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N-Desmethylclozapine, a Major Metabolite of Clozapine, Increases Cortical Acetylcholine and Dopamine Release *In Vivo* Via Stimulation of M_1 Muscarinic Receptors

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The active moiety of clozapine, the prototypical antipsychotic drug, consists of clozapine and its major metabolite, *N*-desmethylclozapine (NDMC). Previous studies have suggested that NDMC may be more important than the patent compound itself for the improvement in cognition in patients with schizophrenia treated with clozapine. While the pharmacology of clozapine and NDMC are similar in most respects, NDMC has been shown to be an M_1 muscarinic receptor partial agonist whereas clozapine is an M_1 antagonist *in vitro* and *in vivo*. We hypothesized that NDMC may improve cognition by increasing dopamine (DA) and acetylcholine (ACh) release in medial prefrontal cortex (mPFC) via direct stimulation of M_1 receptors, whereas both NDMC and clozapine itself would do so by other mechanisms as well, and that clozapine would inhibit the M1 agonist effect of NDMC. In the present study, using microdialysis in awake, freely moving rats, we found that NDMC at doses of 10 and 20, but not 5 mg/kg, significantly increased DA and ACh release in the mPFC and HIP, but not in the nucleus accumbens (NAC). The M_1-preferring antagonist, telenzepine (3 mg/kg), completely blocked NDMC (10 mg/kg)-induced increases in cortical DA and ACh release. Clozapine (1.25 mg/kg), which by itself had no effect on DA or ACh release in the cortex, blocked NDMC (10 mg/kg)-induced ACh, but not DA, release in the mPFC. The 5-HT_{1A} receptor antagonist, WAY100635 (0.2 mg/kg) blocked NDMC (20 mg/kg)-induced cortical DA but not ACh release. These findings suggest that: (1) NDMC is an M_1 agonist while clozapine is an M_1 antagonist *in vivo*; (2) M_1 agonism of NDMC can contribute to the release of cortical ACh and DA release; (3) NDMC, because of its M_1 agonism, may more effectively treat the cognitive impairments observed in schizophrenia than clozapine itself; and (4) M_1 receptor agonism may be a valuable target for the development of drugs that can improve cognitive deficit in schizophrenia, and perhaps other neuropsychiatric disorders as well.
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INTRODUCTION

Acetylcholine (ACh) plays an important role in motor function and various domains of cognition, for example attention, learning, and memory (Winkler *et al*, 1995; Perry *et al*, 1999). Cholinergic dysfunction has been shown to be central to the pathophysiology of Alzheimer's disease (Cummings and Benson, 1987) and has also been postulated to contribute to the cognitive deficits of various neuropsychiatric disorders, including schizophrenia (Tandon and Greden, 1989; Sarter and Bruno, 1998). There are five known (M_1–M_5) muscarinic acetylcholine receptors in the human genome (Kubo *et al*, 1986; Bonner *et al*, 1987; Brann *et al*, 1993). Of these, the M_1 receptor has been most closely linked to schizophrenia. The M_1 receptor subtype is the most abundant of the muscarinic receptors in the cortex and hippocampus (Levey *et al*, 1991; Wei *et al*, 1994), brain regions crucial to cognitive function. Decreased M_1 receptor binding has been reported in postmortem studies of the prefrontal cortex, hippocampus, and striatum from patients with schizophrenia (Dean *et al*, 1996; Crook *et al*, 2000, 2001; Katerina *et al*, 2004); and decreased M_1-receptor cDNA levels in the frontal cortex have also been reported (Mancama *et al*, 2003). This has contributed to the suggestion that enhancement of central cholinergic neurotransmission by M_1 agonists might be useful to treat the cognitive impairments of schizophrenia (Sur *et al*, 2003; Weiner *et al*, 2004).

Clozapine, the prototypical atypical antipsychotic drug (APD), was the first APD shown to be effective in treating the cognitive dysfunction of schizophrenia (Hagger *et al*, 1993), a finding which has been replicated, and is shared

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by other compounds with a similar pharmacology, for example, olanzapine, quetiapine, risperidone, and ziprasidone (Woodward *et al*, 2005). Clozapine and olanzapine have been reported to have antimuscarinic properties (Herrling and Misbach-Lesenne, 1982; Bymaster *et al*, 1996). Clozapine has nanomolar affinity for all five cloned muscarinic receptors (Bolden *et al*, 1992). It has been reported to act as an antagonist at M_1 receptor (Bolden *et al*, 1992; Zorn *et al*, 1994; Sur *et al*, 2003; Weiner *et al*, 2004) and $M_{2/3/5}$ receptor (Bymaster *et al*, 1996; Michal *et al*, 1999). However, clozapine is also reported as $M_{1/2/4}$ partial agonist (Zorn *et al*, 1994; Fritze and Tilmann, 1995; Zeng *et al*, 1997; Olianas *et al*, 1997). The discrepancy from these studies may be due to the methodology difference as these experiments involved CHO cells. To add to the complexity, *N*-desmethylclozapine (NDMC), the major active metabolite of clozapine in rodent and man (Aravagiri and Marder, 2001; Baldessarini *et al*, 1993; Weigmann *et al*, 1999), has its own unique muscarinic receptor pharmacology. Clozapine is rapidly metabolized to NDMC in rats and, thus, high serum levels of NDMC are seen after oral administration of clozapine, producing brain levels comparable to serum levels (Baldessarini *et al*, 1993; Weigmann *et al*, 1999). NDMC has been reported to be a potent M_1 agonist *in vivo* (Sur *et al*, 2003; Weiner *et al*, 2004) and, like clozapine, to have high affinities for 5-HT_{2A} and 5-HT_{2C}, and weaker, but still significant affinities, for D_2 receptors (Kuoppamaki *et al*, 1993, Weiner *et al*, 2004). This receptor-binding profile is similar to clozapine, suggesting that NDMC might have antipsychotic properties. NDMC also demonstrates a high affinity for M_4 and M_5 receptors, comparable to that observed for M_1 receptors (Weiner *et al*, 2004). Acute administration of NDMC, like clozapine, significantly increases c-Fos expression in the medial prefrontal cortex (mPFC) and nucleus accumbens (NAC), consistent with its atypical APD pharmacologic profile (Young *et al*, 1998).

