

Atypical, but Not Typical, Antipsychotic Drugs Increase Cortical Acetylcholine Release without an Effect in the Nucleus Accumbens or Striatum

Junji Ichikawa, M.D., Ph.D., Jin Dai, M.D., Ian A. O'Laughlin, B.S., Wiley L. Fowler, M.S., and Herbert Y. Meltzer, M.D.

The role of acetylcholine (ACh) in the action of antipsychotic drugs (APDs) was studied by microdialysis, without AChEsterase inhibition, to facilitate the interpretation of any observed drug effects. The atypical APDs, clozapine (2.5–20 mg/kg), olanzapine (10 mg/kg), risperidone (1 mg/kg), and ziprasidone (3 mg/kg) significantly increased ACh release in rat medial prefrontal cortex (mPFC), whereas the typical APDs, haloperidol (0.1–1 mg/kg), S(-)-sulpiride (10–25 mg/g), and thioridazine (5–20 mg/kg) did not. None of seven APDs increased ACh release in the nucleus accumbens or striatum at the doses effective in the mPFC. Thus, atypical and typical APDs may differ in the ability to increase cortical ACh release, a possible factor contributing to

cognitive improvement in schizophrenia. After perfusion with neostigmine, an AChEsterase inhibitor, clozapine, but not haloperidol, increased ACh release in all three aforementioned brain regions with an enhanced effect in the mPFC, indicating the importance of studying ACh release in the absence of AChEsterase inhibition. Clozapine, and perhaps other atypical APDs, alone or in combination with an AChEsterase inhibitor, may improve cognition in schizophrenia, and perhaps other cognitive disorders, e.g., early Alzheimer's disease, by enhancing cortical cholinergic transmission.

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Acetylcholine (ACh) plays an important role in motor function and various domains of cognition, e.g., attention (Perry et al. 1999) and working memory (Winkler et

al. 1995), and thus, may be important to either the cognitive dysfunction of schizophrenia or the ability of antipsychotic drugs (APDs) to improve some or all aspects of that cognitive deficit (Meltzer and McGurk 1999). The atypical APDs, clozapine, olanzapine, risperidone, and quetiapine, have, in fact, been reported to improve some domains of cognitive dysfunction (see Meltzer and McGurk 1999; Purdon 1999 for reviews), in addition to psychopathology (Leucht et al. 1999) in schizophrenia, more so than typical APDs, e.g., haloperidol and phenothiazines. The basis for these advantages is unclear.

The atypical APDs share in common a relatively more potent antagonist action at serotonin (5-HT)_{2A} compared to dopamine (DA) D₂ receptors (Meltzer et

From the Departments of Psychiatry and Pharmacology, Vanderbilt University School of Medicine, Nashville, TN, USA.

Address correspondence to: Dr. J. Ichikawa, 1601 23rd Avenue South, The First Floor Laboratory R-1117, The Psychiatric Hospital at Vanderbilt, Nashville, TN 37212, USA.

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al. 1989; Schotte et al. 1996) and α_1 - and α_2 -adrenoceptors (Hertel et al. 1999). These features have been related to their ability to increase DA release in the mPFC (Hertel et al. 1999; Kuroki et al. 1999; Ichikawa et al. 2001). However, a possible role for ACh in mediating these effects is receiving increasing attention. Clozapine and olanzapine, but not risperidone, quetiapine, ziprasidone, or iloperidone (Schotte et al. 1996; Bymaster et al. 1999), have significant direct agonist or antagonist effects upon various subtypes of muscarinic ACh receptors (mAChRs) (Tandon 1999), whereas the typical APDs, thioridazine and mesoridazine, are also potent mAChR antagonists (Niedzwiecki et al. 1989a,b; Bolden et al. 1992). Furthermore, trihexyphenidyl and biperiden (Bolden et al. 1992), mAChR antagonists which reduce typical APD-induced extrapyramidal symptoms (EPS), have been reported to cause complex clinical effects in patients with schizophrenia, e.g., working memory impairment (King 1990) and worsening of psychosis while decreasing negative symptoms (Tandon et al. 1992). It is possible that clozapine and olanzapine influence clinical symptoms in schizophrenia via a direct effect upon main cholinergic transmission (Zorn et al. 1994; Bymaster et al. 1996).

As has been demonstrated in rodents (Everitt and Robbins 1997) and primates (Mrzljak et al. 1995), the nucleus basalis magnocellularis (NBM) or the basal forebrain consisting of the substantia innominata, the nucleus of Meynert, and the horizontal limb of the diagonal band, project cholinergic neurons to the cortex, whereas cholinergic interneurons exist mostly in the striatum (STR) and nucleus accumbens (NAC) (Kawaguchi et al. 1995). These three regions are involved in various actions of APDs (Ichikawa and Meltzer 1999): 1) the medial prefrontal cortex (mPFC) has been suggested to be involved in the neural circuitry important for negative symptoms and cognition, e.g., working memory (Goldman-Rakic and Selemon 1997); 2) the STR is centrally involved in motor function; and 3) the NAC plays a key role in both psychosis and reward (Davis et al. 1991; Kalivas and Nakamura 1999).

We have reported that some atypical APDs, e.g. clozapine, olanzapine, and risperidone, but not typical APDs, e.g., haloperidol and S(-)-sulpiride, increase DA release in the mPFC (Kuroki et al. 1999). However, drugs of other classes, e.g., antidepressants, which increase the release of DA (Tanda et al. 1994), but not ACh (Ichikawa et al. unpublished data) in the mPFC, are not effective in the treatment of cognition and negative symptoms in schizophrenia (Plasky 1991). Thus, we postulate that the increases in ACh and DA release in the mPFC are responsible, in part, for the ability of these atypical APDs to improve cognition and negative symptoms in schizophrenia.

It has been reported that clozapine increases ACh release in mPFC, NAC, and STR, using microdialysis with neostigmine to increase basal ACh to detectable

levels (Parada et al. 1997). Other investigators have also reported that clozapine (Imperato et al. 1993), haloperidol (Imperato et al. 1993, 1994; De Boer and Abercrombie 1996; Steinberg et al. 1998), and S(-)-sulpiride (Imperato et al. 1993, 1994; Ikarashi et al. 1997) increases ACh release in the STR. However, these effects of haloperidol (Osborne et al. 1994) and (\pm)- or S(-)-sulpiride (De Boer et al. 1990; Westerink et al. 1990; Ueda et al. 1995) were not replicated. Neither haloperidol nor S(-)-sulpiride has been reported to increase ACh release in mPFC or NAC (Moore et al. 1999).

It should be noted that all of these microdialysis studies employed various concentrations of AChesterase inhibitors, such as physostigmine and neostigmine, in the perfusion medium, in order to achieve reliable detection of ACh in the dialysate samples (see Ichikawa et al. 2000 for review). However, AChesterase inhibitors increase ACh concentrations in the extracellular space 30–100 fold (Maura and Raiteri 1986; Kawashima et al. 1994) which would be expected to cause persistent stimulation of muscarinic and nicotinic AChRs, and should alter the physiology of cholinergic transmission, making interpretation of the data problematic (Ichikawa et al. 2000). As discussed by Fujii et al. (1997), high concentrations of ACh stimulate feedback mechanisms, which inhibit ACh release via presynaptic mACh autoreceptors on cholinergic neuron terminals, and may also affect the activities of non-cholinergic neurons. These considerations provide a strong rationale for conducting microdialysis experiments in the absence of AChesterase inhibition, when possible, to determine the effect of various types of APDs on ACh release under more physiological conditions (De Boer and Abercrombie 1996; Di Chiara et al. 1996).

We have now developed a highly sensitive method to measure extracellular ACh in rat brain dialysate in the absence of AChesterase inhibitors (Ichikawa et al. 2000). With this method, we examined the effect of four atypical APDs (clozapine, olanzapine, risperidone, and ziprasidone), and three typical APDs (haloperidol, S(-)-sulpiride, and thioridazine), on ACh release in the mPFC, NAC, and STR.

