Available online at www.sciencedirect.com

SCIENCE DIRECT

European Journal of Pharmacology 506 (2005) 209–219

www.elsevier.com/locate/ejphar

The role of muscarinic receptor antagonism in antipsychotic-induced hippocampal acetylcholine release☆

David E. Johnson[a], Frank M. Nedza[b], Douglas K. Spracklin[b], Karen M. Ward[a], Anne W. Schmidt[a], Philip A. Iredale[a], Dennis M. Godek[c], Hans Rollema[a,*]

[a]Department of Neuroscience, Pfizer Global Research and Development, Eastern Point Road, Groton, CT 06340, USA
[b]Department of Pharmacokinetics, Dynamics and Metabolism, Pfizer Global Research and Development, Groton, CT 06340, USA
[c]Global Project Management, Pfizer Global Research and Development, Groton, CT 06340, USA

Received 24 June 2004; received in revised form 22 October 2004; accepted 10 November 2004
Available online 15 December 2004

Abstract

Olanzapine and clozapine produce robust increases in hippocampal acetylcholine release during acetylcholinesterase inhibition, while other antipsychotics, including thioridazine, have only small effects. Since thioridazine binds with similar high affinities to muscarinic receptors as olanzapine and clozapine, muscarinic autoreceptor blockade was ruled out as a primary mechanism [Neuropsychopharmacology 26 (2002) 583]. This study compared in vitro binding affinities and functional activities of olanzapine, clozapine, thioridazine, ziprasidone, risperidone, chlorpromazine and scopolamine at muscarinic M_2 receptors with their in vivo potencies to increase acetylcholine release in the rat hippocampus. We found that scopolamine, olanzapine and clozapine, but also high doses of thioridazine and chlorpromazine, markedly increase acetylcholine release. The reduced in vivo potencies of thioridazine and chlorpromazine are consistent with their significantly weaker functional antagonist activity at human muscarinic M_2 receptors, while thioridazine's reduced binding affinity for rat muscarinic M_2 receptors and lower brain exposure, may further contribute to its weak in vivo potency compared to olanzapine. The excellent correlation between in vitro antagonist activities of antipsychotics at muscarinic M_2 receptors and their in vivo potencies to increase acetylcholine release, suggests that olanzapine, clozapine, as well as thioridazine and chlorpromazine, increase acetylcholine release via blockade of terminal muscarinic M_2 autoreceptors.

Keywords: Olanzapine; Clozapine; Thioridazine; Acetylcholine; Muscarinic receptors

1. Introduction

Several typical and atypical antipsychotics, including olanzapine, clozapine and thioridazine, have high binding affinities for the five muscarinic (M_{1-5}) receptors (Bolden et al., 1992; Bymaster et al., 1996) and are thought to act as antagonists at all subtypes, with the possible exception of the muscarinic M_4 receptor (Zorn et al., 1994). While interaction of antipsychotics with muscarinic receptors may have beneficial effects (Bymaster et al., 2003), muscarinic receptor blockade can also impair memory and cognitive function, as exemplified by the effects of the potent non-selective muscarinic antagonist, scopolamine (Mintzer and Griffiths, 2003). Assessing the beneficial vs. detrimental effects of atypical antipsychotics with muscarinic antagonist properties is complicated by the fact that antipsychotics can increase acetylcholine release in the prefrontal cortex (Parada et al., 1997; Ichikawa et al., 2002; Li et al., 2003) and hippocampus (Shirazi-Southall et al., 2002; Chung et al., 2003). Since elevated acetylcholine levels following antipsychotic treatment can activate postsynaptic cholinergic receptors, the net result of the opposing effects of increased acetylcholine levels and postsynaptic muscarinic receptor blockade will depend on both the magnitude of the acetylcholine increase and the functional potencies at

☆ Portions of this study were presented in poster form at the ACNP 42nd Annual Meeting, December 7–11, 2003, San Juan, Puerto Rico.

* Corresponding author. Tel.: +1 860 441 6374; fax: +1 860 715 8357.
E-mail address: hans_rollema@groton.pfizer.com (H. Rollema).

0014-2999/$ - see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.ejphar.2004.11.015

postsynaptic muscarinic receptor subtypes (Kennedy et al., 2001; Dean et al., 2003). Therefore, it is important to know the potencies of antipsychotics to increase acetylcholine release in relation to their functional potencies at muscarinic receptor subtypes.

A recent report (Shirazi-Southall et al., 2002) showed that clozapine and olanzapine, but not haloperidol, ziprasidone, thioridazine or chlorpromazine, produce large, 5–15-fold, increases in extracellular hippocampal acetylcholine levels, measured by in vivo microdialysis in the presence of an acetylcholinesterase inhibitor. Since thioridazine, an antipsychotic with similar high binding affinities for muscarinic receptors as olanzapine and clozapine, failed to increase acetylcholine release at comparable doses, the authors concluded that muscarinic antagonism plays a minimal role in the acetylcholine increase. In addition, it was suggested that the ability of these drugs to increase rat hippocampal acetylcholine levels could translate into cognitive benefits in schizophrenic patients.

Two considerations prompted us to re-examine the potential role of muscarinic antagonism in antipsychotic-induced acetylcholine release. First, while thioridazine has potent binding affinities for all muscarinic subtypes, it has been reported to have very weak functional antagonist activity (Herrling and Misbach-Lesenne, 1982; Niedzwiecki et al., 1989), which could account for its failure to increase acetylcholine release in vivo. Second, the magnitude of the acetylcholine increase reported for olanzapine and clozapine in the presence of an acetylcholinesterase inhibitor seems to be more compatible with blockade of release regulating muscarinic autoreceptors than with any other reported receptor mediated mechanism.

This study determined in vitro binding affinities and functional potencies of olanzapine, clozapine, thioridazine, ziprasidone, risperidone, chlorpromazine and scopolamine at the cloned human muscarinic M_1 and M_2 receptor subtypes and studied the dose-dependency of their effects on hippocampal acetylcholine release after systemic administration using in vivo microdialysis. In addition, local application of olanzapine, clozapine, thioridazine, risperidone and scopolamine by retrodialysis into either the cholinergic cell body or terminal area was used to obtain information on their site of action. Finally, brain and plasma levels of olanzapine, thioridazine and ziprasidone were measured in order to determine if differences in drug exposure might contribute to reduced in vivo potencies of thioridazine and ziprasidone.

We report here that olanzapine and clozapine, but also high doses of thioridazine and chlorpromazine, markedly increase extracellular acetylcholine levels in rat hippocampus and that this effect occurs locally in the hippocampus and is not mediated by direct activation of cell bodies in the septum. Compared with olanzapine, thioridazine is functionally an order of magnitude less potent as a muscarinic antagonist, binds with lower affinity to rat muscarinic M_2 receptors and has lower brain exposure, which fully account for the reduced in vivo potency of thioridazine to increase acetylcholine release via antagonism at presynaptic muscarinic M_2 autoreceptors.

2. Methods

Principles of laboratory animal care (Guide for the Care and Use of Laboratory Animals, National Academy Press 1996) were followed and the Animal Care and Use Committee of Pfizer Global Research and Development approved all protocols.

