methylhistamine (0.3–3 mg/kg p.o.) increased the release of acetylcholine, noradrenaline, and dopamine in the anterior cingulate cortex and acetylcholine in the dorsal hippocampus. Functional antagonism of central $H_3$ receptors was demonstrated by blockade of R-α-methylhistamine-induced diisoprogens in rats (ID$_{50}$ = 0.03 mg/kg p.o.). GSK189254 significantly improved performance in rats in diverse cognition paradigms, including passive avoidance (1 and 3 mg/kg p.o.), water maze (1 and 3 mg/kg p.o.), object recognition (0.3 and 1 mg/kg p.o.), and attentional set shift (1 mg/kg p.o.). These data suggest that GSK189254 may have therapeutic potential for the symptomatic treatment of dementia in Alzheimer’s disease and other cognitive disorders.

Progressive decline in cognitive performance is a key characteristic of Alzheimer’s disease (AD) and related dementias, and improving cognitive function in these diseases represents a complex challenge, given the involvement of numerous neurotransmitter systems and brain regions (Corey-Bloom, 2002). Current therapies, such as cholinesterase inhibitors, provide only minimal benefit to a subset of patients and for a limited period, so a number of alternative approaches have been explored. One such approach is targeting histamine H3 receptors, which are present in the central nervous system and play a role in cognitive function. Histamine H3 receptor antagonists may provide a novel therapeutic target for improving cognitive performance in AD and related dementias.

ABBREVIATIONS: AD, Alzheimer’s disease; $H_3$, histamine $H_3$; 5-HT, 5-hydroxytryptamine; MK-801, 5-(N-(4-(1-methyl-4-yl)-phenethyl)phenoxy)phenylcyclopropyl)methanone; A-317920, N-(1R)-2-[4-(4-(cyclopropylcarbonyl)phenoxy)propyl]-1-piperazinyl)-1-methyl-2-ox-ethyl]-2-furamidine; 24587, 2-[4-(3-piperidin-1-yl-propoxy)-benzyl]-piperidine; SCH 79687, N-(3,5-dichlorophenyl)N’-[4-(1H-imidazol-4-yl)methyl]phenyl]-methyl]-urea; NNC 38-1049, 1-cyclopentyl-4-(4-(4-chlorophenyl)-4-oxobutanoyl)piperazine.
symptomatic strategies are being pursued, including the development of selective histamine H3 receptor antagonists (Johnson et al., 2004).

The H3 receptor is one of four receptor subtypes (H1, H2, H3, and H4) that mediate the diverse biological effects of the neurotransmitter histamine (Brown et al., 2001). H3 receptors are widely expressed in the mammalian brain, particularly in areas involved in cognitive processes and arousal, such as the cerebral cortex, hippocampus, basal ganglia, and hypothalamus (Martinez-Mir et al., 1990; Pollard et al., 1993). Activation of H3 autoreceptors results in the inhibition of histamine synthesis and release from histaminergic neurons (Arrang et al., 1983), whereas activation of H3 heteroreceptors leads to the inhibition of release of other neurotransmitters such as acetylcholine, noradrenaline, dopamine, and 5-HT from nonhistaminergic neurons (Blandina et al., 1996; Schlicker and Kathmann, 1998; Brown et al., 2001). Conversely, blockade of H3 receptors with selective antagonists can increase the release of neurotransmitters involved in cognitive processes (Fox et al., 2005).

Selective H3 antagonists have been shown to improve performance in a diverse range of rodent cognition paradigms, including object recognition, olfactory recognition, water maze, radial maze, and passive avoidance, with most pronounced effects being observed in models where a cognitive deficit is present such as in aged animals or following a pharmacological challenge with agents such as scopolamine or MK-801 (Hancock and Fox, 2004; Witkin and Nelson, 2004). H3 antagonists can also increase wakefulness in preclinical studies, consistent with the pivotal role of histamine in the sleep-wake cycle (Brown et al., 2001). These preclinical studies have generated considerable interest in the development of H3 antagonists as novel treatments for cognitive deficits in conditions such as Alzheimer’s disease, other dementias, mild cognitive impairment, and schizophrenia as well as for disorders of sleep and attention such as narcolepsy and attention deficit hyperactivity disorder (Passani et al., 2004).

First generation imidazole-based H3 antagonists, such as thioperamide, clobenpropit, and ciproxifan, were discovered several years ago before the molecular characterization of the H3 receptor (Celanire et al., 2005). However, these compounds proved to be undevelopable as therapeutic agents for humans due to a number of liabilities, including cytochrome P450 inhibition, low affinity for human compared with rat H3 receptors, lack of selectivity, or suboptimal brain penetration. Interest in H3 receptor antagonists as potential therapeutic agents was re-ignited following the cloning of the H3 receptor in 1999 (Lovenberg et al., 1999) and the subsequent discovery of several nonimidazole-based compounds (Celanire et al., 2005; Leurs et al., 2005).

The detailed in vitro and in vivo pharmacological profile for some of these more recent nonimidazole H3 antagonists has been reported, including compounds from Abbott Laboratories such as nonimidazole ABT-239 (Esbenshade et al., 2005; Fox et al., 2005), A-304121, and A-317920 (Esbenshade et al., 2003; Fox et al., 2003), which show proisocognitive effects in a number of rodent models. Other H3 antagonists such as JNJ-5207852 (Barbier et al., 2004), NNC 38-1049, and SCH 79687 have been shown to exert wake promoting effects, antiobesity properties, and nasal decongestant activity (when dosed in combination with an H1 antagonist), respectively (Celanire et al., 2005). A number of nonimidazole H3 antagonists are currently in clinical trials, but to date no efficacy data have been reported in patient populations. In the late 1990s, the imidazole-based compound GT-2331 (Cipraливant, Perceptin) was the first H3 antagonist to reach phase I clinical trials, but its further development has not been reported (Celanire et al., 2005).

In the current study, we describe the in vitro and in vivo pharmacological properties of GSK189254 (Fig. 1) (Bamford et al., 2004), a structurally novel, highly potent, and selective nonimidazole H3 receptor antagonist that exhibits efficacy across a broad range of rodent cognition paradigms. Preliminary data have been presented previously (Wilson, 2005; Medhurst, 2006).

Materials and Methods

Animals. All experimental procedures involving animals (except passive avoidance and water maze studies) were conducted in compliance with the Home Office Guidance on the operation of the Animals (Scientific Procedures) Act 1986 under the authority granted in personal and project licenses, and procedures were reviewed and approved by the GlaxoSmithKline Procedures Review Panel. Passive avoidance and water maze studies were approved by the Animal Research Ethics Committee of University College Dublin, and these studies were carried out under license issued by the Irish Department of Health.

Drugs. GSK189254 and ABT-239 were synthesized at GlaxoSmithKline (Harlow, UK). R-α-Methylhistamine, imetit, thioperamide, and clobenpropit (Celanire et al., 2005) were obtained from Tocris Cookson Inc. (Bristol, UK). All tissue culture media, supplements, and other chemicals were purchased from Invitrogen (Paisley, UK) or Sigma-Aldrich (Gillingham, UK) unless otherwise stated.

H3 Receptor Cloning and Preparation of Membranes. H3 receptors were cloned by polymerase chain reaction from human, rat, and mouse brain cDNAs. Translated protein sequences from these clones were identical to previously published sequences for human (Nakamura et al., 2000), rat (Lovenberg et al., 2000), and mouse (Chen et al., 2003) H3 receptors.