METHODS

Animals

Male Sprague-Dawley albino rats (Zivic-Miller, Porterville, PA) weighing 250–350 g were housed two to three per cage and maintained in a controlled 12–12-h light-dark cycle and under constant temperature at 22°C, with free access to food and water.

Surgery

Rats were anesthetized with an intraperitoneal injection (i.p.) of a combination of xylazine (13 mg/kg; Rompun;

Shawnee Mission, KS) and ketamine hydrochloride (87 mg/kg, Ketaset; Fort Dodge Lab, Fort Dodge, IA). One stainless guide cannula with a dummy probe was placed and fixed onto the cortex dorsal to either mPFC, NAC, or STR. Stereotaxic coordinates of each probe when implanted was A+3.2, L+0.8 (10° inclination), V−5.5 mm for the mPFC; A+0.5, L−4.0, V−5.5 for the STR and A+2.0, L+1.5~1.7, V−7.5 mm for the NAC, respectively, relative to bregma; incision bar level: −3.0 mm, according to the atlas of Paxinos and Watson (1986).

Microdialysis

Three to five days following cannulation surgery, a concentric-shaped dialysis probe (2 mm length, polyacrylonitrile/sodium methallylsulfonate polymer membrane, AN69H; Hospal) was implanted into either mPFC, NAC, or STR under slight anesthesia with methoxyflurane (Metofane; Pitman-Moore, Mundelein, IL). Perfusion flow rate of the implanted probes was set at 1.5 μ l/min and then dialysate samples (45 μ l) were collected every 30 min. The perfusion medium was Dulbecco's phosphate buffered saline solution (Sigma) including Ca^{2+} (NaCl 138 mM, Na_2HPO_4 8.1 mM, KCl 2.7 mM, KH_2PO_4 1.5 mM, MgCl 0.5 mM, CaCl_2 1.2 mM, pH 7.4). After stable baseline values in the dialysates were obtained, each drug or vehicle was administered to the rats. The effect of drugs was followed for at least another 180 min. Dialysate samples were directly applied onto a HPLC with electrochemical detection assisted by a chromatography manager (Millennium; Waters, Milford, MA), and analyzed for ACh. The location of dialysis probes was verified after each experiment by brain dissection with 100 μ m brain slices (OTS-4000; FHC, Bowdoinham, MD). The procedures applied in these experiments were approved by the Institutional Animal Care and Use Committee of Vanderbilt University in Nashville, TN, where we completed the present studies.

Biochemical Assay

We utilized a highly sensitive method to measure endogenous ACh in extracellular space in the absence of AChesterase inhibition (Ichikawa et al. 2000). In brief, dialysate samples were directly injected onto the liquid chromatography/electrochemistry (LCEC) system assisted by a chromatography manager (Millennium; Waters, Milford, MA), and analyzed for ACh. ACh was separated on a coiled cation exchanger ACh column (analytical column) (Sepstik 10 μ m ID 530 \times 1.0 mm; BAS, West Lafayette, IN), followed by the post-IMER (immobilized enzyme reactor) (BAS), which consists of choline oxidase (ChO)/AChesterase. ACh was hydrolyzed by AChesterase to form acetate and choline in the post IMER. Then, choline was oxidized by ChO to produce betaine and hydrogen peroxide (H_2O_2). H_2O_2 was

detected and reduced to H_2O on an Unijet amperometric detector cell with a peroxidase-redox-coated glassy carbon electrode (MF-2098; BAS), set at +100 mV (LC-4C; BAS) versus Ag/AgCl reference electrode.

This reduction was analyzed with the detector (LC-4C; BAS) as signal indicating ACh in the chromatogram. However, it is difficult in most cases to separate the ACh peak from the huge choline peak with the analytical column. Therefore, a pre-IMER (BAS), which consists of ChO and catalase, was added prior to the analytical column. Choline in the dialysate samples was oxidized by ChO to form betaine and H_2O_2 by the pre-IMER. The H_2O_2 was then converted by the catalase to H_2O before entering the analytical column. This procedure eliminates interference of the ACh peak in the chromatogram by choline, thereby decreasing the detection limit of ACh. The ACh peak is clearly resolvable and can be identified in all chromatograms (Figure 1A). The area and height of ACh peak showed a linear correlation with concentration. Due to the markedly increased signal-to-noise ratio, the detection limit was 2–3 fmol per sample (20 μ l sample loop). The mobile phase (Na_2HPO_4 50 mM, pH 8.2) including ProClin (BAS), a microbiocide, was pumped at 0.14 ml/min by a LC-10AD pump (Shimadzu, Kyoto, Japan). The reagents used were analytical or HPLC grade.

Drugs

Clozapine HCl (Sandoz, East Hanover, NJ), haloperidol (McNeil, Spring House, PA), olanzapine (Eli Lilly, Indianapolis, IN), risperidone (Janssen, Titusville, NJ), S(-)-sulpiride (Research Biochemical Inc., Natick, MA), and M100907 (Hoechst Marion Roussel, Bridgewater, NJ) were each dissolved in a small amount of 0.1 N HCl solution and the pH was adjusted to 6–7 with 0.1 N NaOH. Ziprasidone mesylate (Pfizer, Groton, CT) was dissolved in 45% 2-hydroxypropyl- β -cyclodextrin (HBC) (Research Biochemical Inc., Natick, MA). Thioridazine HCl, S(-)-scopolamine HBr, and oxotremorine sesquifumarate (Research Biochemical Inc., Natick, MA) were dissolved in deionized water. Vehicle or drugs were administered subcutaneously (s.c.). Neostigmine bromide and tetrodotoxin citrate (RBI) were each dissolved in the perfusion medium of Dulbecco's phosphate buffered saline. Receptor affinities of these seven APDs were summarized in Table 1.

Data Analysis

Only results derived from healthy rats with a correctly positioned dialysis probe were included in the data analysis. Repeated measure ANOVA (treatment \times time) followed by Fisher's protected least significant difference post-hoc pairwise comparison procedure and one-way ANOVA were used to determine group dif-