2.1. In vitro receptor binding studies

Radioligand binding assays in cell membranes from Chinese hamster ovary (CHO) cells expressing human muscarinic receptors or in washed rat cerebellar membranes were performed essentially as previously described (Seeger et al., 1995). Membranes were incubated in 50 mM Tris HCl buffer pH 7.4 containing 2 mM $MgCl_2$ for 60 min at room temperature with 0.2 nM [^{3}H]*N*-methyl-scopolamine (82 Ci/mol) and varying concentrations of test compounds (0.32–10,000 nM). Incubations were terminated by rapid filtration onto GF/B filters pre-soaked in 0.5% polyethylenimine. Non-specific binding was determined using a saturating concentration of atropine (10 μM), a potent non-selective muscarinic inhibitor and radioactivity was quantified by liquid scintillation counting. IC_{50} values were determined by linear regression of the concentration–response data and K_i values were calculated according to the Cheng Prusoff equation, $K_i = IC_{50}/(1+(L/K_d))$, where L is the concentration of the radioligand used in the experiment and the K_d value is the dissociation constant for the radioligand (determined previously by saturation analysis for each tissue/receptor). Reported K_i values are the means±S.E.M. of at least three separate experiments performed in duplicate.

2.2. In vitro functional activity studies

Functional antagonist activities were determined in CHO cell lines transfected with human muscarinic M_1 or M_2 receptors by measuring effects on intracellular calcium flux using a Fluorimetric Imaging Plate Reader (FLIPR, Molecular Devices). Cells were maintained in cell medium, containing Dulbecco's Modified Eagles Medium (DMEM) high glucose with L-glutamine/with pyridoxine hydrochloride/without sodium pyruvate, 500 μg/ml G418, 100 μM non-essential amino acids, 10 μM HEPES buffer, 2 mM L-glutamine, 10% fetal bovine serum (heat inactivated, Gibco) and harvested 48 h prior to the assay, counted and seeded at a density of 12,500 cells/well into black 384 well, clear bottom, collagen coated-1 plates (Becton Dickinson) and incubated at 37 °C (with CO_2). Blank wells contained assay buffer of the following composition: 145 mM NaCl, 10 mM glucose, 5 mM KCl, 1 mM $MgSO_4$, 10 mM HEPES and 2

mM $CaCl_2$ (pH 7.4). On the day of the assay, dye medium was prepared by adding 22 μl dimethyl sulfoxide, 22 μl 20% pluronic acid, 110 μl probenecid solution and 11 μl of serum-free DMEM medium to a 50 μg vial of Fluo-4 AM dye. The dye medium was added (25 μl/well) and the plates incubated for 75 min at 37 °C (with CO_2). The medium was then removed and the cells washed three times with 50 μl of cell wash buffer (2.5 mM probenecid in assay buffer). Excess dye was separated from the cells by incubation in 30 μl cell wash buffer for 45 min at 37 °C. Assay plates were then loaded onto the FLIPR and read at excitation and emission wavelengths of 488 and 516 nm, respectively. When looking for antagonists, the order of addition necessitates the identification of compounds that act as agonists, since agonist depletion of internal calcium stores can mimic the effect of an antagonist. Therefore, test compounds were added and a FLIPR reading was taken to identify possible agonists prior to the addition of the known agonist. Finally, the muscarinic agonist carbachol (20 nM for M_1, 1 μM for M_2) was added and the reduction in the carbachol signal was measured to determine the antagonist activity of the test compounds. IC_{50} values were estimated by non-linear regression of concentration–response data and K_b values were calculated using the Cheng Prusoff equation. K_b values are the means of four separate experiments each performed in triplicate.

2.3. In vivo microdialysis studies

Microdialysis was performed in freely moving male Sprague–Dawley rats (300–350 g), according to standard procedures. Briefly, guide cannulas (Bioanalytical Systems) were implanted into the hippocampus (AP −5.2 from bregma, ML ±5.0, DV −3.3 from dura) under isoflurane anesthesia and fixed to the skull. For dual probe experiments, a second guide cannula was targeted at the septum (AP +0.5 from bregma, ML +1.0, DV −5.0 from dura, at a 9° angle from vertical toward midline). After guide cannula implantation, animals were housed separately in a Perspex rat cage inside an isolation box with free access to food and water and a 12-h light–dark cycle (7 a.m. lights on, 7 p.m. lights off). One day after surgery, a 4-mm microdialysis probe was inserted into the guide cannula and perfused overnight at 0.3 μl/min with artificial cerebral spinal fluid (aCSF: NaCl, 147 mM; $CaCl_2$, 1.3 mM; KCl, 2.7 mM; $MgCl_2$, 1.0 mM). For dual probe experiments, a 2-mm probe was inserted into the septal guide. Around 7:30 a.m. on the day after probe insertion, 100 nM neostigmine-bromide was added to the aCSF, the flow rate was increased to 2 μl/min and hippocampal dialysates were collected in a 30-μl sample loop and analyzed by on-line high performance liquid chromatography with electrochemical detection at 17-min intervals. For dual probe experiments, drugs were dissolved in aCSF (without neostigmine) and perfused into the septum at 2 μl/min, while acetylcholine was measured in hippocampal dialysate. Acetylcholine in microdialysates was assayed using a modification of an acetylcholine–choline assay kit from BAS (Bioanalytical Systems). Briefly, acetylcholine was separated at 1 ml/min at 28 °C over two 10-cm BAS polymer MF 6150 Acetylcholine Analytical Columns, connected in series, using a mobile phase with 35 mM Na_2HPO_4, 0.1 mM EDTA and 0.005% ProClin adjusted to pH 8.5 with phosphoric acid. Acetylcholine was then converted in a post-column acetylcholine–choline immobilized enzyme reactor (BAS MF-6151 Acetylcholine/Choline IMER) to hydrogen peroxide, which was detected electrochemically at a platinum electrode at a potential of +0.5 V vs. Ag/AgCl. (Antec DECADE, Leyden, The Netherlands).

Once basal acetylcholine levels had stabilized (after 8–10 samples), drugs were administered and the effects on acetylcholine monitored for at least 3 h. Drug-induced changes in extracellular acetylcholine levels were expressed as percentages of baseline (mean of last five to six basal samples)±S.E.M. (n=4–8) and used to construct time courses. Since handling the animals and s.c. injection of vehicle consistently caused a small, rapid and short lasting acetylcholine increase, which returned to basal levels within 50 min, the average response over 51–102 min after drug administration was used for the dose–response curves. For a comparison of in vivo potencies, we estimated the dose of each compound that produces a four-fold acetylcholine increase from the s.c. dose–response curves ($ED_{400\%}$), since responses to these doses are on the ascending part of the dose–response curves and data bracket the four-fold change. Limited solubility prevented perfusions with sufficiently high concentrations to reach four-fold increases for all compounds, so we choose the concentrations that produce a three-fold acetylcholine increase ($EC_{300\%}$) for potency comparisons, again with responses on the ascending part of the concentration–response curves and data bracketing the three-fold change.

2.4. HPLC/mass spec analysis of ziprasidone, thioridazine and olanzapine

At 0.5, 1, 2 and 4 h after s.c. administration of 10 mg/kg olanzapine, 10 mg/kg thioridazine or 3.2 mg/kg ziprasidone to male Sprague–Dawley rats (300–350 g), plasma and whole brain were collected. Brain tissue, homogenized in three mass equivalents H_2O, and plasma samples were subjected to liquid–liquid extraction using the following method: 200 μl of each matrix was sampled and spiked with 20 ng of an internal standard (different for each compound). To this mixture, 100 μl of 1 M $NaHCO_3$ was added. The mixture was vortexed with 0.5-ml methyl-t-butylether for 5 min. The organic and aqueous phases were then separated by centrifugation at 2000×g for 10 min. The organic layer was removed and evaporated to dryness under nitrogen at ambient temperature. The residue was reconstituted in 200 μl of 70% methanol in H_2O and analyzed by HPLC-MS. Standards for plasma, brain and CSF analysis were prepared from identically treated blank sample matrices, which were

subsequently spiked with the analyte of interest. An Agilent HP-1100 quaternary pump system (Agilent, Palo Alto, CA, USA), and a CTC PAL autosampler (CTC Analytics, Zwingen, Switzerland) were used to separate compounds and internal standards on a 50×2.1 mm I.D. Ace C-8 column (Advanced Chromatography Technologies, Aberdeen, Scotland) at ambient temperature. Mobile phase "A" consisted of 10 mM ammonium formate adjusted to either pH 3.0 with formic acid (ziprasidone, thioridazine) or left pH unadjusted (olanzapine). Mobile phase "B" consisted of 100% acetonitrile. A linear gradient from 85% "A" to 5% "A" over 1.5 min at a flow rate of 0.4 ml/min was run for all analyses. Eluants were detected using a Micromass Quattro Ultima mass spectrometer equipped with an electrospray ionization source (Waters, Milford, MA, USA) operated in positive ion MRM mode. Reported brain and plasma concentrations represent the mean±S.D. (n=4).