Human embryonic kidney-293 cells stably expressing the macrophage scavenger receptor class II (HEK-293-MSR-II) were maintained in minimum essential medium supplemented with Earle’s salts, 2 mM l-glutamine, 1% nonessential amino acids, 400 μg/ml Geneticin (G-418; Invitrogen), and 10% fetal bovine serum at 37°C, 5% CO2 in a humidified environment. Exponentially growing cells were transiently transfected with human, rat, or mouse recombinant H3 receptor cDNA by using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions, and then they were incubated under normal growth conditions for 24 h. Cells were harvested in phosphate-buffered salme (PBS) and pelleted by centrifugation at 200g for 5 min at room temperature. Then, the supernatant was discarded, and the pellets were stored at −80°C before membrane preparation. For preparation of membranes, cell pellets were homogenized in ice-cold 50 mM Tris-HCl buffer, pH 7.7, at 25°C for approximately 10 s using an Ultra-Turrax homogenizer (Jankel and Kunkel, Staufen, Germany). The homogenates were centrifuged at 50,000g for 20 min at 4°C, and the resulting pellet was rehomogenized and centrifuged as described above. The membranes were

\[ \text{Fig. 1. Chemical structure of GSK189254.} \]
finally resuspended in the same buffer at a concentration of approximately 4 μg protein/ml, and they were stored at −80°C until use. Cerebral cortical tissues from human (three nonidentifiable individuals aged 17 to 93 years, whose cause of death was non-neurological, from Peterborough Hospital, Peterborough, UK; approved by a local ethics committee), rat (males, Sprague-Dawley, 200–250 g; Charles River, Margate, Kent, UK), mouse (CD1, 25–40 g; Charles River), dog (beagle, 10–15 kg; 20 months old; bred by GlaxoSmithKline), or pig (Yorkshire/Landrace, 40 kg) were homogenized, and membranes were prepared as described above for the HEK-293-MSR-II cells, with the inclusion of an additional wash step following incubation of the homogenate at 37°C for 20 min after the first centrifugation.

Radioligand Binding Assays. H₂ receptor binding studies were carried out on membranes derived from cerebral cortical tissues or HEK-293-MSR-II cells transiently transfected with H₂ receptors (see above) in 50 mM Tris-HCl, pH 7.7, at 25°C-containing 5 mM EDTA. Assays consisted of 50 μl of displacing compound or buffer, 400 μl of membrane suspension (corresponding to approximately 15 μg of protein/well for the recombinant cells and 50 μg of protein/well for the brain tissue), and 50 μl of R-(−)-α-methyl-(imidazole-2,5-n)-[3H]histamine dihydrochloride (100 nM; [3H]-α-methylhistamine; specific activity, 24 Ci/μmol; GE Healthcare, Little Chalfont, Buckinghamshire, UK).

In competition binding experiments, 10 concentrations of the competing ligands were tested (concentration range, 0.03 nM–1 μM), with a final [3H]-α-methylhistamine concentration of 2 nM. Nonspecific bindings with [3H]-α-methylhistamine were for 45 min at 30°C. Nonspecific binding was determined in the presence of 10 μM imetit. The experiments were terminated by rapid filtration through Whatman GF/B filters (Whatman, Maidstone, UK) (presoaked in 0.3% (v/v) polyethyleneimine), and then the filters were washed through with 4 × 5 ml of ice-cold buffer containing 50 mM Tris-HCl, pH 7.7, at 25°C, and 5 mM MgCl₂. Filters were dried and added to vials each containing 4 ml of Ultima Gold MV scintillation fluid (Hewlett Packard, Palo Alto, CA). Radioactivity was determined by liquid scintillation spectrometry using a Packard Tri-Carb 2500TR liquid scintillation counter (PerkinElmer Life and Analytical Sciences, Boston, MA). Protein concentrations were determined using the Bradford protein assay method (Bio-Rad Protein Assay kit; Bio-Rad, York, UK) with bovine serum albumin as a standard.

[3H]GSK189254 (specific activity, 81 Ci/mmol) was prepared through a contract with GE Healthcare. Saturation binding experiments in human and rat cerebral cortex membranes were performed at concentrations of [3H]GSK189254 between 0.03 and 10 nM. Nonspecific binding was determined in the presence of 10 μM imetit.

cAMP Accumulation Assays. Human embryonic kidney 293 cells stably expressing G protein Goα (HEK-293-Goα) were maintained in similar conditions to HEK-293-MSR-II cells but without 1% nonessential amino acids. Exponentially growing cells were transduced with BacMam virus (Condreay et al., 1999) encoding the human recombinant H₂ receptor as follows. Cells were detached from flask with PBS and collected by centrifugation at 200g for 5 min at room temperature. The cells were then resuspended in growth media containing virus at a multiplicity of infection of 50, replaced, and then incubated under normal growth conditions for 24 h. Cyclic AMP levels in cells were determined by radioimmunoassay (FlashPlate adenyl cyclase activation assay system; PerkinElmer, Beaconsfield, UK). Cells were washed once with PBS and centrifuged at 200g for 5 min at room temperature. The supernatant was discarded, and the pellet was resuspended in manufacturer's stimulation buffer and washed by centrifugation (as described above). The pellet was then resuspended in stimulation buffer containing 3-isobutyl-1-methyloxanthine at a cell density of 2 million cells/ml, and 100,000 cells were added to the appropriate wells of the FlashPlates. The wells contained 10 μl forskolin together with a concentration range of the H₂ agonist imetit (1 μM–100 μM) in the absence or presence of GSK189254 (3, 10, 30, or 100 nM). Plates were incubated for 30 min at 37°C, before the addition of the manufacturer's detection buffer containing 125I-cAMP tracer (0.16 μCi/ml) to the wells. Plates were covered and left for 24 h at room temperature before counting on a Packard TopCount scintillation counter.

Guanosine 5′-O-(3′-[3H]thio)triphosphate Binding Assays. GTPγS binding assays were used to investigate potential inverse agonist properties of GSK189254 in HEK-293-Goα cells transduced with human H₂ receptor-encoding BacMam virus as described above but using a multiplicity of infection of 100. After overnight incubation, cells were collected into 10 ml of PBS and spun at 200g for 5 min. After removal of the supernatant, the pellet was resuspended and homogenized in 20 mM HEPES, pH 7.4, containing 3 mM MgCl₂ and 100 mM NaCl, centrifuged at 50,000g for 20 min, and then homogenized and centrifuged again. The membrane pellet was then resuspended and assayed for protein concentration.

Cell membranes were diluted to ~1 mg/ml in assay buffer (20 mM HEPES, 100 mM NaCl, and 10 mM MgCl₂, pH 7.4) and incubated with wheat germ agglutinin scintillation proximity assay beads (GE Healthcare) for 45 min, after which 40 μM GDP was added. Various concentrations of GSK189254 (0.3 nM–10 μM in half-log increments) were added to a 96-well plate along with 10 μl of assay buffer. Nonspecific binding was determined by the inclusion of 0.6 mM GTPγS. Then, 60 μl (~55 μg protein/well) of membranes/wheat germ agglutinin scintillation proximity assay beads/GDP mix was added to each well, and the plate was incubated on an orbital shaker for 30 min at room temperature. [35S]GTPγS (0.3 nM) was then added to each well, the plate was incubated on the shaker for a further 30 min, and bound [35S]GTPγS was determined by scintillation counting on a 1450 Microbeta Trilux counter (PerkinElmer Wallac, Gaithersburg, MD).

In Vitro H₂ Receptor Autoradiography. Autoradiography studies were carried out based on previous methods (Roberts et al., 2004), with human tissues obtained following approval of local ethics committees and where the cause of death was non-neurological. Frozen sections (20 μm) of human cerebral cortex (female, aged 93; obtained from Institute of Neurology, London, UK), hippocampus (female, aged 76 diagnosed with AD for 5 years; obtained from Peterborough Hospital), medial temporal gyrus (control, female, aged 52; AD Braak stages I–VI, male or female, ages 72–90; obtained from the Netherlands Brain Bank, Amsterdam, The Netherlands), or rat (Sprague-Dawley; Charles River) whole brain were thaw-mounted on to gelatin-coated slides, and slides were stored at −80°C until time of assay. Sections were incubated in assay buffer (50 mM Tris-HCl, pH 7.7, and 5 mM EDTA) containing 1 nM [3H]GSK189254 for 60 min at room temperature (22°C). On anatomically adjacent sections, nonspecific binding was determined in the presence of 10 μM imetit. After incubation, all sections were rinsed five times for 3 min at 4°C in Tris-HCl buffer with the addition of 5 mM MgCl₂. The sections were then quickly dipped in distilled water at 4°C to remove buffer salts, and they were dried in a stream of cool air. Once dried, the sections were analyzed by digital autoradiography using a Beta-Imager 2000 instrument (Biospace, Paris, France). Some sections were later emulsion dipped in LM-1 emulsion to permit cellular resolution (Roberts et al., 2004). Adjacent sections were also stained with cresyl fast violet to allow for anatomical orientation. Alzheimer’s disease plaque pathology was confirmed in adjacent sections using monoclonal E8 (1:1000) antibody raised against the 13 to 27 fragment of β-amyloid as described previously (Howlett et al., 2004).