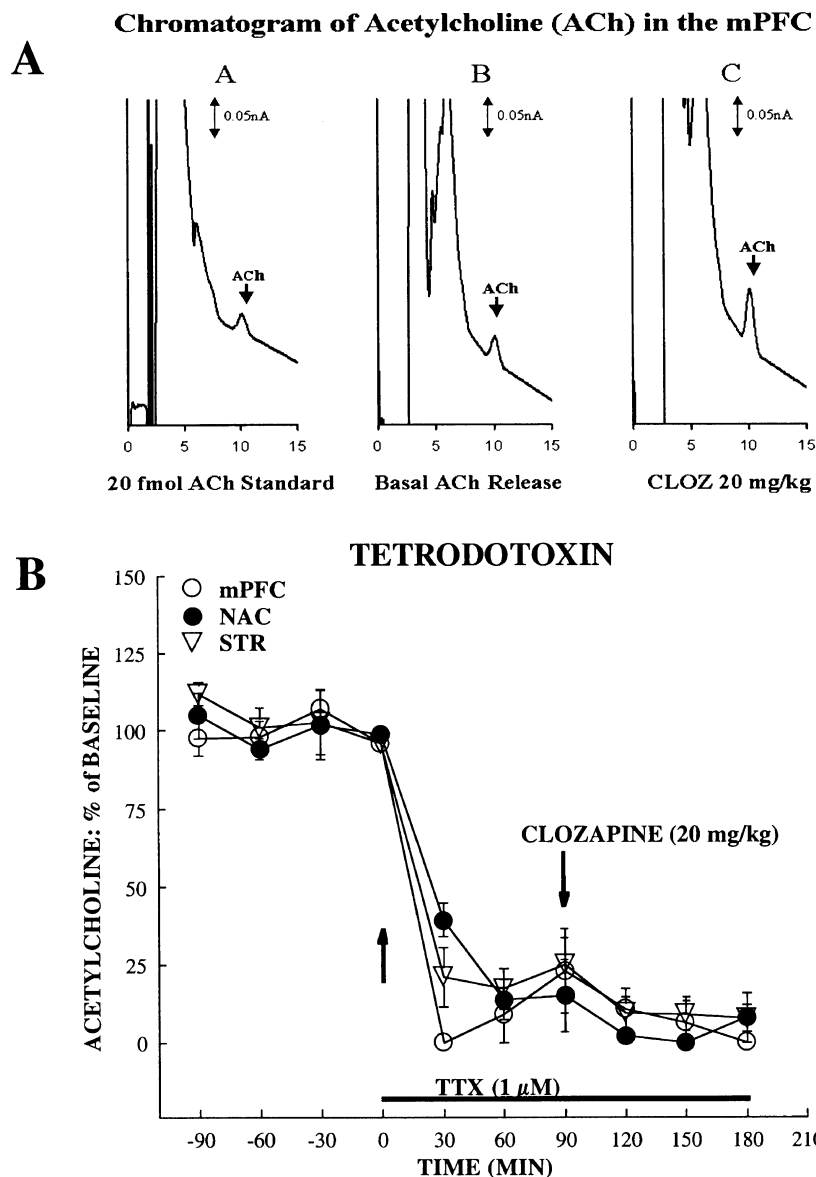


Figure 1. (A) Separation of ACh peak on the chromatograph obtained from authentic standard solution (20 fmol) and dialysate samples in the medial prefrontal cortex (mPFC). ACh peak was clearly separated and reliably detected in the chromatogram. Clozapine (CLOZ, 20 mg/kg, s.c.) significantly increased the height and area of ACh peak compared to the basal peak. Detection limit was less than 2–3 fmol per sample. Vertical axis indicates nA and horizontal axis indicates time after injection (min), respectively. (B) Perfusion of tetrodotoxin (TTX, 1 μ M) into the dialysis probe immediately decreased basal dialysate ACh concentrations to approximately 10% of the baseline levels ($N = 4$). Administration of clozapine (20 mg/kg, s.c.) did not affect the TTX-induced decreases in ACh concentrations in the medial prefrontal cortex (mPFC, \circ , $N = 4$), nucleus accumbens (NAC, \bullet , $N = 4$), and striatum (STR, \blacktriangledown , $N = 4$).

ferences (StatView 4.5 for the Macintosh). Mean values of four consecutive pre-drug dialysate levels were designated as 100%. The net-AUC was calculated from the absolute net increase for a 0–180-min period (six samples) or a 30–180-min period (five samples) after subtracting each pre-drug baseline value. The % net-AUC is the net-AUC value expressed as a percentage of each baseline AUC value. A $p < .05$ was considered significant in this study. All results are given as mean \pm S.E.

RESULTS

Basal Levels

Basal dialysate ACh concentrations in the absence of AChEsterase inhibition were 19.5 ± 0.7 (fmol/20 μ l) in

the mPFC, 27.7 ± 1.7 in the NAC, and 54.7 ± 3.4 in the STR, respectively, and were not significantly different between treatment groups in each region (Table 2). The sodium channel blocker tetrodotoxin (TTX, 1 μ M), given in the perfusion medium, rapidly decreased dialysate ACh concentrations to approximately 10% of basal levels in all regions. Clozapine (20 mg/kg, s.c.) had no effect on dialysate ACh concentrations in the mPFC, NAC or STR in the presence of TTX (1 μ M) (Figure 1B).

Effects of Scopolamine and Oxotremorine in the Absence of AChEsterase Inhibition

The non-selective mAChR antagonist S(-)-scopolamine (0.16, 0.5, and 1.5 mg/kg) increased dialysate ACh concentrations in the mPFC (Figure 2A), whereas S(-)-scopolamine (0.5 mg/kg) had no effect in the NAC or STR

Table 1. Receptor Binding Affinity (K_i, nM) of Antipsychotic Drugs

	D ₂	5-HT _{1A}	5-HT _{2A}	mACh		Mixed	α ₁	α ₂
				m1	m2			
Clozapine	150e	180e	3.3e	1.9d 3.1a	10d 48a		7d	8d
Olanzapine	2.1f	7100f	1.9f	1.9d	18d		19d	230d
Risperidone	3.3e	250e	0.16e	>10000d	>10000d		2d	3d
Ziprasidone	2.8f	37f	0.25f			>10000f	12e	>1000e
Haloperidol	1.4e	3080e	25e	1475d	1200d		46d	360d
Thioridazine	16b		4.2b	2.7a	14a			
S(-)-sulpiride	15c							

The K_i values for D₂, 5-HT_{1A}, 5-HT_{2A}, m1, m2 and mixed mACh receptors in human and rat subtypes are from: a = Bolden et al. (1992); b = Leysen et al. (1993); c = Seeman and van Tol (1994); d = Bymaster et al. (1996); e = Schotte et al. (1996); and f = Arnt and Skarsfeldt (1998), respectively.

(Figures 2B and 2C). Oxotremorine (0.5, 1.5, but not 0.16, mg/kg), a non-selective mAChR agonist, decreased dialysate ACh concentrations in the mPFC, and in the NAC and STR as well, at 0.5 mg/kg (Figures 2D, 2E, and 2F).

Effects of Antipsychotics in the Absence of AChesterase Inhibition

Atypical APDs, clozapine (2.5, 5, 20, but not 1.25, mg/kg, s.c.), olanzapine (10, but not 1 or 3, mg/kg, s.c.), risperidone (1, but not 0.1 or 0.3, mg/kg, s.c.), and ziprasidone (3, but not 1, mg/kg, s.c.), in the absence of AChesterase inhibition, significantly increased dialysate ACh concentrations in the mPFC compared with vehicle controls (Figure 3). However, typical APDs, haloperidol (0.1 and 1 mg/kg, s.c.), S(-)-sulpiride (10 and 25 mg/kg, s.c.), and thioridazine (5 and 20 mg/kg, s.c.) had no significant effect on dialysate ACh concentrations in the mPFC (Figure 4) compared with vehicle controls. On the other hand, none of these seven APDs at the effective doses in the mPFC had any significant effect on dialysate ACh concentrations in the NAC or

STR compared with vehicle controls (Figure 5). (Time-course effects were not shown in the NAC and STR).

The effect of APDs on dialysate ACh concentrations in the mPFC, NAC, and STR was also analyzed with the % net-AUC (area under the curve) to clearly show a regional difference (Figure 5). In order to avoid the influence of an injection artifact (see below), a 30–180-min period (five samples) analysis is also provided. The pattern of results obtained with the 0–180-min and the 30–180-min periods were virtually identical.

Effects of Neostigmine

Inclusion of neostigmine (0.03 and 0.3 μM), an AChesterase inhibitor, in the perfusion medium markedly increased dialysate ACh concentrations in the mPFC, NAC, or STR (Table 2). The increase in the NAC and STR was dose-dependent (not fully studied in the mPFC because we had already found that clozapine increases ACh release in the absence of neostigmine). The ability of 0.3 μM neostigmine itself to increase ACh concentrations in the mPFC was almost equipotent to that of lower dose neostigmine (0.03 μM) in the NAC and STR.

Table 2. Basal Dialysate Concentrations

Region	Neostigmine (μM)	n	ACh (fmol/20 μl)	Ratio
mPFC	0	127	19.5 ± 0.7	
	0.3	12	616 ± 55	31.5
NAC	0	45	27.7 ± 1.7	
	0.03	4	623 ± 69	
	0.3	11	2537 ± 334	91.6
STR	0	45	54.7 ± 3.4	
	0.03	4	513 ± 30	
	0.3	12	4035 ± 687	73.8

Abbreviations: mPFC = medial prefrontal cortex; NAC = nucleus accumbens; STR = striatum.
Ratio: ACh at 0.3/0 μM Neostigmine.