2.5. Data handling and statistics

To determine statistical differences between treatments, data were analyzed in SAS Version 8 (SAS Institute, Cary, NC, USA), using a mixed effects repeated measures model with autoregressive order 1 variance. The data were repeated by time nested within dose. Multiple comparisons of the time course data were done using the Western Electric procedure for runs (Small, 1956) to control the family-wise and experiment-wise error rates. All data were analyzed together to obtain the most accurate estimate of error. F-values were used to determine if time nested within dose was significant for each compound and then t-values were used to determine which times within doses were significant. To determine statistical differences between doses, or concentrations, a mixed effect model was used with animal as a random effect and with compound symmetric variance. Multiple comparisons were done using Dunnett's procedure, which produces adjusted p values that were considered significant when less than 0.05. All data were again analyzed together to obtain the most accurate estimate of error. Overall F-values were used to determine if there was a difference in concentrations and then t-values from Dunnett's procedure determine which concentrations differed from baseline. To determine estimates of $ED_{400\%}$ and $EC_{300\%}$ values, the percentage of baseline was first regressed onto the doses, or the concentrations, that bracketed the four-fold or three-fold change, respectively, and then using inverse prediction. A 95% inverse prediction interval was then calculated for each estimate. The regression analysis, inverse predictions and inverse prediction intervals were performed using R Version 1.8.1 (The R Foundation for Statistical Computing).

2.6. Drugs and chemicals

Ziprasidone and olanzapine were synthesized at Pfizer Global Research and Development (Groton, CT, USA). Scopolamine, thioridazine, clozapine, haloperidol, risperidone, chlorpromazine, 7-chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1*H*-3-benzazepine (SCH-23390) and 2-hydroxypropyl-β-cyclodextrin were purchased from Sigma (St. Louis, OH); [^{3}H]*N*-methyl-scopolamine from Perkin Elmer (Boston, MA); methanol from J.T. Baker (Philipsburg, NJ); and all other analytical grade chemicals from Fluka Chemika-BioChemika (Ronkonkoma, NY). CHO cell lines expressing the human muscarinic receptors were obtained from Dr. Thomas Bonner (NIH). Ziprasidone and thioridazine were dissolved in 40% 2-hydroxypropyl-β-cyclodextrin, all other compounds in 5.5% glucose acidified with acetic acid unless otherwise noted. Drugs were administered subcutaneously (s.c.) in a volume of 1 ml/kg. For local drug perfusions via the microdialysis probe, stock solutions of the compounds in aCSF were diluted with aCSF to the appropriate concentration.

3. Results

3.1. In vitro binding affinities and functional activities at muscarinic M_1 and M_2 receptors

Thioridazine binds to human muscarinic M_1 and M_2 receptors expressed in CHO cells with similar high affinities as olanzapine and clozapine (K_i 1.4–14 nM, Table 1), but its functional muscarinic M_2 antagonist potency (K_b=188 nM) is 10-fold less than that of olanzapine and 5-fold less than clozapine (Table 2). Chlorpromazine binds with moderate affinity to human muscarinic M_2 receptors (K_i=150 nM) and is functionally about 35-fold less potent than olanzapine as a muscarinic M_2 antagonist (K_b=601 nM). Scopolamine has the highest affinity (K_i=0.1–0.2 nM) and functional antagonist activity (K_b=0.16–0.09 nM) at muscarinic M_1 and M_2 receptors. Ziprasidone, risperidone and haloperidol have no appreciable affinity at human muscarinic M_1 or M_2 receptors. Binding affinities of the compounds to native muscarinic receptors in rat cerebellar membranes, which are

Table 1
In vitro binding affinities for human M_1 and M_2 receptors expressed in CHO cells and for native rat M_1 and muscarinic M_2 receptors in rat cortex and cerebellum, respectively

Drug	*h* M_1 affinity	*h* M_2 affinity	*r* M_1 affinity	*r* M_2 affinity
	K_i (nM)		K_i (nM)	
Olanzapine	2.5±0.3[a]	13±0.8[a]	1.9±0.1[a]	84±7
Clozapine	1.4±0.3[a]	7±1[a]	1.9±0.4[a]	120±13
Scopolamine	0.1±0.1	0.2±0.1		1.8±0.2
Thioridazine	2.7±0.3[b]	14[b]±3		454±89
Chlorpromazine	25±3[b]	150[b]±14		868±22
Ziprasidone	>2000	>2000		>3000
Haloperidol	>1500	>2000	>1500[a]	
Risperidone	>10,000[a]	>10,000[a]	>10,000[a]	>3000

[a] K_i values from Bymaster et al. (1996).
[b] K_i values from Bolden et al. (1992).

Table 2
In vitro functional potencies at human M_1 and M_2 receptors expressed in CHO cells (K_b in nM) and in vivo potencies to increase acetylcholine release in rat hippocampus ($ED_{400\%}$ = estimated dose required to increase acetylcholine four-fold; $EC_{300\%}$ = estimated concentration required to increase acetylcholine three-fold)

Drug	In vitro		In vivo	
	h M_1 potency, K_b (nM)	*h* M_2 potency, K_b (nM)	$ED_{400\%}$ (mg/kg s.c.)	$EC_{300\%}$ (μM perfused)
Olanzapine	8.7±3.4	17.2±3.8	6.6	8.6
Clozapine	12.2±2.9	35.4±4.0	16.3	50.5
Scopolamine	0.16±0.05	0.09±0.04	0.05	0.01
Thioridazine	86±27	188±44	97	129
Chlorpromazine	220±56	601±258	>100[a]	
Ziprasidone	>2000	>3000	≫32[b]	
Haloperidol	>2000	>3000	≫1[c]	
Risperidone	>2000	>3000		>100[d]

[a] Two-fold increase at 100 mg/kg s.c.
[b] 1.4-fold increase at 32 mg/kg s.c.
[c] No effect at 1 mg/kg s.c.
[d] 1.5-fold increase at 100 μM.

85% muscarinic M_2 receptors (Cohen et al., 2000), are generally lower than affinities for human M_2 receptors (Table 1), while thioridazine and chlorpromazine bind with 5–10-fold lower affinity than olanzapine to native rat M_2 receptors.

3.2. Effects of systemically administered test compounds on acetylcholine release

The mean basal acetylcholine concentration in microdialysates from rat hippocampus collected in the presence of 100 nM neostigmine was 10.1±0.4 nM (300 fmol/30 μl sample) and ranged from 3.7 to 16.8 nM (n=60). Vehicle administration (acidified 5.5% glucose or 40% cyclodextrin) did not change acetylcholine levels except for a rapid, transient 1.5-fold increase that returned to baseline within 50 min. The average vehicle response over the time period 51–102 min, used to construct dose–response curves (96±5% for acidified glucose and 110±7% for 40% 2-hydroxypropyl-β-cyclodextrin), were not significantly different from basal levels. Administration of high doses of the test compounds caused mild and transient sedative effects.