Ex Vivo Binding Assays. Ex vivo binding studies were carried out to determine CNS H₂ receptor occupancy. Adult male rats (Lister Hooded, 200–250 g; Charles River) received vehicle [1% (w/v) aqueous methylcellulose] or GSK189254 (0.1, 0.3, 1, or 3 mg/kg) by oral gavage (n = 3 per group). Animals were sacrificed 0.5, 1, 2, 4, 6, 8, or 12 h following oral dosing. In other studies, male CD rats (270–350 g; Charles River) were administered GSK189254 (0.3, 1, and 10 mg/kg), twice daily by oral gavage (n = 3 per group), and sacrificed 6 h after the first daily dose on days 1, 4, and 8. In addition, other male rats (Sprague-Dawley, 87–164 g; Charles River) were admin-
istered single doses of GSK189254 (5, 20, and 50 mg/kg) and sacrificed 3 h postdose (n = 6). Terminal blood samples were collected and brains rapidly removed in all studies. Cerebral cortex tissue was dissected from half of each brain (for ex vivo binding), and the other half of the brain was kept for pharmacokinetic analysis of GSK189254 brain concentrations. All dissected tissue samples were snap-frozen in liquid nitrogen and stored at −80°C until use. The tissues were rapidly thawed and homogenized in approximately 30 volumes of ice-cold buffer containing 50 mM Tris-HCl, pH 7.7, at 25°C and 5 mM EDTA. This crude homogenate (600–800 µg/well) was then used to measure H3 receptor binding as described previously with [3H]R-α-methylhistamine as radioligand. Blood and brain samples were extracted using protein precipitation. Rat brains were homogenized with sterile water [50/50 (v/v)]. Diluted blood (50 µl + 50 µl of water) or brain homogenate (50 µl of water) samples were extracted using protein precipitation with acetonitrile containing an internal standard. The samples were mixed and centrifuged, and an aliquot of the supernatant was analyzed for GSK189254 concentrations using a method based on protein precipitation with acetonitrile containing an internal standard. The samples were mixed and centrifuged, and an aliquot of the supernatant was analyzed for GSK189254 by high-performance liquid chromatography (HPLC)-tandem mass spectrometry by using a heat-assisted electro-spray interface in positive ion mode. Samples were assayed against calibration curves prepared in the appropriate matrix within the range 0.014 to 12.27 µM (0.005–5.0 µg/ml).

Pharmacokinetic Studies in the Rat. Pharmacokinetic studies with GSK189254 were conducted in conscious male Sprague-Dawley rats (257–304 g) using a crossover design with a period of at least 2 days between dose administrations, with each animal receiving GSK189254 on both dosing occasions. Animals were prepared with a cannula inserted via the femoral vein into the vena cava (for compound administration) and via the jugular vein (for blood sampling). The cannulae were exteriorized at the back of the neck, and the animals were placed in cages with tethers and housed in plastic-bottom cages in facilities with a 12 h dark/light cycle. Each animal had free access to food and water. Following postoperative recovery, animals received an intravenous infusion of GSK189254 (n = 3) administered at a nominal dose level of 1 mg of free base/kg for 1 h via the femoral vein cannula (10 ml/h/kg). GSK189254 was dissolved in 0.9% (w/v) saline at a target concentration of 0.1 mg free base/ml and filtered with a 0.22-mm Millipore GF filter (Millipore, Billerica, MA) before administration. After a washout of at least 2 days, the same rats received a single oral administration of GSK189254 by gastric gavage to achieve a target dose of 2 mg of free base/kg. GSK189254 was formulated in 1% (v/v) aqueous methylcellulose at a target concentration of 0.4 mg free base/ml. Serial blood samples were collected from the jugular vein cannula predose and at intervals up to 12 h after the start of the intravenous infusion and up to 3 h after oral dose into tubes containing potassium EDTA as anticoagulant. Diluted samples (1:1 with deionized water) were analyzed for GSK189254 concentrations using a method based on protein precipitation and HPLC-tandem mass spectrometry analysis. The lower limit of quantification was 0.014 µM (0.005 µg/ml). Noncompartmental pharmacokinetic parameters were obtained from the blood concentration-time curves using WinNonlin Professional version 3.3 (Pharsight, Mountain View, CA). Oral bioavailability was calculated as the ratio of the area under the blood concentration versus time curve after oral and intravenous doses after normalizing for dose.

Immunohistochemistry-c-Fos Activation. c-Fos immunohistochemistry was used as an indicator of in vivo neuronal activation using methodology described previously (Jennings et al., 2006). Briefly, habituated male CD rats (250–300 g, n = 4 per group; Charles River) were administered GSK189254 (0.3, 1.0, or 3.0 mg/kg) or vehicle (1% aqueous methylcellulose) by oral gavage. Two hours after dosing, rats were transcardially perfused with freshly depolymerized 4% paraformaldehyde in phosphate-buffered saline (PBS, pH 7.4, at 4°C), and their brains were removed. After postfixation in paraformaldehyde for 4 h at 4°C and cryoprotection (30% sucrose in PBS overnight at 4°C), 50-µm coronal cryosections were prepared and stored free-floating in immunobuffer (IB) as described previously (Jennings et al., 2006). After quenching endogenous peroxidase activity (1% hydrogen peroxide in IB for 30 min), washing (IB 2 × 10 min), and blocking (2% serum in IB for 45 min), sections were incubated (4 h at 4°C with gentle agitation) with rabbit-raised primary c-Fos antiseraum (1:2,000; Oncogene, Edenbridge, Kent, UK). Washed (IB 3 × 10 min) sections were then processed for peroxidase immunostaining using an ATP-binding cassette amplification system (Vector Laboratories, Peterborough, UK) according to manufacturer’s instructions. Sections were then mounted onto Superfrost polished slides (BDH, Poole, UK), air-dried, and covered with DPX mountant (BDH). Immunohistochemistry was performed on digitized images captured at a final magnification of 150× using Microcomputer Imaging Device Elite software (Imaging Research Inc., St. Catharines, ON, Canada).

In Vivo Microdialysis. Microdialysis studies were carried out in male CD rats (250–275 g; Charles River) based on methodology described in detail previously (Hughes and Dawson, 2004). Animals were anesthetized with isoflurane and microdialysis guide cannulae (CMA 11; CMA Microdialysis, Sunderland, UK) were implanted for sampling from the anterior cingulate subregion of the medial prefrontal cortex and the dorsal hippocampus, using implantation coordinates of AP, +2.7 mm, ML, −1.6 mm with a 12° angle, DV, −2.0 mm, and AP, +3.5 mm, ML, −2.0 mm, DV −2.0 mm, respectively (Paxinos and Watson, 1986). Rats were monitored until they regained their righting reflex, and they received at least 5 days postoperative care.

Rats were moved into microdialysis cages 18 h before the start of the dialysis experiment to allow for overnight acclimatization. On the following morning, pins were removed from guide cannulae and replaced with microdialysis probes (CMA 11, 10/02; Linton Instruments, Pal-grave, Norfolk, UK; 2-mm active membrane). Probes were perfused with artificial cerebral spinal fluid containing 145.0 mM NaCl, 2.7 mM KCl, 1.0 mM MgCl2, 1.2 mM CaCl2, and 2.0 mM Na2HPO4 at 1 µl/min. Following a 2-h equilibration time, four basal microdialysate samples (30 min each) were collected. Animals (n = 6–9 per group) were then dosed acutely with GSK189254 (0.3, 1.0, and 3.0 mg/kg; 2 µl/kg p.o.) or vehicle (1% methylcellulose; 1 µl/kg p.o.), and microdialysate samples were collected every 30 min for 4 h postdose. At the end of each experimental day, animals were returned to their home cage and reused in a randomized crossover design, allowing at least 7-day drug washout before subsequent use. After the completion of the final microdialysis experiment, animals were euthanized, and their brains were removed and stored in formalin solution for probe placement verification.