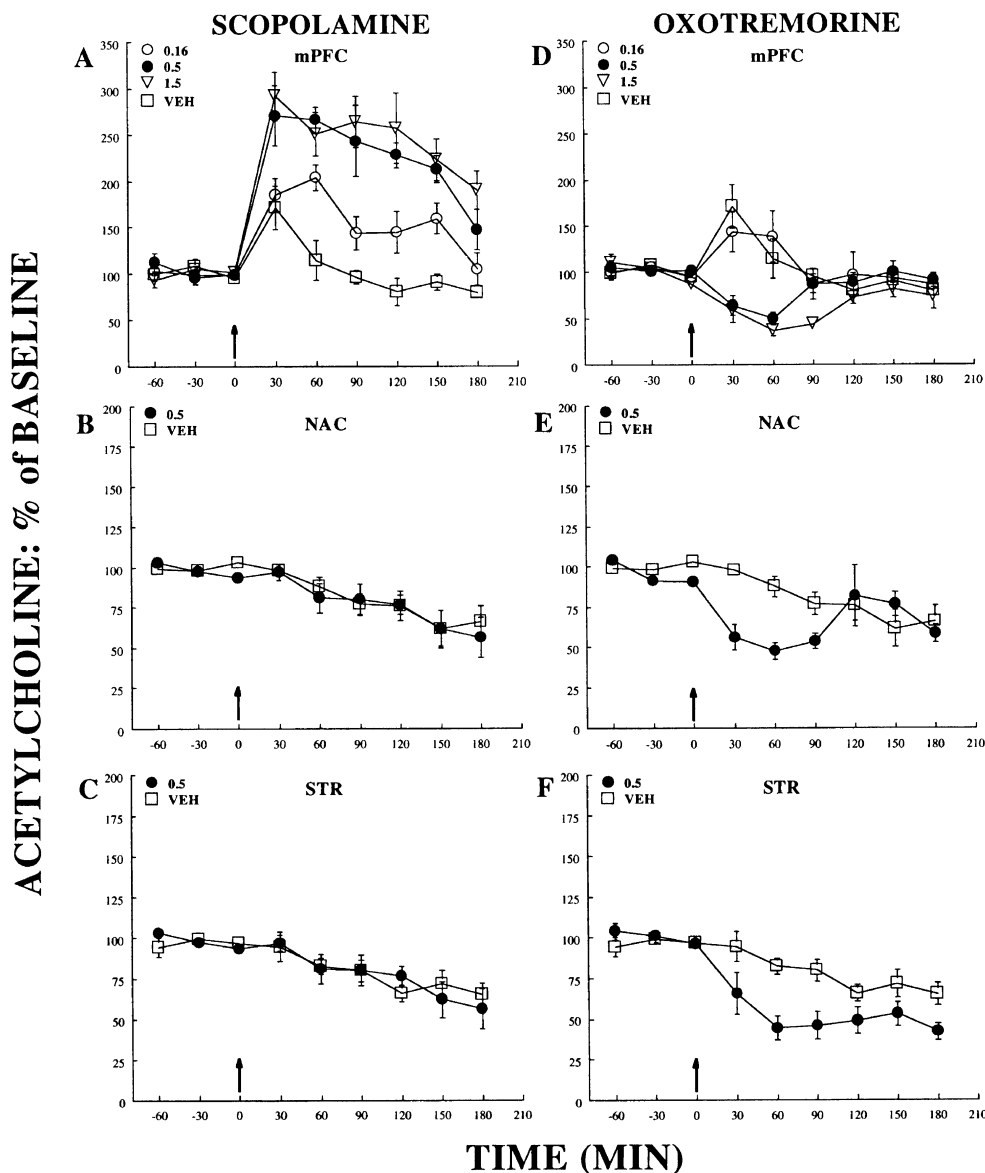


Figure 2. Time-course effects of S(-)-scopolamine and oxotremorine, non-selective mAChR antagonist and agonist, respectively, compared with vehicle controls (VEH, □, $N = 8$) in the absence of AChE inhibition. **(A)** S(-)-scopolamine (0.16, 0.5, and 1.5 mg/kg s.c.; ○, ●, and ▼, respectively) dose-dependently increased dialysate ACh concentrations in the mPFC ($N = 5-6$) ($F(1,11) = 18.99, p = .001$; $F(1,12) = 85.98, p < .001$; and $F(1,10) = 63.67, p < .001$, respectively). **(B)** and **(C)** S(-)-scopolamine (0.5 mg/kg, ●) did not affect dialysate ACh concentrations in the NAC ($N = 6$) or STR ($N = 6$) ($F(1,11) = 0.05, p = .82$; and $F(1,10) = 0.11, p = .75$, respectively). **(D)** Oxotremorine (0.5, 1.5, but not 0.16, mg/kg, s.c.; ●, ▼, and ○, respectively) significantly decreased dialysate ACh concentrations in the mPFC ($N = 5-6$) ($F(1,11) = 5.27, p = .042$; $F(1,11) = 13.37, p = .004$; and $F(1,12) = 0.02, p = .89$, respectively). **(E)** Oxotremorine (0.5 mg/kg, ●) decreased them at the first three samples in the NAC ($N = 6$) ($F(1,11) = 9.43, p = .011$), although it had no significant effect in the six samples ($F(1,11) = 3.38, p = .09$). **(F)** Oxotremorine (0.5 mg/kg, ●) significantly decreased dialysate ACh concentrations in the STR ($N = 6$) ($F(1,11) = 32.14, p < .001$).

Effects of Antipsychotics in the Presence of AChE Inhibition

In the presence of 0.3 μM neostigmine, clozapine (20 mg/kg, s.c.) significantly increased dialysate ACh concentrations in the NAC and STR, as well as the mPFC, compared with vehicle controls (Figures 6A, 6B, and 6C). The increase in dialysate ACh concentrations in the

mPFC produced by clozapine (20 mg/kg, s.c.) was potentiated two-to-three fold by the presence of neostigmine (0.3 μM), compared to the increase in its absence (Figure 3). Neostigmine 0.3 μM given in the perfusion medium did not affect the inability of haloperidol (1 mg/kg, s.c.) to increase dialysate ACh concentrations in the three regions in the absence of neostigmine, com-