Time courses for effects of 10 mg/kg of atypical antipsychotics show that olanzapine and clozapine produce robust and sustained increases in acetylcholine levels in the rat hippocampus to seven-fold ($t_{[13]}$=7.95, P<0.0001) and four-fold ($t_{[16]}$=7.3, P<0.0001) over basal levels, respectively, at 2 h (Fig. 1). Ziprasidone treatment produced a modest 1.4-fold elevation of acetylcholine release, which was significant at the 6.5% level ($t_{[8]}$=2.51, P=0.064). While 10 mg/kg thioridazine had no effect on acetylcholine release ($t_{[11]}$=0.72, P=0.820) and 32 mg/kg produced a not significant two-fold increase ($t_{[11]}$=1.48, P=0.3636), 100 mg/kg thioridazine significantly increased acetylcholine levels four-fold ($t_{[11]}$=5.87, P=0.0003; Fig. 2). Likewise, a high dose of 100 mg/kg chlorpromazine produced a significant 2.5-fold increase ($t_{[4]}$=8.61, P=0.018) in acetylcholine release (Fig. 3). Haloperidol at 1 mg/kg did not have a significant effect on acetylcholine levels ($t_{[4]}$=−1.24, P=0.284).

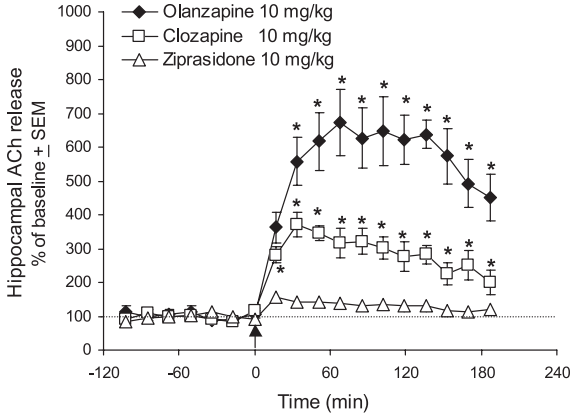

Fig. 1. Time courses for the effects of antipsychotics on extracellular acetylcholine levels in the rat ventral hippocampus. Drugs were administered (arrow, t=0) at a dose of 10 mg/kg s.c. Data are expressed as % of baseline levels and represent the mean±S.E.M. (n=5–8). Ziprasidone was given in 40% cyclodextrin vehicle, the other drugs in acidified 5.5% glucose. *P<0.05 vs. vehicle treatment.

The effects of systemic treatment with antipsychotics on extracellular acetylcholine levels are dose-dependent and dose–response curves of olanzapine, clozapine and thioridazine show a parallel shift to the right compared with scopolamine (Fig. 3). A comparison of the estimated s.c. doses required to increase acetylcholine release to 400% of basal levels shows that olanzapine is the most potent of the antipsychotics tested, with an $ED_{400\%}$ of 6.6±3.2 mg/kg (Fig. 3), being 2.5 and 15 times more potent than clozapine ($ED_{400\%}$=16.3±7.8 mg/kg) and thioridazine ($ED_{400\%}$= 97±37 mg/kg), respectively. Scopolamine, a non-selective muscarinic antagonist with the highest affinity for muscarinic receptors among the test compounds, showed the highest potency in this in vivo model, causing a maximal

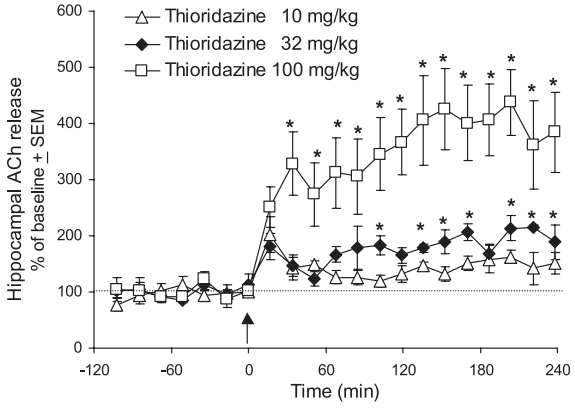

Fig. 2. Time courses for the effects of thioridazine administered at doses of 10, 32 and 100 mg/kg s.c. Data are expressed as % of baseline levels and represent the mean±S.E.M. (n=5). Thioridazine was given in 40% cyclodextrin vehicle. *P<0.05 vs. vehicle treatment.

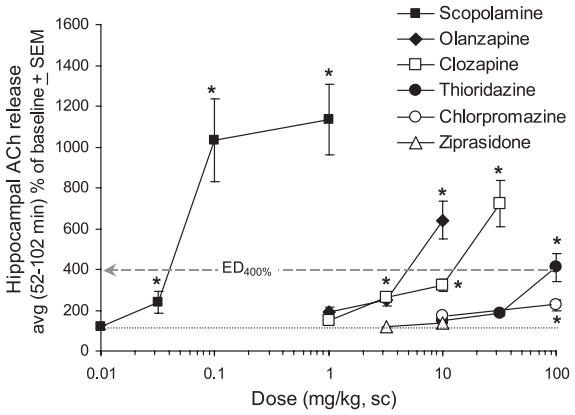

Fig. 3. Dose–response curves for the effects of antipsychotics and scopolamine on acetylcholine release in rat hippocampus after s.c. administration and estimation of $ED_{400\%}$ values (doses that produce a four-fold acetylcholine increase). Data are expressed as % of baseline levels and represent the average of data collected between 51 and 102 min±S.E.M. (n=5–8, scopolamine n=3–4). *P<0.05 vs. vehicle treatment.

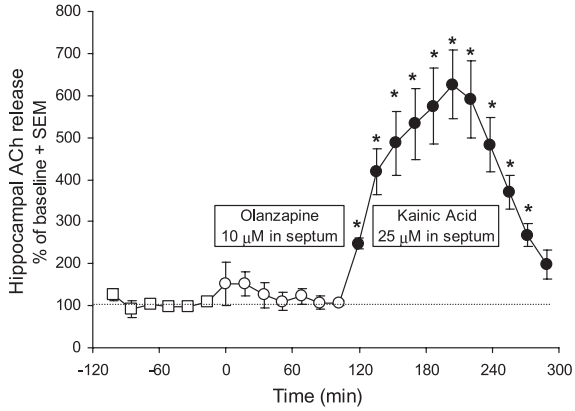

Fig. 4. Time course for the effects of perfusions with olanzapine (10 μM) and kainic acid (25 μM) into the septum on acetylcholine release in rat hippocampus. Compounds were perfused into the septum via the dialysis probe (bars) and the acetylcholine response measured in samples collected from the hippocampus. Data are expressed as % of baseline levels and represent the mean±S.E.M. (n=4). *P<0.05 vs. aCSF.

increase in acetylcholine levels to 10–12-fold over basal values after 1 mg/kg s.c. and is 130 times more potent than olanzapine ($ED_{400\%}$=0.05±0.02 mg/kg, Fig. 3).