Microdialysate samples were analyzed for serotonin, dopamine, and noradrenaline using a HPLC system and electrochemical detection as described in detail previously (Lacroix et al., 2004). Separations were performed using a Capcell Pak SCX UG80 5-µm column (150 × 1.5 mm i.d.; Phenomenex, Macclesfield, UK). Mobile phase was 80:20 (13 mM Na2HPO4, 87 mM NaH2PO4, 5 mM NaCl, and 0.1 mM EDTA Na2, pH 6.0/0.1 M MeOH (methanol 205, super purity; Romil, Cambridge, UK). The eluent was degassed in-line with a DEC-103 in-line degasser (Kontron, Tokyo, Japan) and delivered at a rate of 0.200 ml/min until noradrenaline and dopamine had eluted, and then flow rate was increased to 0.400 ml/min until serotonin was eluted. Eluates were detected at an oxidation potential of 500 mV versus in situ Ag/AgCl reference electrode using a Decade electrochemical detector (Antec, Leiden, Netherlands). Components were identified by retention time, and then they were quantified against a calibration curve generated from standard solutions containing noradrenaline, dopamine, and serotonin.

Concurrently, microdialysate samples were analyzed for acetylcholine using a binary HPLC system. Separation of acetylcholine was performed on a Primesep 200 5-µm HPLC column (2.1 × 150 mm; Hichrom Ltd., Berkshire, UK) thermostatted at 35°C. Mobile phase A was composed of 0.04% (v/v) tetrafluoroacetic acid in water. Mobile phase B was composed of 0.08% (v/v) tetrafluoroacetic acid in acetonitrile. Gradient was run from 100% A to 100% B over 4 min.
Eluates were detected using a Sciex API 4000 triple quadrupole mass spectrometer with a turboionspray source (Applied Biosystems, Warrington, UK) operated in positive ion mode and monitored with molecular reaction monitoring, with parent ion m/z 146.1 and product ion m/z 87.1. Data were acquired and processed using Analyst version 1.3.1 software (Applied Biosystems). Components were identified by molecular reaction monitoring, and retention time was then quantified against a calibration curve generated from standard solutions containing acetylcholine.

**In Vivo Functional Activity: Dipsogenia Assay.** The effect of GSK189254 on H3 receptor agonist-induced water intake in male Lister hooded rats (370–500 g; Harlan UK Limited, Bicester, Oxon, UK) was studied in a dipsogenia model similar to the design previously (Clapham and Kilpatrick, 1993). Groups of individually housed rats with access to water via identical bottles and spouts received either vehicle (1% aqueous methylcellulose; n = 6) or GSK189254 (0.01–0.3 mg/kg p.o.; n = 6), followed 2 h later by either vehicle (0.9% saline; 2 ml/kg) or R-α-methylhistamine (2.5 mg/kg s.c.). Water consumption was determined in each animal for 1 h after administration of the second treatment by weighing each bottle to the nearest 0.1 g.

**Cognition: Passive Avoidance Paradigm.** The ability of acute treatment of GSK189254 to influence memory consolidation in male Wistar rats (350–400 g; Biomedical Facility, University College, Dublin, Ireland) was investigated in a passive avoidance paradigm in which some animals were administered scopolamine to induce a cholinergic deficit using methodology as described previously (Foley et al., 2004). Following a habituation period of 5 days where animals were housed in pairs, vehicle (1% aqueous methylcellulose) or GSK189254 (0.3, 1, or 3 mg/kg p.o.) was administered to rats 2 h before training (n = 6 per group). Analysis of spontaneous behavior in an open-field apparatus was performed 2 days before training and immediately before training as described previously using a 5-min evaluation period each time (Foley et al., 2004). Rats were then trained in a single-trial, step-through, light-dark passive avoidance paradigm as described previously (Foley et al., 2004). Briefly, on the day of training, animals were placed into the light compartment of the apparatus. Their latency to enter the dark chamber was recorded, and having completely entered the dark compartment, a scrambled foot shock (0.5 mA; 5-s duration) was administered to the animal, which immediately returned to the light compartment. Animals were rendered amnesic of the task by administration of scopolamine (0.8 mg/kg i.p.) 6 h after training. Recall of the inhibitory stimulus was evaluated 24 h post-training by returning the animal into the light chamber and recording its latency to enter the dark chamber (with a criterion time of 600 s used).

**Cognition: Water Maze Paradigm.** A water maze spatial learning paradigm was used to determine the potential cognition-enhancing properties of acutely administered GSK189254 on spatial task deficits in aged male Wistar rats (20 months of age, 450–550 g; Biomedical Facility) using methodology as described in detail previously (Foley et al., 2004). Briefly, following a habituation period and assessment of spontaneous behavior (Foley et al., 2004), rats were dosed with GSK189254 (1 and 3 mg/kg p.o.) or vehicle (1% aqueous methylcellulose) 2 h before each training session and 2 h before recall (n = 6 per group). Animals were trained to explore the water maze apparatus (with pool water maintained at 26°C) using extra-maze visual cues, and the time taken to locate a hidden platform within a 90-s criterion was defined as the escape latency time. To minimize temperature changes, animals were quickly dried between trials and rested in a cage with a raised platform so that they were not sitting in cooling runoff water. Rats were trained in four consecutive daily sessions, each consisting of five trials, with an intertrial rest interval of 5 min and escape latencies measured over each trial. Recall of the platform position was assessed by a probe test at 1, 3, and 7 days following the final training session, in which the platform was removed, and rats were allowed to explore the maze for 60 s. The time spent in each quadrant was recorded and used to compare recall of the platform position by each treatment group.

**Cognition: Object Recognition Paradigm.** An object recognition test was used to assess the effects of repeated administration of GSK189254 on recognition memory in rats. This model is based on the greater spontaneous exploration of a novel object, compared with a familiar object, shown by rodents (Ennaceur and Delacour, 1988). Male Lister hooded rats (200–300 g; n = 8–10 per group; Charles River) were assessed for cognitive ability in a test apparatus comprising an “open-field” arena placed in a sound-attenuated room under dimmed lighting. Images of the open-field were captured by a closed circuit television camera, and they were viewed on a monitor in an adjoining room. Each rat was subjected to the procedure separately and care was taken to remove any olfactory/taste cues by cleaning the arena and test objects with alcohol between trials and rats.

After a 3- to 5-min habituation period, each rat was placed into the test arena in the presence of two identical objects (plastic shapes such as pyramids or cylinders). Each rat was placed facing the same direction at the same position in the arena, and the time spent actively exploring the objects during a 3- or 5-min test period (T1) was recorded. The rat was placed in its home cage between tests. After 24 or 48 h, each rat was again placed in the test arena for 3 or 5 min, respectively (T2), in the presence of one of the familiar objects and a novel object, and the time spent exploring both objects was again recorded. The presentation order and position of the objects (left/right) was randomized to prevent bias from order or place preference. Rats were administered GSK189254 (0.3 or 1 mg/kg p.o.) or vehicle (1% aqueous methylcellulose) either twice daily commencing 2 h before T1 until 2 h before T2 (48-h delay paradigm, total of five doses), or for 7 days (twice daily before testing), and then 2 h before T1 and T2 (24-h delay paradigm, total of 16 doses).

**Cognition: Attentional Set Shifting Paradigm.** The effect of repeated oral administration of GSK189254 (1 mg/kg, twice daily for 7 days before testing) was investigated in rats using the attentional set shifting task as a model of executive function using methodology described previously (Hatcher et al., 2005). Briefly, male Lister hooded rats (250–300 g; Charles River) were habituated to the testing area and trained to dig in bowls for a food reward on the day before testing (Hatcher et al., 2005). GSK189254 or vehicle (v = 10 per group) was administered twice daily (approximately 8:00 AM and 5:00 PM) over 7 days. Animals were habituated to test arena and two simple discriminations on day 7 and tested on day 8. In a single test session, rats performed a series of consecutive discriminations. Bowls were presented in pairs, only one of which was baited. In the simple discrimination (SD), the bowls differed only by odor. For the compound discrimination (CD), a second dimension, i.e., digging medium, was introduced, but the previously relevant odor continued to be rewarded irrespective of the digging medium it was presented with. During reversal learning (REV-1, -2, and -3), the exemplars and relevant dimensions remained unchanged, but the previously correct stimulus was now incorrect (Hatcher et al., 2005). New exemplars were used for both the intradimensional (ID) and extradimensional (ED) shift. For the ID shift, the previously relevant dimension, i.e., odor, was still rewarded but for the ED shift the previously irrelevant dimension, i.e., digging medium, was now rewarded (set shift). At each testing stage (i.e., SD, CD, REV-1, ID, REV-2, ED, REV-3), the number of trials to criterion (i.e., for the rat to choose the correct bowl on six consecutive occasions) was recorded for each discrimination.