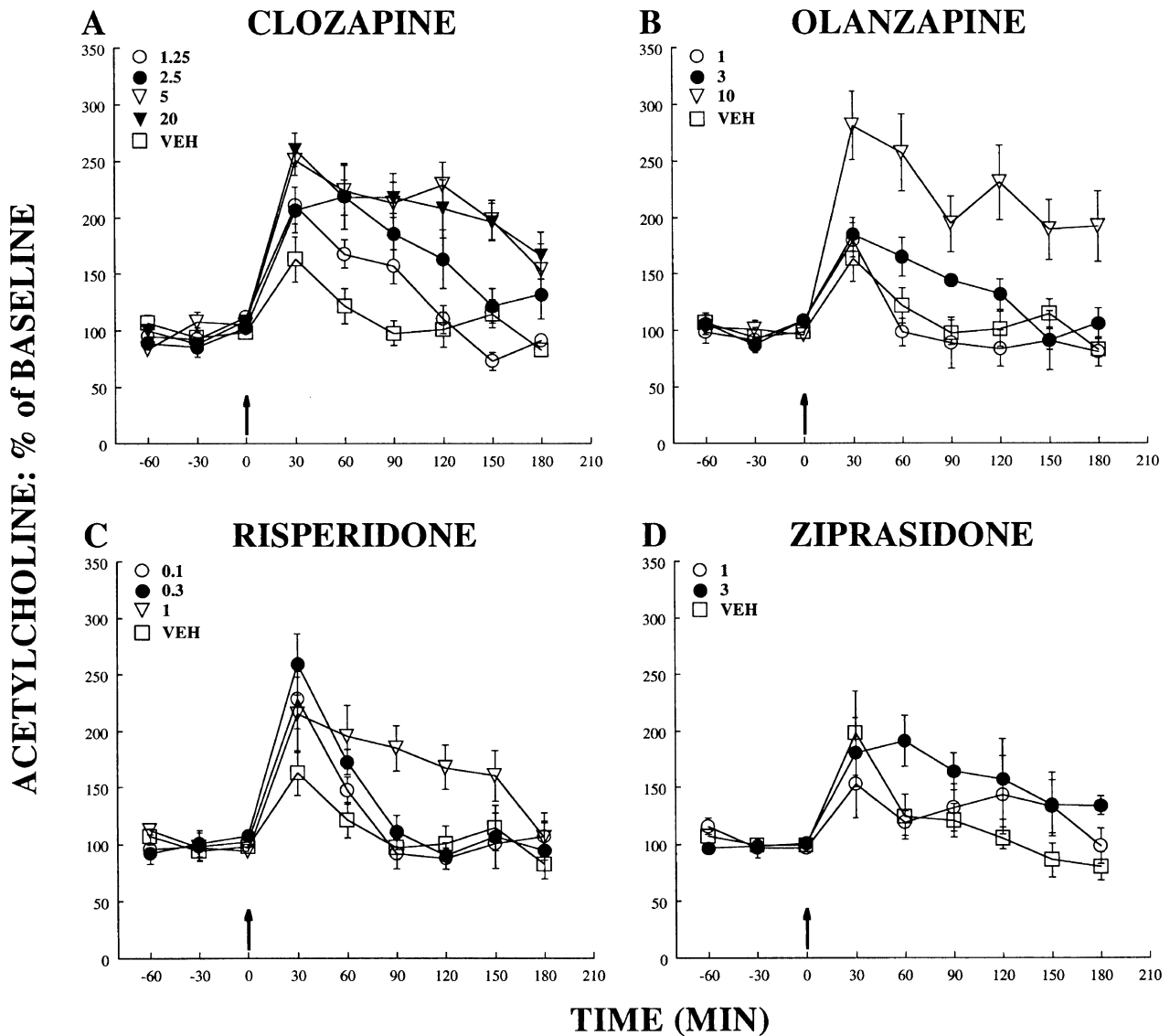


Figure 3. Time-course effects of atypical antipsychotic drugs in the medial prefrontal cortex (mPFC), compared with vehicle controls (VEH, □) (*N* = 6–7) in the absence of AChesterase inhibition. Dialysate ACh concentrations were significantly increased by clozapine (*N* = 6) [(A) 2.5, 5, 20, but not 1.25, mg/kg, s.c.; ●, ▼, ▼, and ○, respectively] ($F(1,10) = 16.78, p = .002$; $F(1,10) = 47.45, p < .001$; $F(1,10) = 34.29, p < .001$; and $F(1,10) = 3.44, p = .09$, respectively), olanzapine (*N* = 6–7) [(B) 10, but not 1 or 3, mg/kg, s.c.; ▼, ○, and ●, respectively] ($F(1,11) = 16.76, p = .002$; $F(1,11) = 0.30, p = .60$; and $F(1,10) = 4.61, p = .058$, respectively), risperidone (*N* = 5–6) [(C) 1, but not 0.1 or 0.3, mg/kg, s.c., ▼, ○, and ●, respectively] ($F(1,10) = 6.19, p = .032$; $F(1,10) = 2.25, p = .16$; and $F(1,9) = 4.53, p = .062$, respectively), and ziprasidone (*N* = 6) [(D) 3, but not 1, mg/kg, s.c., ● and ○, respectively] ($F(1,10) = 7.21, p = .023$; and $F(1,10) = 0.49, p = .50$, respectively)

pared with vehicle controls (Figures 6D, 6E, and 6F). Since preliminary data demonstrated that clozapine (20 mg/kg) had no significant effect on dialysate ACh concentrations in the NAC or STR in the presence of a lower dose of neostigmine (0.03 μM), we did not further examine the effect of 0.03 μM neostigmine on the ability of clozapine (20 mg/kg) to increase dialysate ACh concentrations in the mPFC, or the ability of haloperidol (1 mg/kg) to increase dialysate ACh concentrations in the mPFC, NAC, or STR.

DISCUSSION

The major finding in this study is that, in the absence of AChesterase inhibition, the atypical APDs, clozapine, olanzapine, risperidone, and ziprasidone significantly increased ACh release in the mPFC, but had no effect in the NAC or STR at doses effective in the mPFC. None of the typical APDs, haloperidol, S(-)-sulpiride, or thioridazine significantly increased ACh release in these three regions. Perfusion of neostigmine enhanced the