3.3. Effect of locally perfused test compounds in cell bodies or terminals by retrodialysis

To examine whether olanzapine exerts its effect on hippocampal acetylcholine release in cell bodies or terminals, it was delivered directly into the terminal area (hippocampus) or cell body area (septum) by retrodialysis, while monitoring effects on acetylcholine levels in the hippocampus. Local perfusion of 10 μM olanzapine into cell bodies did not increase acetylcholine release in the hippocampus ($t_{[6]}$=0.13, P=0.9876). To verify correct probe placement and proper functioning of the cholinergic system, the glutamatergic agonist kainic acid (25 μM) was subsequently perfused into the septum as a positive control and was found to increase hippocampal acetylcholine release to six-fold over basal levels ($t_{[6]}$=14.65, P<0.0001; Fig. 4). In contrast, local application of olanzapine into the hippocampus (Fig. 5) produced a non significant 1.8-fold increase in acetylcholine release after 1 μM ($t_{[8]}$=1.77, P=0.2636) and a significant 3.5-fold increase after 10 μM ($t_{[8]}$=5.20, P=0.0022), while a 100 μM olanzapine perfusion produced a similar seven-fold acetylcholine increase ($t_{[8]}$=12.11, P<0.0001), as observed after systemic administration of 10 mg/kg olanzapine (Figs. 3 and 6). Hippocampal acetylcholine levels were also significantly increased after local perfusions of 0.01 μM scopolamine ($t_{[4]}$=4.88, P=0.0142), 10 μM clozapine ($t_{[8]}$=4.32, P=0.0068) and 100 μM thioridazine ($t_{[9]}$=4.97, P=0.0021) into the terminal area (Fig. 6). A comparison of the perfusate concentrations required to cause a 300% increase in acetylcholine release shows that olanzapine ($EC_{300\%}$=8.6±2.7 μM) is approximately 5 and 15 times more potent than clozapine ($EC_{300\%}$=50.5±47.2 μM) and thioridazine ($EC_{300\%}$=129±108 μM), respectively. Scopolamine is also the most potent compound after local perfusion and with an $EC_{300\%}$ of 0.01±0.02 μM it is approximately 850 times more potent than locally applied olanzapine (Fig. 6). It should be noted that these are drug concentrations in the perfusate and that the amount of drug that actually reaches the tissue, typically 5–10% of the perfusate concentration, is unknown.

Linear regression analysis shows that in vivo potencies of the test compounds to increase acetylcholine release in the rat hippocampus ($ED_{400\%}$ or $EC_{300\%}$) are highly correlated with their in vitro functional potencies (K_b) at muscarinic M_2 receptors. The correlation coefficients for log K_b vs. log $ED_{400\%}$ and vs. log $EC_{300\%}$ are 0.998 and 0.985, respectively, and the slopes from the fitted regression equations are not significantly different from 1.

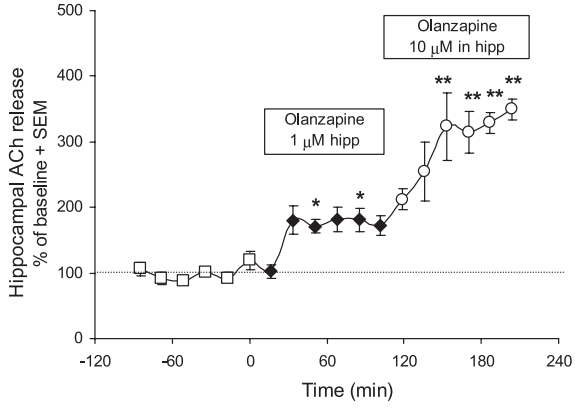

Fig. 5. Time course for the effect of olanzapine perfused directly into the hippocampus on acetylcholine release in rat hippocampus. Olanzapine (1 and 10 μM) was added to the perfusing solution (bars), while monitoring acetylcholine levels in rat hippocampus. Data are expressed as % of baseline levels and represent the mean±S.E.M. (n=4). *P<0.05 vs. aCSF, **P<0.05 vs. 1 μM olanzapine.

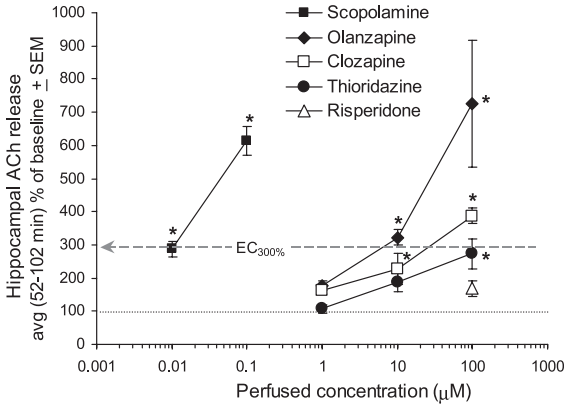

Fig. 6. Concentration–response curves for the effects of antipsychotics and scopolamine perfused via the dialysis probe into rat hippocampus on acetylcholine release in hippocampus and estimation of $EC_{300\%}$ values (concentrations that produce a three-fold acetylcholine increase). Increases in acetylcholine release were calculated by averaging the values from three samples collected between 51 and 102 min after the start of the drug perfusion. Data are expressed as % of baseline levels and represent the mean±S.E.M. (n=3–5). *P<0.05 vs. aCSF.

3.4. Brain and plasma levels of olanzapine, thioridazine and ziprasidone

After a single 10 mg/kg dose, brain levels of olanzapine increased rapidly reaching a C_{max} of 7500 ng/g within 0.5–1 h and decreased to 2600 ng/g after 4 h. Olanzapine plasma levels showed a similar time course with a C_{max} of 2960 ng/ml at 0.5 h and decreasing to 790 ng/ml at 4 h. Brain and plasma levels after 10 mg/kg thioridazine were lower and reached maximum levels slower than olanzapine, with C_{max} values of 2,050 ng/g and 250 ng/ml, respectively, at 2 h and remaining steady until 4 h. Ziprasidone brain and plasma levels after 3.2 mg/kg were 287 ng/g and 422 ng/ml, respectively, at 1 h after administration.

4. Discussion

4.1. Increases in acetylcholine release via muscarinic antagonism

Our data confirm the ability of olanzapine and clozapine to produce robust increases in extracellular acetylcholine levels in rat hippocampus in the presence of neostigmine, as previously reported by Shirazi-Southall et al. (2002). The results from our and previous studies provide several lines of evidence that the large increases in acetylcholine release, produced during acetylcholinesterase inhibition by antipsychotics with muscarinic antagonist properties, including thioridazine and chlorpromazine, are mediated via blockade of muscarinic M_2 autoreceptors.

First, olanzapine and clozapine are potent functional antagonists at muscarinic M_2 receptors and produce increases in extracellular acetylcholine levels during acetylcholinesterase inhibition that are of the same order of magnitude as those following administration of the muscarinic antagonist scopolamine, which enhances acetylcholine release by blocking muscarinic M_2 autoreceptors (Douglas et al., 2001; Stillman et al., 1996; Kitaichi et al., 1999a,b). Recent data showing that clozapine- and olanzapine-induced increases in acetylcholine release are dependent on the acetylcholinesterase inhibitor concentration (Ichikawa et al., 2002; Chung et al., 2003) corroborate a muscarinic antagonist mechanism, since the local cholinergic tone significantly influences the magnitude of muscarinic receptor antagonist-induced acetylcholine increases. Second, local perfusions with a high concentration of olanzapine produce four-fold increases in terminal acetylcholine release when administered into the terminal hippocampal area, but have no effect when perfused into the cell bodies in the septum, in agreement with the lack of effects following local perfusions with the muscarinic antagonist atropine (Moor et al., 1995). Third, the in vivo potencies of compounds to increase acetylcholine release after systemic or intracerebral administration correlate highly with their in vitro functional potencies at muscarinic M_2 receptors. Finally, antipsychotics that share several receptor affinities with olanzapine and clozapine, but are devoid of muscarinic M_2 antagonist properties, only modestly increase acetylcholine release during acetylcholinesterase inhibition.