**Data Analysis.** The concentration of drug inhibiting specific radioligand binding by 50% (IC50) was determined by iterative curve fitting and pKi values (the negative log10 of the molar Kd) for receptor binding were determined from the IC50 values using the Cheng and Prusoff (1973) approximation. In saturation binding studies, Kd and Bmax values were calculated using Prism (GraphPad Software Inc., San Diego, CA). Drug concentration-response curves from CAMP
In ex vivo binding studies, specific radioactivity in the samples was corrected for protein, and data were expressed as inhibition of H3 binding (percent of control) as a surrogate marker of CNS H3 receptor occupancy. ED_{50} values (dose required to produce a 50% reduction in ex vivo radio ligand binding) were determined by plotting the log_{10} of the oral dose against percentage of specific binding using Grafit 5.0.8 software (Erithacus Software).

In the passive avoidance study, data were analyzed by Mann–Whitney U test and by ANOVA followed by the Bonferroni post hoc test. Another test compound group was included in the design and statistical analysis, but it is not included in the figures.

### Results

#### In Vitro Characterization of GSK189254

Radioligand binding studies with {[H]}Rα-methylhistamine revealed that GSK189254 had high affinity for both recombinant H3 receptors expressed in HEK-293-MSR-II cells and native H3 receptors expressed in the cerebral cortex of several species. pK_{i} values for GSK189254 and reference imidazole (thioperamide, clobenpropit, and ciprofloxin) and nonimidazole (ABT-239) H3 antagonists are shown in Table 1. GSK189254 generally exhibited higher affinity for human and pig H3 receptors compared with rat, mouse, and dog H3 receptors. GSK189254 (1 μM) was tested in a commercial battery of approximately 50 receptors, ion channels, and other drug targets (Cerep, Celle L'Evescault, France), and it showed less than 50% inhibition at any of these targets (data not shown).

In functional assays with HEK-293-Gao cells expressing the human H3 receptor the selective H3 receptor agonist imetit induced a concentration-dependent decrease in forskolin-stimulated cAMP accumulation (pIC_{50} = 8.92 ± 0.04; n = 12). GSK189254 (3, 10, 30, and 100 nM) produced a dose-dependent rightward shift in the concentration-effect curve to imetit with a pA_{2} of 9.06 ± 0.02 (n = 3). GTPγS binding studies in HEK-293-Gao cells expressing the human H3 receptor showed that GSK189254 exhibited inverse agonist properties, a common observation with other structurally distinct H3 antagonists such as ABT-239 (Esbenshade et al., 2005). Basal GTPγS binding (in the absence of H3 agonist) was inhibited by GSK189254 (>70%) in a dose-dependent manner with a pIC_{50} of 8.20 ± 0.12 (n = 3). GSK189254 showed minimal activity at human recombinant H1 (pK_{B} = 5.6; fluorescent imaging plate reader assay), H2 (pK_{B} < 5.5; cAMP assay) and H4 (pK_{B} < 5.5; fluorescent imaging plate reader assay) receptors (data not shown).

#### In Vitro Autoradiography

To assess direct binding interactions with the H3 receptor in the CNS, {[H]}HGSK189254 was synthesized for autoradiography studies. In preliminary radioligand binding characterization studies in human (n = 1) and rat (n = 4; Fig. 2a) cortex homogenates, saturation binding analysis with {[H]}HGSK189254 yielded B_{max} values of 93.3 and 282.8 ± 8.1 fmol/mg and K_{d} values of 0.08 and 0.47 ± 0.05 nM, respectively. Autoradiographic analysis of coronal (Fig. 2b) and sagittal (Fig. 2c) rat brain sections revealed heterogeneous, extensive specific {[H]}HGSK189254 binding within the cerebral cortex, corpus striatum, nucleus accumbens, Islands of Calleja, olfactory tubercle, hippocampus, hypothalamus, and substantia nigra, whereas binding levels were negligible following coincubation with 10 μM imetit to define nonspecific binding. Specific labeling with {[H]}HGSK189254 was also observed in sections of nondiseased brain regions.

### Table 1

<table>
<thead>
<tr>
<th>Human Cortex</th>
<th>Rat Cortex</th>
<th>Mouse Cortex</th>
<th>Dog Cortex</th>
<th>Pig Cortex</th>
<th>Human Recombinant</th>
<th>Rat Recombinant</th>
<th>Mouse Recombinant</th>
</tr>
</thead>
<tbody>
<tr>
<td>GSK189254</td>
<td>9.59 ± 0.15</td>
<td>8.51 ± 0.11</td>
<td>8.89 ± 0.13</td>
<td>8.83 ± 0.09</td>
<td>9.61 ± 0.11</td>
<td>9.90 ± 0.18</td>
<td>9.17 ± 0.09</td>
</tr>
<tr>
<td>Thioperamide</td>
<td>N.D.</td>
<td>8.48 ± 0.10</td>
<td>8.30 ± 0.15</td>
<td>7.91 ± 0.10</td>
<td>8.25 ± 0.09</td>
<td>7.12 ± 0.10</td>
<td>8.62 ± 0.06</td>
</tr>
<tr>
<td>Clobenpropit</td>
<td>9.11 ± 0.11</td>
<td>9.53 ± 0.11</td>
<td>9.57 ± 0.09</td>
<td>9.94 ± 0.12</td>
<td>10.08 ± 0.20</td>
<td>9.47 ± 0.19</td>
<td>9.59 ± 0.09</td>
</tr>
<tr>
<td>Ciprofloxin</td>
<td>6.72 ± 0.14</td>
<td>8.91 ± 0.04</td>
<td>9.10 ± 0.16</td>
<td>7.46 ± 0.10</td>
<td>8.03 ± 0.16</td>
<td>8.96 ± 0.06</td>
<td>9.29 ± 0.05</td>
</tr>
<tr>
<td>ABT-239</td>
<td>N.D.</td>
<td>7.92 ± 0.11</td>
<td>7.96 ± 0.08</td>
<td>8.61 ± 0.20</td>
<td>8.46 ± 0.16</td>
<td>9.51 ± 0.10</td>
<td>8.52 ± 0.08</td>
</tr>
</tbody>
</table>

N.D., not determined.
human cortex, with silver grains observed over neuronal cell bodies following high-resolution autoradiography by emulsion dipping and counterstaining with cresyl fast violet (Fig. 3a). Specific $[^{3}H]$GSK189254 binding was also observed in the hippocampal CA1 region from an individual diagnosed with Alzheimer’s disease for 5 years (Fig. 3b) as well as in samples of medial temporal cortex from mild-to-severe cases of Alzheimer’s disease as defined by Braak stage plaque pathology and confirmation with antibody to total $\beta$-amyloid (Fig. 3c).

Fig. 2. a, saturation binding of $[^{3}H]$GSK189254 to $H_3$ receptors in rat cortex homogenates. In vitro autoradiography of $[^{3}H]$GSK189254 (1 nM) binding to $H_3$ receptors in coronal (b) and sagittal (c) sections of rat brain. Cortex (ctx), striatum (str), hypothalamus (hyp), substantia nigra (sn), thalamus (Th), dentate gyrus of hippocampus (DG), and cerebellum (cer) are indicated. Nonspecific binding (NSB) was determined in the presence of 10 $\mu$M imetit. Scale bar, 2 mm, except coronal NSB (1 mm).