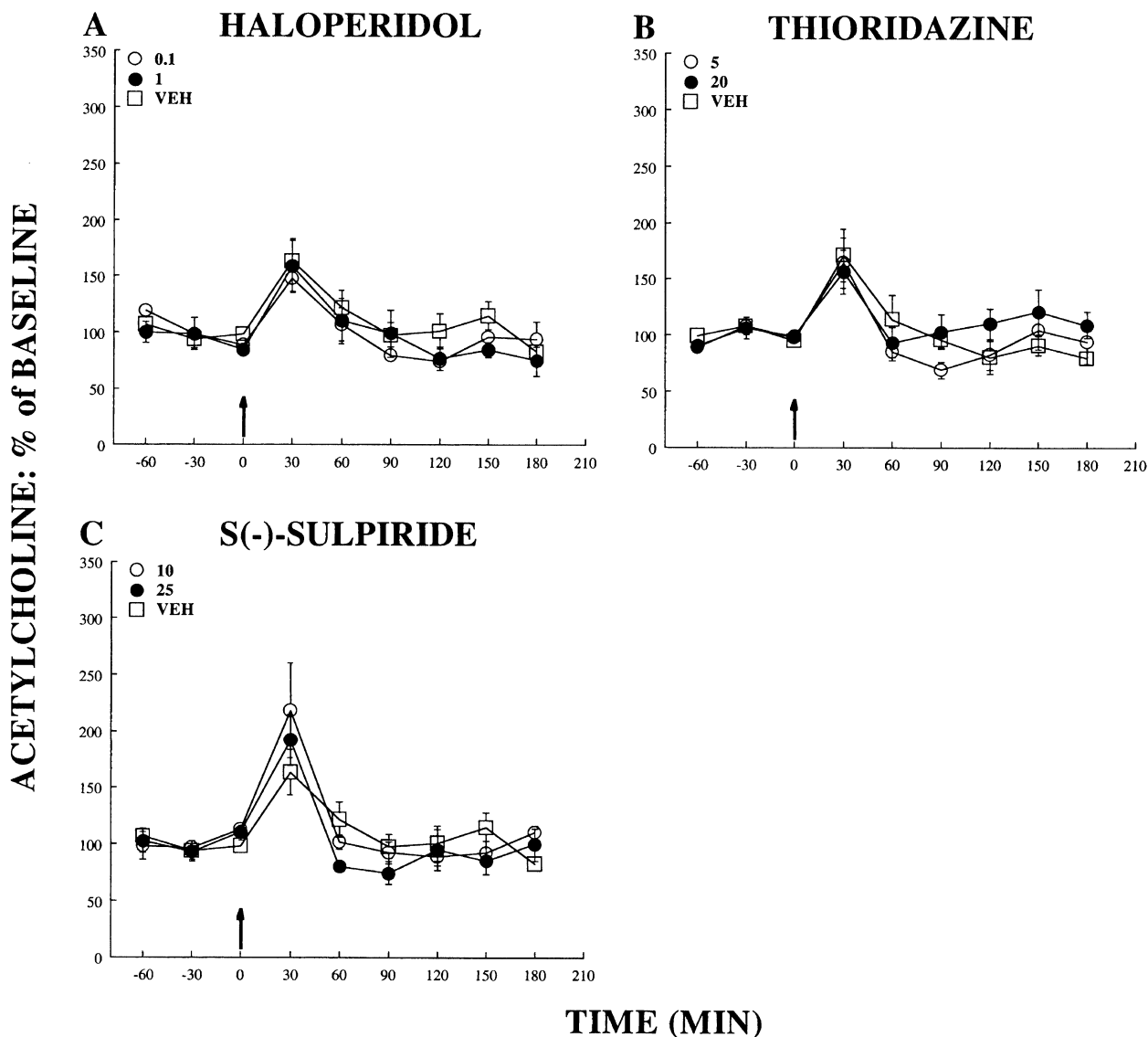


Figure 4. Time-course effects of typical antipsychotic drugs in the medial prefrontal cortex (mPFC), compared with vehicle controls (VEH, \square) ($N = 6-8$) in the absence of AChEsterase inhibition. Dialysate ACh concentrations were not affected by either haloperidol ($N = 6$) [(A) 0.1 and 1 mg/kg, s.c., \circ and \bullet , respectively], thioridazine ($N = 6$) [(B) 5 and 20 mg/kg, s.c., \circ and \bullet , respectively], or S(-)-sulpiride ($N = 5$) [(C) 10 and 25 mg/kg, s.c., \circ and \bullet , respectively].

ability of clozapine, but not haloperidol, to increase ACh release in these three regions.

The method employed here to measure ACh in the absence of AChesterase inhibition has been described in detail elsewhere (Ichikawa et al. 2000). Tetrodotoxin (TTX), a sodium channel blocker, almost eliminated dialysate ACh before and after clozapine (20 mg/kg) in the mPFC, NAC, and STR (Figure 1B), indicating a neuronal origin of dialysate ACh and supporting the conclusion that it reflects ACh release (Di Chiara et al. 1996). The transient increase in ACh release in the mPFC following vehicle may be an injection artifact, which was not found after an indwelling subcutaneous catheter for administering drugs was introduced (data

not shown). This injection artifact does not affect the results reported here since the data with or without the putative injection artifact during 30–180-min or 0–180-min period, respectively, were not significantly different (Figure 5). Our preliminary data showed that haloperidol (1 mg/kg), S(-)-sulpiride (25 mg/kg), and thioridazine (20 mg/kg), administered via the indwelling subcutaneous catheter, also had no effect on ACh release in the mPFC, compared to the vehicle (data not shown).

The mechanism(s) by which the anticholinergic drugs and atypical APDs increase cortical ACh release will now be discussed. Blockade of inhibitory M_2 autoreceptors (Quirion et al. 1994; Billard et al. 1995; Still-

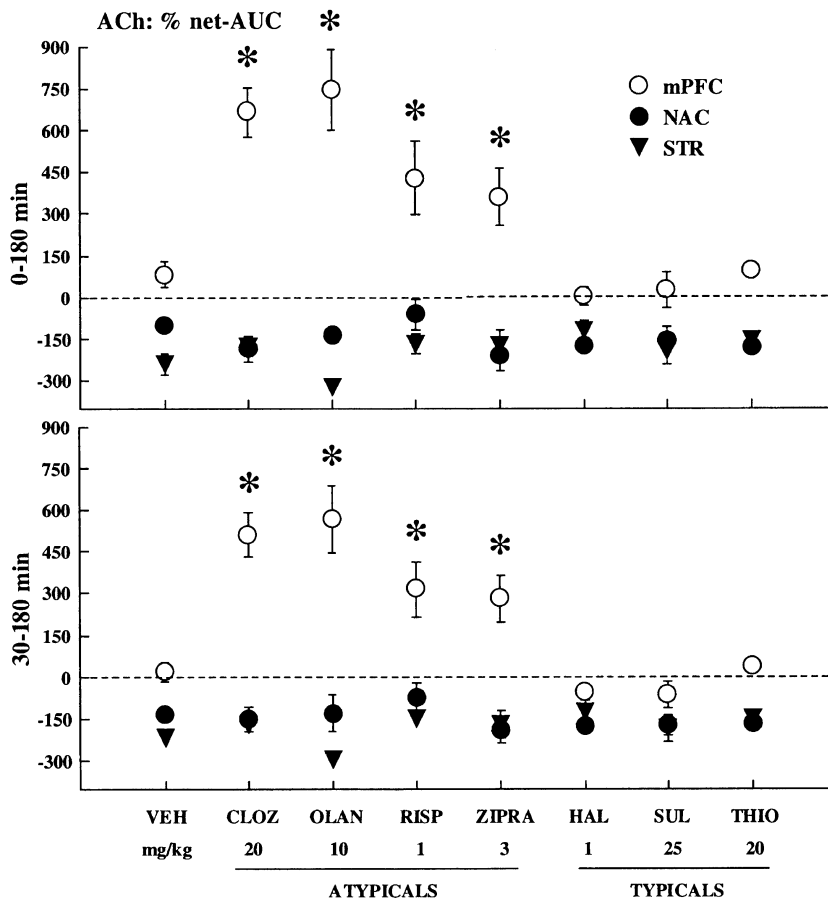


Figure 5. Analysis of % net-AUC (area under the curve) showed a clear regional difference in the effect of atypical drugs [clozapine (CLOZ), olanzapine (OLAN), risperidone (RISP), and ziprasidone (ZIPR)], and typical antipsychotic drugs (APDs) [haloperidol (HAL), S(-)-sulpiride (SUL), and thioridazine (THIO)], at the highest doses tested in the absence of AChesterase inhibition. Atypical APDs preferentially increased dialysate ACh concentrations in the mPFC, compared to the NAC or STR, whereas typical APDs had no effect. None of these APDs affected dialysate ACh concentrations in either the NAC or STR. Data excluding the effect of injection artifact at 30 min following injection (lower panel) are virtually identical to the data of a 0–180-min period (upper panel) with respect to the effect on dialysate ACh concentrations. *N* = 3–8.

man et al. 1996; Kitaichi et al. 1999) and stimulation of M₁ receptors (Ogane et al. 1990; Murakami et al. 1996; Svensson et al. 1996; Suzuki et al. 1998) have been reported to increase brain ACh release. Thus, it is most likely that the non-selective mAChR agonist oxotremorine decreased ACh release in the mPFC, NAC and STR via activation of inhibitory M₂ autoreceptors, as previously suggested for the STR (De Boer et al. 1990; Westerkink et al. 1990; Marshall and Wurtman 1993; Pramanik and Ögren 1993). On the other hand, scopolamine (0.5 mg/kg), a non-selective mAChR antagonist, which increased ACh release only in the mPFC, may have done so via blockade of M₂ autoreceptors in the mPFC (Vannucchi et al. 1997), and possibly the NBM as well, because direct administration of scopolamine (10 µg) into the NBM was reported to increase cortical ACh release (Bertorelli et al. 1991).

The failure of scopolamine to increase ACh release in the NAC and STR, where M₂ receptors are present (Flynn and Mash 1993; Adem et al. 1997), contrasts with the previous report that scopolamine (0.1–1 mg/kg) increased ACh release in the NAC (Pfister et al. 1994) and STR (Pramanik and Ögren 1993; Pfister et al. 1994; Scali et al. 1995; Murakami et al. 1996). However, these latter studies were conducted in the presence AChesterase inhibition. The discrepancy suggests possible mecha-

nisms. The increased ACh following scopolamine is most likely due to blockade of M₂ autoreceptors, and may be rapidly hydrolyzed by AChesterase. Based upon the ratios of increased ACh release (Table 2), AChesterase activity may be sufficiently higher in the NAC and STR compared to the mPFC, to hydrolyze any ACh released by scopolamine in the former two regions, preventing any net increase in extracellular ACh concentrations.