Nevertheless, in previous reports, muscarinic antagonism was not considered to be the predominant mechanism, mainly because thioridazine and chlorpromazine, antipsychotics with high to moderate binding affinity for muscarinic M_2 receptors, failed to produce robust acetylcholine increases after systemic doses of 10–30 mg/kg (Shirazi-Southall et al., 2002; Ichikawa et al., 2002; Li et al., 2003; Bymaster et al., 2003). However, while 10 mg/kg thioridazine failed to significantly increase acetylcholine, a higher dose of 100 mg/kg produced a marked increase in acetylcholine release to four-fold over basal levels. Likewise, chlorpromazine also has the ability to increases acetylcholine release, producing a 2.5-fold increase after 100 mg/kg. Moreover, intra-hippocampal perfusions demonstrate that thioridazine, like olanzapine, clozapine, ziprasidone and scopolamine, increase acetylcholine levels via a local action in the hippocampus, consistent with muscarinic antagonism at terminal presynaptic muscarinic M_2 receptors.

The reduced in vivo potencies of thioridazine and chlorpromazine can be largely explained by their surprisingly weak muscarinic M_2 antagonist potencies, since thioridazine and chlorpromazine are respectively 10- and 35-fold less potent M_2 receptor antagonists than olanzapine. In addition, while thioridazine and olanzapine bind with equal affinities to human M_2 receptors, thioridazine has a five-fold lower affinity than olanzapine for native rat brain muscarinic M_2 receptors, which seems more relevant for effects that occur in rat brain than binding affinities at human or rat heart receptors. The weaker functional activity

plus the reduced binding affinity for native rat muscarinic M_2 receptors explain why much higher doses and concentrations of thioridazine are required to produce acetylcholine responses in vivo similar to olanzapine and clozapine. Such a disparity between the functional activity and binding affinity of thioridazine at muscarinic receptors was previously demonstrated by Niedzwiecki et al. (1989), who showed that thioridazine is several orders of magnitude less potent than atropine in antagonizing carbachol-induced acetylcholine overflow in rabbit striatal slices. These large differences between binding affinities and functional activities are likely due to differences in assay conditions (e.g. assay buffer, assay temperature, cell membranes vs. whole cells).

Recent data showing that olanzapine increases acetylcholine release to the same extent in M_2/M_4 knockout mice as in wild-type mice, whereas scopolamine shows an attenuated response in knockout mice (Bymaster et al., 2003), seem to argue against an involvement of muscarinic M_2 receptors. However, without the strong inhibitory control supplied by muscarinic M_2 autoreceptors, the otherwise small contributions of non-muscarinic receptor mechanisms to the acetylcholine increase, such as seen without a cholinesterase inhibitor, could be amplified in genetically altered mice and cause similar olanzapine-induced acetylcholine increases in knockout as in wild-type mice. In contrast, the effects of the muscarinic receptor antagonist scopolamine are exclusively mediated by muscarinic M_2 receptor blockade and thus attenuated in M_2 deficient mice. Taken together with the striking differences between cholinergic effects in mice and rats (drug-induced acetylcholine increases are 5–10-fold lower in mice than in rats), the data in M_2/M_4 knockout mice are not inconsistent with an important role of muscarinic autoreceptors in the rat acetylcholine release model.

4.2. Other possible mechanisms of antipsychotic-induced acetylcholine release

Given the fact that antipsychotics have high affinities for multiple receptors and receptor subtypes, it is likely that other receptor mechanisms contribute to the acetylcholine increase produced by some antipsychotics during acetylcholinesterase inhibition. However, there are few known receptor-mediated mechanism that can account for the magnitude of the increase in acetylcholine release observed after olanzapine and clozapine treatment. Ichikawa et al. (2002) and Shirazi-Southall et al. (2002) have extensively discussed receptors that are possibly involved in mediating the acetylcholine increase, such as 5-HT_{1A}, 5-$HT_{2A/C}$, 5-HT_6, dopamine D_2 receptors and $\alpha_{1/2}$ adrenoceptors. However, blockade of these receptors only results in modest increases in acetylcholine release and likely mediates the small acetylcholine increases produced by all atypical antipsychotics in the absence of an acetylcholinesterase inhibitor (Ichikawa et al., 2002; Chung et al., 2003). For instance, activation of 5-HT_{1A} receptors, known to increase hippocampal acetylcholine levels by 1.5–2-fold (Shirazi-Southall et al., 2002; Erb et al., 1997) could explain the small acetylcholine increase following ziprasidone and could also contribute to the clozapine-induced acetylcholine release, since both compounds are 5-HT_{1A} receptor agonists, but does not play a role in the effects of olanzapine, which has poor affinity for 5-HT_{1A} receptors (Bymaster et al., 1996).

There are two other receptor mechanisms that are known to cause pronounced increases in hippocampal acetylcholine release. One is receptor-mediated activation of cholinergic cell bodies located in the septum that can enhance hippocampal acetylcholine release five-fold (Moor et al., 1996). However, when olanzapine is directly applied via the microdialysis probe into the septum, at a concentration that produces a robust acetylcholine increase when perfused into the hippocampus, extracellular acetylcholine levels in the ipsilateral hippocampus are not affected, suggesting that cell body receptor activation is not involved in the olanzapine-induced acetylcholine increase, consistent with the observation that the muscarinic antagonist atropine does not activate receptors in the septum after local infusion (Moor et al., 1995). Another mechanism causing robust acetylcholine increases is activation of a permissive dopamine synapse dependent pathway, recently described by Rao et al. (2003), who reported that a nicotinic agonist-induced 20-fold increase in acetylcholine release was completely blocked by the nicotinic antagonist mecamylamine, as well as by the dopamine D_1 receptor antagonist SCH-23390. In addition, direct activation of the dopamine D_1 receptor by the dopamine D_1 receptor agonist dihydrexidine increased acetylcholine levels in rat frontal cortex three-fold (Steele et al., 1997). Since we found that the effect of olanzapine on acetylcholine release was not attenuated by pretreatment with SCH-23390 (data not shown), neither activation of a permissive dopamine synapse dependent pathway nor of the dopamine D_1 receptor is involved in the effects of olanzapine.

4.3. Pharmacokinetic considerations

Since differences in drug exposures can also contribute to reduced in vivo potencies of certain antipsychotics, we measured plasma and brain levels of olanzapine, thioridazine and ziprasidone after single s.c. doses. The results show that while olanzapine and thioridazine both have high brain exposure after 10 mg/kg s.c., thioridazine levels are substantially lower than those of olanzapine, which may partially contribute to thioridazine's inability to increase extracellular levels of acetylcholine at low doses. Since ziprasidone reaches sufficiently high brain levels to elicit a pharmacological effect after a 3.2 mg/kg dose, its small effect on acetylcholine release is due to lack of muscarinic antagonist properties rather than to poor brain penetration.

4.4. Muscarinic antagonism of olanzapine and clozapine in other in vivo models

Several in vivo pre-clinical studies have demonstrated functional anti-muscarinic activity by olanzapine and clozapine, despite a reportedly low occupancy of muscarinic receptors in rat brain by olanzapine and clozapine (Zhang and Bymaster, 1999). For instance, clozapine blocks carbachol- and acetylcholine-induced, but not glutamate-induced, firing in hippocampal neurons in anesthetized cats (Herrling and Misbach-Lesenne, 1982). Also, olanzapine and clozapine block oxotremorine-induced tremors in mice at doses comparable to those used in this study, olanzapine being 4 times more potent than clozapine (Moore et al., 1992). Since oxotremorine-induced tremors are considered to be associated with muscarinic M_2 receptor activation (Gomeza et al., 2001), these data are consistent with the relative potencies of the two compounds to increase acetylcholine release via muscarinic M_2 receptor blockade. Furthermore, olanzapine and clozapine block a muscarinic M_1 receptor mediated effect, pilocarpine-induced phosphoinositide hydrolysis in rat hippocampus (Zhang and Bymaster, 1999), in agreement with their high affinities and functional antagonist activities at muscarinic M_1 receptors. Although it is not known what level of receptor occupancy by muscarinic antagonists is necessary to manifest central nervous system effects, these data demonstrate the potential for in vivo muscarinic antagonism during olanzapine treatment.