Fig. 3. In vitro autoradiography of $[^{3}H]$GSK189254 binding to human brain $H_3$ receptors in nondiseased cortex, with specific binding shown in left panel (scale bar, 1 mm), NSB signal shown in middle panel (scale bar, 1 mm), and silver grains indicated by arrows over neuronal cell bodies following emulsion dipping shown in right panel (scale bar, 50 $\mu$m) (a); hippocampus of individual diagnosed with AD for 5 years with specific binding signal shown in top left panel (scale bar, 1 mm), NSB shown in top right panel (scale bar, 1 mm), anatomical orientation (cresyl violet stained) of CA1 hippocampal region shown in bottom left panel (scale bar, 50 $\mu$m), and amyloid plaques stained with 1E8 antibody to total $\beta$-amyloid shown in bottom right panel (scale bar, 100 $\mu$m) (b); and medial temporal cortex from different Braak stages of Alzheimer’s disease with top panels showing increasing plaque pathology across Braak Stages (scale bar, 25 $\mu$m) and bottom panels showing specific $H_3$ receptor binding maintained despite severe plaque pathology (scale bar, 1 mm).
Ex Vivo Binding. GSK189254 (0.1, 0.3, 1, and 3 mg/kg p.o. 2 h postdose) dose-dependently inhibited specific [³H]Rα-methylhistamine binding in the ex vivo binding assay in rat cerebral cortex (Fig. 4a). Iterative curve fitting of these data gave an estimated mean ED₅₀ value of 0.17 ± 0.03 mg/kg (n = 3). With increasing dose (0.1–3 mg/kg), brain concentrations of GSK189254 increased from 0.036 ± 0.006 to 0.615 ± 0.334 μM and blood concentrations increased from 0.032 ± 0.012 to 0.680 ± 0.385 μM, corresponding to a brain/blood concentration ratio in the range 0.9 to 1.2:1, consistent with good brain penetration and confirming that brain and blood concentrations of GSK189254 exhibited a linear relationship with dose across the range tested in efficacy models. Nonlinear regression analysis of the GSK189254 brain concentration and receptor occupancy data at 2 h postdose estimated the brain EC₅₀ of GSK189254 to be approximately 60 nM (Fig. 4b). In a GSK189254 time course study (0.3 mg/kg p.o.; Fig. 4c), the inhibition of ex vivo bind-

![Graph](image_url)

**Fig. 4.** Inhibition of [³H]Rα-methylhistamine ex vivo binding in the rat cortex following oral administration of GSK189254. a, dose-dependent inhibition with 0.1, 0.3, 1, and 3 mg/kg GSK189254 2 h after dosing (n = 3). b, relationship between total brain concentration (micromolar) of GSK189254 and H₃ receptor occupancy in individual rats. c, time course of inhibition (0.5 to 12 h after dosing) obtained with 0.3 mg/kg GSK189254. Data points in a and c represent mean ± S.E.M. from three rats per group, and they are expressed as H₃ receptor occupancy (percentage of control).

**TABLE 2**

<table>
<thead>
<tr>
<th>Dose (mg/kg)</th>
<th>Time Postdose (h)</th>
<th>% Inhibition of EVB (&quot;Occupancy&quot;)</th>
<th>Blood Conc. (μM)</th>
<th>Brain Conc. (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1</td>
<td>2</td>
<td>37 ± 13</td>
<td>0.032 ± 0.012</td>
<td>0.036 ± 0.006</td>
</tr>
<tr>
<td>0.3</td>
<td>2</td>
<td>70 ± 4</td>
<td>0.103 ± 0.008</td>
<td>0.125 ± 0.031</td>
</tr>
<tr>
<td>1</td>
<td>2</td>
<td>80 ± 9</td>
<td>0.327 ± 0.038</td>
<td>0.293 ± 0.021</td>
</tr>
<tr>
<td>3</td>
<td>2</td>
<td>90 ± 2</td>
<td>0.680 ± 0.385</td>
<td>0.615 ± 0.334</td>
</tr>
<tr>
<td>0.3</td>
<td>0.5</td>
<td>67 ± 4</td>
<td>0.190 ± 0.059</td>
<td>0.254 ± 0.146</td>
</tr>
<tr>
<td>0.3</td>
<td>1</td>
<td>73 ± 2</td>
<td>0.181 ± 0.046</td>
<td>0.286 ± 0.028</td>
</tr>
<tr>
<td>0.3</td>
<td>2</td>
<td>68 ± 5</td>
<td>0.215 ± 0.075</td>
<td>0.225 ± 0.067</td>
</tr>
<tr>
<td>0.3</td>
<td>3</td>
<td>63 ± 3</td>
<td>0.197 ± 0.033</td>
<td>0.208 ± 0.054</td>
</tr>
<tr>
<td>0.3</td>
<td>6</td>
<td>47 ± 2</td>
<td>0.079 ± 0.010</td>
<td>0.076 ± 0.004</td>
</tr>
<tr>
<td>0.3</td>
<td>8</td>
<td>30 ± 7</td>
<td>0.053 ± 0.026</td>
<td>0.043 ± 0.015</td>
</tr>
<tr>
<td>0.3</td>
<td>12</td>
<td>6 ± 6</td>
<td>0.020 ± 0.004</td>
<td>N.Q.</td>
</tr>
<tr>
<td>5</td>
<td>3</td>
<td>N.D.</td>
<td>0.806 ± 0.135</td>
<td>1.015 ± 0.157</td>
</tr>
<tr>
<td>10</td>
<td>3</td>
<td>N.D.</td>
<td>3.593 ± 0.425</td>
<td>5.189 ± 1.086</td>
</tr>
<tr>
<td>50</td>
<td>3</td>
<td>N.D.</td>
<td>5.661 ± 1.146</td>
<td>9.210 ± 1.822</td>
</tr>
<tr>
<td>0.3</td>
<td>6 (day 1)</td>
<td>45 ± 4</td>
<td>0.026 ± 0.006</td>
<td>0.054 ± 0.007</td>
</tr>
<tr>
<td>1</td>
<td>6 (day 1)</td>
<td>65 ± 4</td>
<td>0.076 ± 0.023</td>
<td>0.115 ± 0.028</td>
</tr>
<tr>
<td>10</td>
<td>6 (day 1)</td>
<td>92 ± 3</td>
<td>0.966 ± 0.462</td>
<td>1.051 ± 0.304</td>
</tr>
<tr>
<td>0.3</td>
<td>6 (day 4)</td>
<td>40 ± 13</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>1</td>
<td>6 (day 4)</td>
<td>67 ± 11</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>10</td>
<td>6 (day 4)</td>
<td>88 ± 5</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>0.3</td>
<td>6 (day 8)</td>
<td>55 ± 7</td>
<td>0.115 ± 0.035</td>
<td>0.073 ± 0.024</td>
</tr>
<tr>
<td>1</td>
<td>6 (day 8)</td>
<td>48 ± 2</td>
<td>0.129 ± 0.093</td>
<td>0.069 ± 0.015</td>
</tr>
<tr>
<td>10</td>
<td>6 (day 8)</td>
<td>88 ± 1</td>
<td>1.914 ± 0.833</td>
<td>0.800 ± 0.324</td>
</tr>
</tbody>
</table>

Conc., concentration; N.D., not determined; N.Q., nonquantifiable (below the limit of quantification, which was 0.014 μM).
GSK189254 induced a significant increase in ACh levels. Post hoc analysis of
these effects presented as total neurotransmitter efflux is shown in Fig. 6b. No significant effects were observed on 5-HT release (data not shown).

**Dipsogenia.** R-α-Methylhistamine (2.5 mg/kg s.c.) produced a significant 3-fold increase in water consumption in the rat dipsogenia model. Oral administration of GSK189254 dose-dependently inhibited the dipsogenic response reaching significance at 0.1 and 0.3 mg/kg (Fig. 7). Iterative curve fitting of the data yielded an ID_{50} of 0.05 mg/kg. The reference H_{3} antagonists thioperamide (5 mg/kg p.o.), ciproxifan (10 mg/kg p.o.), and ABT-239 (3 mg/kg p.o.) were also fully efficacious in reversing the dipsogenic response, whereas the H_{1} and H_{2} antagonists mepyramine (3 mg/kg i.p.) and zolantidine (5 mg/kg i.p.) were inactive (data not shown).