It is also possible that scopolamine increases ACh release in the mPFC via blockade of inhibitory somatodendritic M₂ autoreceptors in the NBM (Bertorelli et al. 1991), which projects cholinergic neurons to the mPFC, but not to the NAC or STR (see the Introduction). Alternatively, it could be argued that the ability of M₂ receptors to tonically inhibit ACh release in the STR and NAC is relatively weak compared with that in the mPFC. If so, scopolamine can increase ACh release in the striatum or nucleus accumbens in the presence of AChesterase inhibition that elevates extracellular ACh levels resulting in greater stimulation of M₂ receptors. It would be relevant to this hypothesis that clozapine produced greater increases in ACh release in the mPFC, NAC, and STR in the presence of AChesterase inhibition, compared to its absence (present data). Analogous results have been reported for the D₂ receptor that in-

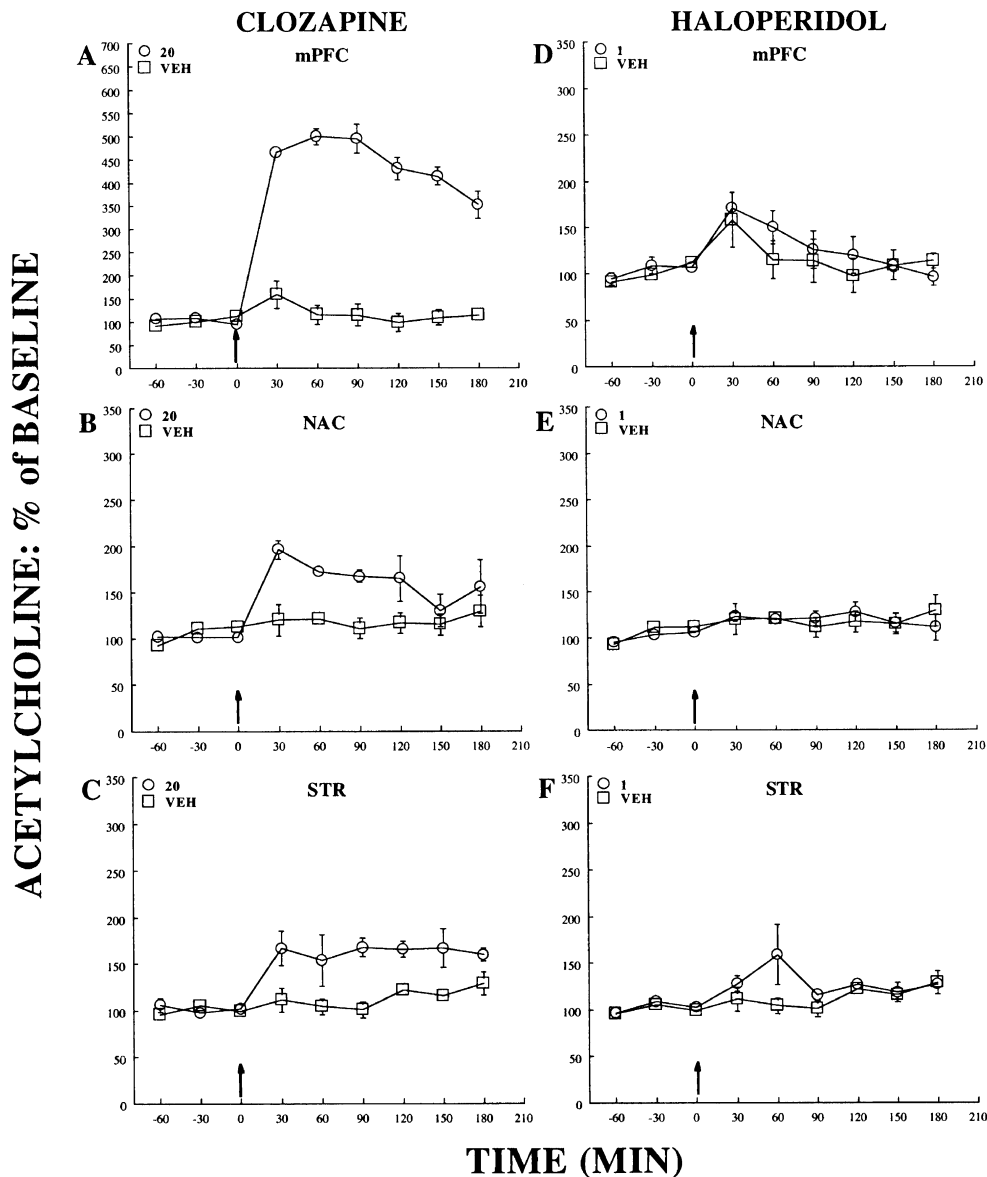


Figure 6. Time-course effects of clozapine and haloperidol in the presence of the AChEsterase inhibitor neostigmine (0.3 μ M) in the perfusion medium throughout the experiment after a probe implantation, compared with vehicle controls (VEH, \square) ($N = 3-4$). **(A, B)** and **(C)** Clozapine (20 mg/kg, s.c., \circ) significantly increased dialysate ACh concentrations in the mPFC ($N = 4$) [**(A)** $F(1,6) = 173.03$, $p < .001$], NAC ($N = 3$) [**(B)** $F(1,5) = 9.35$, $p = .028$], and STR ($N = 4$) [**(C)** $F(1,6) = 16.05$, $p = .007$]. **(D, E)** and **(F)** haloperidol (1 mg/kg, s.c., \circ) had no significant effect on dialysate ACh concentrations the mPFC ($N = 4$) [**(D)** $F(1,6) = 0.24$, $p = .64$], NAC **(E)** ($N = 4$), or STR **(F)** ($N = 4$).

clusion of nomifensine, a DA uptake inhibitor, in the perfusion medium that elevates basal DA levels potentiated the $D_{2/3}$ receptor antagonist raclopride-induced DA release in the mPFC (Westerink et al. 2001), suggesting the relatively weak ability of D_2 receptors to tonically inhibit DA release in that region (Ozaki et al. 1989), compared with that in the NAC or STR. We also considered that potential damage of cholinergic neurons by probe implantation, as suggested by a gradual decrease in basal ACh release, could have contributed to the lack of an effect of scopolamine on ACh release in

the STR or NAC. However, this seems unlikely, since oxotremorine clearly decreased ACh release in the STR and NAC, and TTX nearly eliminated dialysate ACh, both suggesting neuronal origin of a majority of the dialysate ACh.

The M_1 receptor agonists AF102B (Ono et al. 1988) and pilocarpine (Murakami et al. 1996) have been reported to increase ACh release in the STR. Interestingly, pirenzepine, reportedly a M_1 receptor antagonist (Moriya et al. 1999), has also been reported to increase ACh release in the STR (De Boer et al. 1990; Billard et al.

1995; Murakami et al. 1996). Since pirenzepine did not attenuate the effect of pilocarpine, a M_1 receptor agonist, on striatal ACh release (Murakami et al. 1996), pirenzepine may be more potent as a M_2 receptor antagonist, *in vivo*, than a M_1 receptor agonist, particularly regarding the effect on striatal ACh release. It should be noted that all of the studies concerning ACh release mechanisms published to date have used agonists and antagonist that are deficient in selectivity. Thus, additional studies with selective drugs, in the absence of AChE inhibitors, are needed to delineate the role of M_1 and M_2 receptors in the regulation of ACh release in the different brain regions.