4.5. Improvement in cognitive function via muscarinic antagonism?

There is evidence that atypical antipsychotics have beneficial effects on some of the cognitive impairments in schizophrenia, such as deficits in learning, secondary memory, attention and executive functioning (Harvey and Keefe, 2001). Since increased acetylcholine release is known to improve cognitive and memory functions, most likely via postsynaptic muscarinic M_1 receptor activation (Fischer, 2003), it was speculated that the robust effects of olanzapine and clozapine on acetylcholine levels contribute to the improvement in cognitive function (Shirazi-Southall et al., 2002). As olanzapine and clozapine lack muscarinic subtype selectivity, it has to be assumed that the acetylcholine increase produced by these compounds is sufficient to overcome simultaneous M_1 receptor blockade, so that the net outcome of the balance between the opposing effects of olanzapine on acetylcholine release and M_1 receptor blockade is cognitive improvement (Zhang and Bymaster, 1999; Kennedy et al., 2001; Shirazi-Southall et al., 2002; Dean et al., 2003). However, the fact that olanzapine is at least an equipotent muscarinic M_1 and M_2 antagonist in vitro, either in binding affinities or in functional potencies (Tables 1 and 2), argues against this assumption. In addition, in vivo data also indicate that olanzapine is a slightly more potent as M_1 antagonist, blocking a muscarinic M_1 mediated effect, pilocarpine-induced PI hydrolysis (Zhang and Bymaster, 1999), more effectively than muscarinic M_2 mediated effects, such as oxotremorine-induced tremors (Moore et al., 1992) and autoreceptor control of acetylcholine release (this study). Hence, doses of olanzapine that increase acetylcholine release via muscarinic M_2 receptor blockade will most likely also block postsynaptic muscarinic M_1 receptors. Moreover, olanzapine only produces the large acetylcholine increases during acetylcholinesterase inhibition, so that under normal tonic conditions, when olanzapine only moderately enhances acetylcholine release (Ichikawa et al., 2002), much less acetylcholine is available to overcome postsynaptic receptor blockade. Finally, the non-selective muscarinic antagonist scopolamine has a similar muscarinic M_2 to M_1 antagonist potency ratio as olanzapine (Tables 1 and 2), but causes cognitive impairment and amnesia by blocking postsynaptic muscarinic M_1 receptors (Carey et al., 2001), and olanzapine has been shown to produce scopolamine-like cognitive deficits in some animal models (Rasmussen et al., 2001). Therefore, while olanzapine and clozapine have been reported to improve some aspects of cognition, our data suggest that it is unlikely that such an improvement is mediated by increased ACh release, but possibly by other mechanisms that are shared by most atypical antipsychotics, including those that do not produce robust ACh increases in the presence of neostigmine. In fact, if the balance of increased acetylcholine levels and muscarinic M_1 receptor blockade determines effects on cognitive function, one can speculate that antipsychotics that produce only modest acetylcholine increases, but lack affinity for muscarinic receptors and therefore the potential to block postsynaptic M_1 receptors, could improve cognitive deficits via a cholinergic mechanism. Results from recent studies, showing performance in patients improved after switching from olanzapine to ziprasidone (Harvey et al., 2004), are consistent with this notion. Interestingly, it was recently reported (Sur et al., 2003) that *N*-desmethylclozapine, a major clozapine metabolite, binds to an allosteric site and has agonist activity at the muscarinic M_1 receptor. If the muscarinic M_1 receptor agonist activity of N-desmethylclozapine could overcome clozapine's muscarinic M_1 receptor antagonist activity, this could facilitate M_1 mediated cholinergic neurotransmission and could differentiate clozapine's cognitive profile from that of olanzapine.

In conclusion, while the finding that olanzapine and clozapine cause robust acetylcholine increases in rat hippocampus during acetylcholinesterase inhibition is in agreement with the results of Shirazi-Southall et al. (2002), our additional data suggest that this effect is most likely mediated by blockade of muscarinic M_2 autoreceptors. The failure of thioridazine and chlorpromazine to increase acetylcholine levels at doses comparable to those of olanzapine and clozapine is mainly due to their much weaker functional antagonist activities at muscarinic M_2 receptors, while lower

brain levels and lower binding affinities at rat brain muscarinic M_2 receptors further reduce their in vivo potencies. The excellent agreement between in vivo potencies of the antipsychotics to increase acetylcholine release and their in vitro functional activities at muscarinic M_2 receptors is consistent with a muscarinic M_2 antagonist mechanism. While improvements in schizophrenic patients treated with atypical antipsychotics have been reported for a wide range of cognitive parameters, clinical measures that will predict significant functional improvement in patients and the neurochemical mechanisms behind those improvements are still unclear (Harvey and Keefe, 2001). Given their potent antagonist activity at all muscarinic receptors, it is unlikely that the increase in acetylcholine seen with olanzapine or clozapine is responsible for the cognitive improvements reported for these drugs.

Acknowledgement

The authors gratefully acknowledge Dr. Kimberly Crimin (Department of Non-Clinical Biostatics, Pfizer PGRD, Groton) for statistical analyses of the data.

References

Bolden, C., Cusak, B., Richelson, E., 1992. Antagonism by antimuscarinic and neuroleptic compounds at five cloned human muscarinic cholinergic receptors expressed in Chinese hamster ovary cells. J. Pharmacol. Exp. Ther. 260, 576–580.

Bymaster, F.P., Calligaro, D.O., Falcone, J.F., Marsh, R.D., Moore, N.A., Tye, N.C., Seeman, P., Wong, D.T., 1996. Radioreceptor binding profile of the atypical antipsychotic olanzapine. Neuropsychopharmacology 14, 87–96.

Bymaster, F.P., Felder, C.C., Tzavara, E., Nomikos, G.G., Calligaro, D.O., McKinzie, D.L., 2003. Muscarinic mechanisms of antipsychotic atypicality. Progr. Neuro-psychopharmacol. Biol. Psychiatry 27, 1125–1143.

Carey, G.J., Billard, W., Binch, H., Cohen-Williams, M., Crosby, G., Grzelak, M., Guzik, H., Kozlowski, J.A., Lowe, D.B., Pond, A.J., Tedesco, R.P., Watkins, R.W., Coffin, V.L., 2001. SCH 57790, a selective muscarinic muscarinic M2 receptor antagonist, releases acetylcholine and produces cognitive enhancement in laboratory animals. Eur. J. Pharmacol. 431, 189–200.

Chung, Y.C., Li, Z., Dai, J., Meltzer, H.Y., Ichikawa, J., 2003. Clozapine, an atypical antipsychotic increases acetylcholine and dopamine release in rat ventral hippocampus and medial prefrontal cortex, whereas the effect of typical antipsychotic haloperidol is minimal: role of 5-HT_{1A} receptor agonism. Abstr.-Soc. Neurosci. 33, 848.9. (Washington, DC).

Cohen, V.I., Jin, B., McRee, R.C., Boulay, S.F., Cohen, E.I., Sood, V.K., Zeeberg, B.R., Reba, R.C., 2000. In vitro and in vivo m2 muscarinic subtype selectivity of some dibenzodiazepinones and pyridobenzodiazepinones. Brain Res. 861, 305–315.

Dean, B., Bymaster, F.P., Scarr, E., 2003. Muscarinic receptors in schizophrenia. Curr. Mol. Med. 3, 419–426.

Douglas, C.L., Baghdoyan, H.A., Lydic, R., 2001. Muscarinic M_2 muscarinic autoreceptors modulate acetylcholine release in prefrontal cortex of C57BL/6J mouse. J. Pharmacol. Exp. Ther. 299, 960–966.