**Pharmacokinetics of GSK189254.** The pharmacokinetics of GSK189254 was evaluated in male Sprague-Dawley rats. Following a 1-h intravenous infusion of GSK189254 at a target dose of 1 mg free base/kg to the rat, GSK189254 had a moderate blood clearance of 40 ± 5 ml/min/kg (ca. 45% liver blood flow) with a steady-state volume of distribution (V_{ss}) of 4.3 ± 1.1 l/kg, indicating distribution of GSK189254 into tissues, and a terminal half-life of 1.6 ± 0.4 h (Table 3). Following oral administration of GSK189254 at a target dose of 2 mg free base/kg, peak blood concentrations of GSK189254 of 0.498 ± 0.089 μM were achieved between 0.8 and 3.0 h after dosing with an oral half-life of 2.5 ± 0.3 h. The oral bioavailability of GSK189254 in the rat was 83 ± 21% (Table 3).

**c-Fos Expression.** Compared with vehicle-treated animals (Fig. 5a), the density of c-Fos staining was increased in rat forebrain following oral administration of GSK189254 (3 mg/kg; Fig. 5b). Quantification of this effect showed that GSK189254 (n = 4) induced statistically significant increases (p < 0.05) in c-Fos expression in the infralimbic prefrontal cortex and somatosensory cortex (3.0 mg/kg) but not in caudate putamen (Fig. 5c).

**Microdialysis.** Following oral dosing of vehicle, minimal changes in neurotransmitter levels were observed. GSK189254 induced a significant increase in ACh [F(5,43) = 2.95; p < 0.05], dopamine [F(5,35) = 4.18; p < 0.01], and noradrenaline levels [F(5,30) = 2.92; p < 0.05] in the anterior cingulate cortex for up to 4 h (Fig. 6a) and increased ACh in the dorsal hippocampus [F(5,44) = 3.51, p < 0.01; data not shown]. Post hoc analysis revealed that in the anterior cingulate cortex, ACh levels were significantly increased by GSK189254 at 1 mg/kg (p < 0.05) and 3 mg/kg (p < 0.01), whereas in the dorsal hippocampus GSK189254 0.3 mg/kg (p < 0.01) and 1 mg/kg (p < 0.001) significantly increased ACh levels. Post hoc analysis of monoamine responses revealed a significant increase in dopamine by GSK189254 at 1 mg/kg (p < 0.01), whereas noradrenaline levels were increased by GSK189254 at 1 mg/kg (p < 0.05) and 3 mg/kg (p = 0.053). A summary of these effects presented as total neurotransmitter efflux is

![Fig. 5. GSK189254 stimulates c-Fos expression in rat forebrain. Representative photomicrographs illustrating c-Fos immunostaining in the rat somatosensory cortex (S1) following oral administration of vehicle (a) or GSK189254 (3 mg/kg) (b) are shown. Forceps minor corpus callosum (fmi). Scale bar, 100 μm. c, effects of GSK189254 (n = 4 per group; 0.3 mg/kg, grey bars; 1 mg/kg, hatched bars; and 3 mg/kg, black bars) on the density (counts per square millimeter) of c-Fos immunopositive nuclei in infralimbic prefrontal cortex, somatosensory cortex, and caudate putamen (*, p < 0.05; **, p < 0.001) compared with vehicle-treated animals (white bars).](image-url)
**Passive Avoidance.** In comparison with vehicle-treated control animals, scopolamine was effective at rendering animals amnesic, since its administration at the 6 h post-training time point significantly reduced 24-h recall latency (p < 0.001; Bonferroni post hoc analysis). GSK189254 reversed this scopolamine-induced amnesia in a dose-dependent manner, with significant effects being observed after administration of 1 and 3 mg/kg, but not 0.3 mg/kg [Fig. 8; F(3,20) = 5.3; p = 0.008]. GSK189254 (3 mg/kg) administered alone (where animals were treated with vehicle instead of scopolamine at the 6-h post-training time point) did not alter recall of the passive avoidance response compared with vehicle-treated controls. The analysis of spontaneous exploratory behavior in an open-field apparatus, for 2 days before training and again immediately before training, indicated that all animal groups underwent typical habituation to the open-field arena, and no drug-induced changes in exploratory activity were observed (data not shown). In addition, latencies to enter the dark chamber of the passive avoidance apparatus during the training phase (in the absence of shock) were equivalent in rats treated with vehicle or GSK189254.

**Water Maze Spatial Learning Paradigm in Aged Rats.** In the water maze test, acute oral treatment of aged rats with GSK189254 (1 or 3 mg/kg, 2 h before each training session) significantly reduced platform escape latencies [Fig. 9a; F(1,158) = 49.5 and 84.5, p < 0.0001, 1 and 3 mg/kg, respectively] and swim angles [Fig. 9b; F(1,32) = 9.5 and 8.5, p = 0.004 and 0.006, 1 and 3 mg/kg, respectively] on four consecutive daily sessions compared with vehicle-treated controls, but there was no difference in swim speed (Fig. 9c). GSK189254 (3 mg/kg) also significantly improved task recall as measured by significant increases in time spent searching the target quadrant on post-training days 3 and 7 when the escape platform had been removed [Fig. 9d; F(1,54) = 8.6, p = 0.005], consistent with perseveration in the target location.

**Object Recognition.** The effect of repeat dosing with GSK189254 on temporal induced deficits in cognitive performance was investigated in the rat object recognition test. Following either a 24- or 48-h delay, vehicle-treated animals showed no significant preference for the novel object. In contrast, a significant increase (p < 0.05) in time spent exploring the novel object compared with the familiar object was observed following GSK189254 (0.3 and 1 mg/kg p.o.) dosed either twice daily for 7 days (Fig. 10a) or twice daily commencing 2 h before T1 through to 2 h before T2, respectively (Fig. 10b). In a further object recognition study with a 48-h delay, GSK189254 also showed positive effects at 0.1, 1, and 3 mg/kg following twice daily oral dosing for 7 days before T1 (data not shown).

**Attentional Set Shift.** Vehicle-treated rats required more trials to criterion during the first reversal learning phase (REV-1; p < 0.01) and attentional set shifting (ID/ED shift; p < 0.01). Subchronic oral administration of GSK189254 (1 mg/kg, twice daily for 7 days) improved reversal learning as shown by a significant (p < 0.01) reduction in the number of trials to reach criteria on the first reversal (Fig. 11). Moreover, GSK189254 improved...
attentional set shifting, since in contrast with vehicle-treated rats no significant increase in the number of trials to criteria between the ID shift and ED shift was seen (GSK189254 ID/ED shift, \( p > 0.05 \) compared with vehicle ID/ED shift, \( p < 0.01 \), and the number of trials to reach criteria during the ED shift was significantly reduced following GSK189254 (\( p < 0.05 \) compared with vehicle).

**Discussion**

GSK189254 is derived from a novel benzazepine series of \( \mathrm{H}_3 \) receptor antagonists (Bamford et al., 2004) that are structurally distinct from other recently described nonimidazole \( \mathrm{H}_3 \) antagonists (Celanire et al., 2005). Our radioligand binding studies show that GSK189254 had subnanomolar affinity for human \( \mathrm{H}_3 \) receptors, with ~6- to 10-fold lower affinity for rodent \( \mathrm{H}_3 \) receptors. Species differences in \( \mathrm{H}_3 \) antagonist pharmacology have been reported previously (Passani et al., 2004), and our binding data with standard \( \mathrm{H}_3 \) antagonists are consistent with these findings. These pharmacological differences have been attributed to two amino acid differences in the third transmembrane domain where threonine 119 and alanine 122 of the human \( \mathrm{H}_3 \) receptor are replaced with alanine 119 and valine 122 in the rat (Ligneau et al., 2000). Interestingly, the affinity of GSK189254 for the dog receptor (where threonine 119 and valine 122 are present) was similar to the rodent receptors. In contrast, the affinity of GSK189254 for the pig \( \mathrm{H}_3 \) receptor was similar to the human receptor, consistent with partial peptide sequence data for the pig receptor where threonine 119 and alanine 122 are present (H. K. Van der Keyl, unpublished observations).