Direct effects of some APDs on some mAChRs could be important to their ability to increase ACh release. Clozapine has been reported to be: 1) a $M_{1/2}$ partial agonist (Fritze and Tilmann 1995; Michal et al. 1999); 2) a $M_{2/3/5}$ receptor antagonist (Bymaster et al. 1996); and 3) a M_4 receptor full (Zorn et al. 1994; Zeng et al. 1997) or partial agonist (Olianas et al. 1997; Michal et al. 1999). Clozapine and olanzapine may be relatively weaker M_1 receptor antagonists *in vivo* than *in vitro* (Bymaster and Falcone 2000). Thus, M_2 autoreceptor blockade and/or M_1 receptor stimulation by clozapine or M_2 receptor antagonism by olanzapine (Bymaster et al. 1996) may contribute to their ability to increase ACh release in the mPFC. However, thioridazine, despite affinity for M_1 ($K_i = 2.7$ nM) and M_2 receptors (14) comparable to that of olanzapine (1.9 and 18, respectively) (Table 1), did not increase ACh release in the mPFC. There is little question that thioridazine (20 mg/kg) gains access to the brain since it increases striatal DA release at the same or similar doses used here (Gainetidinov et al. 1994; Meltzer et al. 1994; Rayevsky et al. 1995). The lack of an effect of thioridazine contrasts the ability of scopolamine, a relatively non-selective mAChR antagonist, to increase ACh release in the mPFC. Although the discrepancy is not readily reconciled at present, it appears that thioridazine-induced M_1 receptor antagonism may negate its M_2 receptor antagonist effect on ACh release (Niedzwiecki et al. 1989b). Neither M_2 receptor blockade or M_1 receptor stimulation is likely to contribute to the effect of risperidone and ziprasidone because they lack affinity for M_1 or M_2 receptors (Bymaster et al. 1996).

There are many other possible mechanism(s) by which the four atypical APDs, which have a combination of a variety of effects on DA, 5-HT, noradrenergic and other receptors (Table 1), increase ACh release in the mPFC. Potent 5-HT_{2A} relative to weak D_2 receptor antagonism, which is a feature common to the atypical APDs, but not to typical APDs (Meltzer et al. 1989; Schotte et al. 1996), may contribute to the ability of these agents to increase ACh release in the mPFC. However, neither 5-HT_{2A} or D_2 receptor antagonism alone has any effect on increasing ACh release in that region:

1) ketanserin, a 5-HT_{2A} receptor antagonist (Hirano et al. 1995), ritanserin, a 5-HT_{2A/2C} receptor antagonist (Consolo et al. 1996a; Yamaguchi et al. 1997a,b), and M100907, a selective 5-HT_{2A} receptor antagonist (Ichikawa et al. in preparation); and 2) S(-)-sulpiride, a $D_{2/3}$ receptor antagonist (present data). 5-HT_{2C} receptors are also less likely to contribute to the effect on ACh release because the typical and atypical APDs share in common 5-HT_{2C} receptor antagonist properties (Roth et al. 1992; Meltzer, 1999). 5-HT_{1A} receptor agonists have been reported to increase cortical ACh release (Rada et al. 1993; Consolo et al. 1996b; Somboonthum et al. 1997; Koyama et al. 1999). We have recently suggested that potent 5-HT_{2A} and relatively weak D_2 receptor antagonism may promote 5-HT_{1A} receptor agonism (Ichikawa et al. 2001). Clozapine and ziprasidone are also 5-HT_{1A} receptor partial and full agonists, respectively (Newman-Tancredi et al. 1998). Therefore, it is possible that clozapine, olanzapine, risperidone, and ziprasidone may increase ACh release in the mPFC via direct and/or indirect 5-HT_{1A} receptor stimulation.

Clozapine and olanzapine are a D_1 receptor partial agonist (Ahlenius 1999) and antagonist (Bymaster et al. 1999), respectively. ACh release could result from D_1 receptor stimulation secondary to atypical APD-induced DA release in the mPFC (Kuroki et al. 1999; Ichikawa et al. 2001), as has been suggested for the STR (Imperato et al. 1993; De Boer and Abercrombie 1996; Steele et al. 1997). However, this is unlikely in the NAC and STR since the same or similar doses of APDs, clozapine, olanzapine, risperidone, ziprasidone, haloperidol, S(-)-sulpiride, and thioridazine increase DA release in the NAC and STR (Meltzer et al. 1994; Ichikawa and Meltzer 2000; Rollema et al. 2000), but do not increase ACh release in these two regions in the present study. It should also be considered that while clozapine is a D_1 receptor partial agonist (Ahlenius 1999), olanzapine is the antagonist (Bymaster et al. 1999), and both increased cortical ACh release. Antagonism of α_1 (and perhaps α_2) adrenoceptors may also contribute to the increases in ACh release produced by clozapine, olanzapine, risperidone, and ziprasidone, which have relatively high affinity for one or both of these receptors (Table 1). The α_1 adrenoceptor antagonist KF-15372 (Kurokawa et al. 1996) and the α_2 adrenoceptor antagonist idazoxan (Tellez et al. 1997) have both been reported to increase cortical ACh release.

The discrepancy between the present data and some, but not all, previous findings in other laboratories is due, in large part, to the use of AChE inhibitors. For example, clozapine, in the presence of 0.3 μ M neostigmine, significantly increased ACh release in the mPFC and, to a lesser extent, in the NAC and STR (present data), as previously reported by Parada et al. (1997). The failure of haloperidol to increase ACh release in these three regions with or without 0.3 μ M neo-

stigmine (present data), is consistent with the reported inability of haloperidol to increase ACh release in the mPFC, NAC, and STR in the presence of 0.5–1 μ M neostigmine (Osborne et al. 1994; Moore et al. 1999), but is not consistent with an increase in the STR at doses of 0.125–1 mg/kg in the presence of AChEsterase inhibition (Imperato et al. 1993; De Boer and Abercrombie 1996; Steinberg et al. 1998). Similarly, the lack of an effect of S(-)-sulpiride on ACh release in these three regions (present data) is consistent with the lack of an effect of S(-)-sulpiride (1 μ M) locally applied in the STR in the presence of AChEsterase inhibition (Ueda et al. 1995), but is not consistent with an increase in the STR in the presence of AChEsterase inhibition following its systemic (50 mg/kg) (Imperato et al. 1993) and local administration (0.1–10 μ M) (Ikarashi et al. 1997). To date, there have been no other microdialysis data of the effects of olanzapine, risperidone, ziprasidone, or thioridazine on ACh release in rat brain, with or without AChEsterase inhibition, nor data on the effect of clozapine, haloperidol, and S(-)-sulpiride in the mPFC, in the absence of AChEsterase inhibition.

The results reported here demonstrate an important difference in the action of atypical and typical APDs. M_1 receptor agonists have been reported to improve working memory in animals (Aura et al. 1997; McDonald et al. 1998) and xanomeline, a M_1 receptor agonist with minimal M_2 receptor blockade (Bymaster et al. 1994; Shannon et al. 1994, 2000), has also improved cognition, and decreased hallucinatory and delusional symptoms in Alzheimer's disease (Bodick et al. 1997; Bymaster et al. 1997) and perhaps schizophrenia, whereas M_1 receptor antagonists may worsen working memory in patients with schizophrenia (McEvoy 1987; Spohn and Strauss 1989; King 1990) and in animals (Bymaster et al. 1993; Aura et al. 1997; Roldan et al. 1997). The atypical APD-induced cortical ACh release would be expected to increase M_1 receptor stimulation by diminishing M_1 receptor antagonism where it exists. Clozapine-induced cortical ACh release suggests that it could be worthwhile to study the effect of clozapine and other atypical APDs with AChEsterase inhibitors, e.g., donepezil or galanthamine, in the treatment of psychosis and cognitive impairment in senile dementia, Alzheimer's disease, as well as schizophrenia.

In conclusion, the atypical APD, clozapine, olanzapine, risperidone, and ziprasidone, but not the typical APDs, haloperidol, S(-)-sulpiride, or thioridazine, increased ACh release in the mPFC, whereas none did so in the NAC or STR, suggesting cortical ACh may be important to some actions of atypical APDs.

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