Erb, C., Klein, J., Koppen, A., Loffelholz, K., Jeltsch, H., Cassel, J.C., 1997. Modulation of hippocampal acetylcholine release after fimbra-fornix lesions and septal transplantation in rats. Neurosci. Lett. 231, 5–8.

Fischer, A., 2003. M_1 muscarinic agonists can modulate some of the hallmarks in Alzheimer's disease: implications in future therapy. J. Mol. Neurosci. 20, 349–356.

Gomeza, J., Zhang, L., Kostenis, E., Felder, C.C., Bymaster, F.P., Brodkin, J., Shannon, H., Xia, B., Duttaroy, A., Deng, C.X., Wess, J., 2001. Generation and pharmacological analysis of muscarinic M_2 and M_4 muscarinic receptor knockout mice. Life Sci. 68, 2457–2466.

Harvey, P.D., Keefe, R.S.E., 2001. Studies of cognitive change in patients with schizophrenia following novel antipsychotic treatment. Am. J. Psychiatry 158, 1184–1760.

Harvey, P.D., Meltzer, H.Y., Simpson, G.M., Potkin, S.G., Loebel, A., Siu, C., Romano, S.J., 2004. Improvement in cognitive function following a switch to ziprasidone from conventional antipsychotics, olanzapine, or risperidone in outpatients with schizophrenia. Schizophr. Res. 66, 101–113.

Herrling, P.L., Misbach-Lesenne, B., 1982. Effects of clozapine in a selective muscarinic bioassay and on single cells of the rat hippocampus. Naunyn-Schmiedberg's Arch. Pharmacol. 320, 20–25.

Ichikawa, J., Dai, J., O'Laughlin, I.A., Fowler, W.L., Meltzer, H.Y., 2002. Atypical, but not typical, antipsychotic drugs increase cortical acetylcholine release without an effect in the nucleus accumbens or striatum. Neuropsychopharmacology 26, 325–339.

Kennedy, J.S., Zagar, A., Bymaster, F., Nomikos, G., Trzepacz, P.T., Gilmore, J.A., Rotelli, M.D., Breier, A., Tollefson, G., 2001. The central cholinergic system profile of olanzapine compared with placebo in Alzheimer's disease. Int. J. Geriatr. Psychiatry 16 (Suppl. 1), S24–S32.

Kitaichi, K., Day, J.C., Quirion, R., 1999a. A novel muscarinic M_4 receptor antagonist provides further evidence of an autoreceptor role for the muscarinic M_2 receptor sub-type. Eur. J. Pharmacol. 383, 53–56.

Kitaichi, K., Hori, T., Srivastava, L.K., Quirion, R., 1999b. Antisense oligodeoxynucleotides against the muscarinic m_2, but not m_4, receptor supports its role as autoreceptors in the rat hippocampus. Mol. Brain Res. 67, 98–106.

Li, Z., Ichikawa, J., Meltzer, H.Y., 2003. A comparison of the effects of loxapine with ziprasidone and thioridazine on the release of dopamine and acetylcholine in the prefrontal cortex and nucleus accumbens. Psychopharmacology 167, 315–323.

Mintzer, M.Z., Griffiths, R.R., 2003. Lorazepam and scopolamine: a single-dose comparison of effects on human memory and attentional processes. Exp. Clin. Psychopharmacol. 11, 56–72.

Moor, E., DeBoer, P., Auth, F., Westerink, B.H.C., 1995. Characterisation of muscarinic autoreceptors in the septo-hippocampal system of the rat: a microdialysis study. Eur. J. Pharmacol. 294, 155–161.

Moor, E., Auth, F., DeBoer, P., Westerink, B.H.C., 1996. Septal and hippocampal glutamate receptors modulate the output of acetylcholine in hippocampus: a microdialysis study. J. Neurochem. 67, 310–316.

Moore, N.A., Tye, N.C., Axton, M.S., Risius, F.C., 1992. The behavioral pharmacology of olanzapine, a novel "atypical" antipsychotic agent. J. Pharmacol. Exp. Ther. 262, 545–551.

Niedzwiecki, D.M., Cubeddu, L.X., Mailman, R.B., 1989. Comparative anticholinergic properties of thioridazine, mesoridazine and sulforidazine. J. Pharmacol. Exp. Ther. 250, 126–133.

Parada, M.A., Hernandez, L., Puig De Parad, M., Rada, P., Murzi, E., 1997. Selective action of acute systemic clozapine on acetylcholine release in the rat prefrontal cortex by reference to the nucleus accumbens and striatum, J. Pharmacol. Exp. Ther. 281, 582–588.

Rao, T.S., Reid, R.T., Correa, L.D., Santori, E.M., Gardner, M.F., Sacaan, A.I., Lorrain, D., Vernier, J.M., 2003. In vivo pharmacological characterization of (+/−)-4-[2-(1-methyl-2-pyrrolidinyl)ethyl]thiophe-

nol hydrochloride (SIB-1553A), a novel cholinergic ligand: microdialysis studies. Brain Res. 986, 71–81.

Rasmussen, T., Fink-Jensen, A., Sauerberg, P., Swedberg, M.D.B., Thomsen, C., Sheardown, M.J., Jeppesen, L., Calligaro, D.O., DeLapp, N.W., Whitesitt, C., Ward, J.S., Shannon, H.E., Bymaster, F.P., 2001. The muscarinic receptor agonist BuTAC, a novel potential antipsychotic, does not impair learning and memory in mouse passive avoidance. Schizophr. Res. 49, 193–201.

Seeger, T.F., Seymour, P.A., Schmidt, A.W., Zorn, S.H., Schulz, D.W., Lebel, L.A., et al., 1995. Ziprasidone (CP-88,059): a new antipsychotic with combined dopamine and serotonin receptor antagonist activity. J. Pharmacol. Exp. Ther. 275, 101–113.

Shirazi-Southall, S., Rodriguez, D.E., Nomikos, G.G., 2002. Effects of typical and atypical antipsychotics and receptor selective compounds on acetylcholine efflux in the hippocampus of the rat. Neuropsychopharmacology 26, 583–594.

Small, B.B., 1956. Statistical Quality Control Handbook. Western Electric, Indianapolis, IN.

Steele, T.D., Hodges, D.B., Levesque, T.R., Locke, K.W., 1997. D_1 agonist dihydrexidine releases acetylcholine and improves cognitive performance in rats. Pharmacol. Biochem. Behav. 58, 477–483.

Stillman, M.J., Shukitt-Hale, B., Galli, R.L., Levy, A., Lieberman, H.R., 1996. Effects of muscarinic M_2 antagonists on in vivo hippocampal acetylcholine levels. Brain Res. Bull. 41, 221–226.

Sur, C., Mallorga, P.J., Wittmann, M., Jacobson, M.A., Pascarella, D., Williams, J.B., Brandish, P.E., Pettibone, D.J., Scolnick, E.M., Conn, P.J., 2003. *N*-Desmethylclozapine, an allosteric agonist at muscarinic 1 receptor, potentiates *N*-methyl-D-aspartate receptor activity. Proc. Natl. Acad. Sci. 100, 13674–13679.

Zhang, W., Bymaster, F.P., 1999. The in vivo effects of olanzapine and other antipsychotic agents on receptor occupancy and antagonism of dopamine D_1, D_2, D_3, $5HT_{2A}$ and muscarinic receptors. Psychopharmacology 141, 267–278.

Zorn, S.H., Jones, S.B., Ward, K.M., Liston, D.R., 1994. Clozapine is a potent and selective muscarinic M_4 agonist. Eur. J. Pharmacol. 269, R1–R2.