In functional studies, GSK189254 exhibited potent antagonist and inverse agonist properties at the human recombinant \( \mathrm{H}_3 \) receptor, similar to other nonimidazole \( \mathrm{H}_3 \) antagonists such as ABT-239 (Esbenshade et al., 1993; Barbier et al., 2004). \( \left[{\mathrm{H}}\right]\)GSK189254 also labeled specific \( \mathrm{H}_3 \) receptor binding sites in human cortex sections, where cellular resolution of \( \mathrm{H}_3 \) receptors following autoradiography, although in other neurodegenerative conditions such as Parkinson’s disease, \( \mathrm{H}_3 \) receptor binding is either increased or unchanged (Anichtchik et al., 2001). In the current study, although quantitative comparisons of binding in AD versus control brains was not possible due to limited availability of samples, specific \( \mathrm{H}_3 \) receptor binding seemed prominent in the CA1 hippocampal region from an individual diagnosed with AD for 5 years, and in medial temporal cortex samples from various individuals with different Braak stages of plaque pathology. This suggests that the expression of \( \mathrm{H}_3 \) receptors is still prevalent even in severe late stages of AD, an important observation given the aim of targeting these receptors as a potential novel therapeutic approach.

Several studies demonstrated the in vivo activity of GSK189254 following oral dosing. GSK189254 potently inhibited cortical ex vivo \( \mathrm{H}_3 \) receptor binding, consistent with good CNS penetration and \( \mathrm{H}_3 \) receptor occupancy, whereas increased cortical neuronal activation was demonstrated using c-Fos immunoreactivity, similar to previous reports with other \( \mathrm{H}_3 \) antagonists (Hancock et al., 2006). It is well established that \( \mathrm{H}_3 \) receptors can modulate the release of multiple neurotransmitters (Blandina et al., 1996; Fox et al., 2005), and the current microdialysis study with GSK189254 demonstrated increases in acetylcholine, noradrenaline, and dopamine release in the cortex, consistent with blockade of \( \mathrm{H}_3 \) heteroreceptors. In addition, GSK189254 potently blocked \( \mathrm{H}_3 \) agonist-induced dipsogenia, consistent with functional blockade of \( \mathrm{H}_3 \) receptors in vivo.

The effects of \( \mathrm{H}_3 \) antagonists in rodent cognition models
including passive avoidance, object recognition, and water maze have been reviewed in detail, and most studies support pro-cognitive effects of H₃ antagonists (Witkin and Nelson, 2004). In the current study, GSK189254 was efficacious in a diverse battery of cognition models in rats when dosed acutely or repeatedly, and it showed similar effects in these models to cholinesterase inhibitors (A. Foley, unpublished observations) and 5-HT₆ receptor antagonists (Foley et al., 2004; Hatcher et al., 2005; J. Gart- 

don, unpublished observations).

First, GSK189254 reversed amnesia induced by the cholinergic antagonist scopolamine in a passive avoidance paradigm, consistent with facilitation of cholinergic transmission following blockade of H₃ heteroreceptors. Second, GSK189254 reduced platform escape latencies in an aged rat water maze test, and it improved task recall in the probe trial where the platform was removed. Third, in the object recognition model of spontaneous recognition memory and novelty detection (Ennaceur and Delacour, 1988), GSK189254 increased the time spent exploring the novel compared with familiar objects, consistent with an improvement in recognition memory. Finally, GSK189254 showed efficacy in the attentional set shift paradigm by improving both reversal learning and attentional set shifting, and to our knowledge, this is the first report of an H₃ antagonist showing efficacy in a model of cognitive flexibility and executive function, suggesting that GSK189254 may be useful not only for AD, but cognitive dysfunction in schizophrenia. Although the translational relevance of these animal models to human cognition is uncertain, GSK189254 nevertheless showed broad efficacy across all models, increasing confidence that a beneficial effect may be seen in humans. The pro-cognitive effects of GSK189254 are unlikely to be due to nonspecific effects on behavior or other unforeseen confounds, because there were no changes in motor coordination (accelerating rotarod), seizure threshold (maximal electroshock test), or other general behaviors (such as eating, drinking and locomotor activity) following oral doses of up to 50 mg/kg. The effects of GSK189254 on cognition are also unlikely to be due to

![Fig. 9.](image-url)

Fig. 9. Effect of oral administration of vehicle (open circles) or GSK189254 (1 and 3 mg/kg, open triangles and open squares, respectively; n = 6) on spatial learning (a), swim angle (b), and swim speed (c) of aged rats in the water maze task. Data represent mean ± S.E.M. escape latency (seconds) averaged over the five trials of each training session (a), mean ± S.E.M. swim angle (degrees) (b), and mean ± S.E.M. swim speed (meters per second) (c) averaged over the five trials of each training session. Significant difference from vehicle-treated animals is indicated by an asterisk; Bonferroni post hoc analysis, p < 0.05). d, effect of vehicle (white bars) or GSK189254 (1 and 3 mg/kg, hatched bars and black bars, respectively; n = 6) on recall of a water maze spatial task in aged rats. Data represents ratio of time swimming in the target quadrant versus the opposite quadrant of the water maze during probe trials 1, 3, and 7 days post-training. Significant differences from vehicle-treated animals is indicated by an asterisk (Mann–Whitney U test post hoc analysis, p < 0.05).
changes in thermoregulation, because body temperature was unaffected following oral doses of up to 30 mg/kg (T. Stean, unpublished observations).

GSK189254 exhibited broad efficacy across the different cognition models, with activity being generally observed with doses between 0.3 and 3 mg/kg, which equated to >60% H₃ receptor occupancy (ex vivo binding), maximum inhibition in the dipsogenia assay, and brain exposures of 300 to 600 nM. These efficacious doses in the cognition assays were somewhat higher than the minimal effective doses in ex vivo binding and dipsogenia assays. This may be because complete functional blockade of H₃ receptors is required to observe robust efficacy in cognition models that involve complex pathways and multiple neurotransmitters. In contrast, the dipsogenia and ex vivo binding assays reflect specific interactions between an H₃ agonist and antagonist and therefore less H₃ receptor blockade may be required for an effect to be observed, particularly if the agonist is occupying only a small fraction of the receptors. Estimated efficacious brain concentrations of GSK189254 were also higher than rat cortex binding affinities, possibly due to nonspecific binding in the brain and/or because of methodological differences. Affinities were determined in cerebral cortex membranes and reflect specific competition binding at the H₃ receptor, whereas brain concentrations were measured in whole brain samples (unbound and bound drug), and the drug concentration at the receptor level is unknown. However, plasma protein binding is low in the rat for GSK189254 (61%), so this is unlikely to completely account for the mismatches observed.

It should be noted that these pharmacokinetic/pharmacodynamic discrepancies are not unique to GSK189254, because higher doses of other H₃ antagonists seem to be required to exhibit efficacy in cognition or sleep/wake models compared with dipsogenia or ex vivo binding assays (Fox et al., 2003; Barbier et al., 2004). In addition, despite the high affinity of ciproxifan for rat cortex H₃ receptors (pKᵢ = 8.9, i.e., comparable with GSK189254), a dose of 3 mg/kg has routinely been used to demonstrate positive effects in cognition assays (Fox et al., 2005). We have demonstrated that this dose results in brain concentrations of ~3 μM at 2 h following oral dosing, suggesting similar mismatches between pKᵢ and brain drug concentrations to those observed with GSK189254 (M. Briggs, unpublished observations).

Few studies have investigated the effects of repeat dosing of H₃ antagonists, particularly on behavioral parameters, and have generally focused on biochemical readouts (Morisset et al., 2000). Recently, Pan et al. (2006) demonstrated that the effects of ciproxifan on food intake and locomotor activity were susceptible to tolerance, whereas its effects on body weight were not. In the current study, we observed positive effects on cognitive function with GSK189254 in object recognition and attentional set shift paradigms following twice-daily dosing for 7 days, suggesting that the procognitive effects were not subject to tolerance. In addition, receptor occupancy and CNS exposures of GSK189254 were similar following acute dosing or twice-daily dosing for up to 8 days, supporting a lack of tolerance or accumulation. A retention of procognitive efficacy has also been observed with the nonimidazole H₃ antagonist ABT-239 in the spontaneously hypertensive rat...
5-trial inhibitory avoidance model following up to 5 days dosing (Fox et al., 2005). These observations are clearly important when considering potential long-term drug treatment in chronic CNS disorders.

In summary, GSK189254 is a novel potent, selective, and brain-penetrant H₃ receptor antagonist that shows positive effects in a broad range of rodent cognition models; therefore, it may have therapeutic potential for diseases where cognitive deficits occur such as AD, other dementia, and schizophrenia. Clinical studies are underway to investigate the effects of GSK189254 in humans.

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