Recently, Weiner *et al* (2004), using a cell-based functional assay, compared the effects of NDMC and clozapine on muscarinic receptors, and observed that NDMC displayed high potency and significant agonist efficacy at multiple muscarinic receptor subtypes, most notably the M_1 receptor. By contrast, clozapine behaved as an antagonist. Moreover, the M_1 agonist activity of NDMC was blocked by both atropine and clozapine. Furthermore, NDMC, but not clozapine, increased the phosphorylation of mitogen-activated protein kinase (MAP kinase) in the CA1 regions of mouse HIP, a response consistent with M_1 and not M_2–M_5-receptor activation (Berkeley *et al*, 2001). These results suggest that NDMC is a potent M_1 agonist, whereas clozapine displays potent M_1 antagonist actions *in vivo*. NDMC is the only commonly used antipsychotic agent that has been reported to have M_1 agonist activity (Weiner *et al*, 2004).

The ability of APDs to improve some or all aspects of the cognitive deficit in schizophrenia (Meltzer and McGurk, 1999; Woodward *et al*, 2005) has been attributed, in part, to their ability to preferentially increase the release of dopamine (DA) (Imperato and Angelucci, 1989; Moghaddam and Bunney, 1990; Kuroki *et al*, 1999) and ACh in the cortex and HIP (Ichikawa *et al*, 2002a, b; Shirazi-Southall *et al*, 2002; Chung *et al*, 2004), while the anticholinergic activity of clozapine, olanzapine, thioridazine, and mesoridazine has been suggested to interfere with memory (Eitan *et al*, 1992; Adler *et al*, 2002; McGurk *et al*, 2004). The increased DA release induced by the atypical APDs may be due, in part, to blockade of serotonin 5-HT_{2A} and D_2 receptors, and direct or indirect stimulation of 5-HT_{1A} receptors (Ichikawa *et al*, 2001). The mechanism by which clozapine increases ACh release in the mPFC is distinct from the mechanism by which clozapine increases cortical DA release, since 5-HT_{1A} receptor stimulation is not a factor in clozapine-induced ACh release (Ichikawa *et al*, 2002a).

In order to test the hypothesis that NDMC is an M_1 agonist and that the M_1-antagonist effect of clozapine may diminish the M_1-agonist effect of NDMC, the present study examined the effect of NDMC alone, and following pretreatment with telenzepine, an M_1-preferring antagonist (Schudt *et al*, 1988; Noronha-Blob *et al*, 1988), or low-dose clozapine, on DA and ACh release in the mPFC and, in some experiments, the NAC and HIP as well. We have previously found that telenzepine inhibited the ability of clozapine to increase DA and ACh release in rat mPFC (Ichikawa *et al*, 2004). We also examined the ability of WAY100635, a 5-HT_{1A}-receptor antagonist reported to block the effects of clozapine on DA but not ACh release (Ichikawa *et al*, 2002a), to inhibit the effect of NDMC on mPFC DA and ACh release.

MATERIALS AND METHODS

Animals

Male Sprague–Dawley albino rats (Zivic-Miller Laboratories, Porterville, PA) weighing 250–350 g were housed two 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. Animals used in this study were cared for in accordance with the guidelines of the Institutional Animal Care and Use Committee of Vanderbilt University. 'Principles of laboratory animal care' (NIH Publication No. 85-23, revised 1985) were followed.

Surgery and Microdialysis

Rats were anesthetized with the modified Equithesin mixture (810 mg pentobarbital, 4.3 g choral hydrate, 2.12 mg $MgSO_4$, 14 ml ethanol, and 29 ml propylene glycol were dissolved in saline and the final volume was 100 ml), and mounted in a stereotaxic frame (Stoetling, Wood Dale, IL). Stainless guide cannula (21-gauge) with a dummy probe were placed and fixed by cranioplastic cement (Plastic One, Roanoke, VA) onto the cortex dorsal to both the mPFC and the NAC. Rats received dual probe implantation for the mPFC, NAC, or HIP (coordinates: A +3.2, L +0.8 (10°C inclination), V −5.5 mm; A +2.0, L +1.5 to +1.7, V −7.5 mm; and A +5.6, L +5.0, V −7.0 mm, respectively, relative to bregma). The incision bar level was 3.0 mm, according to the atlas of Paxinos and Watson (1998).

The microdialysis probes were constructed in our laboratory. A silica-glass capillary tube (150 μm o.d., 75 μm i.d., Polymicro Technologies, Phoenix, AZ) was inserted through the inner bore of a 25 G stainless tube. The stainless tube was inserted into a 28 G Teflon tubing and then the Teflon tubing was inserted into the inner bore

of a 18 G stainless tube. The hollow fiber dialysis membrane (polyacrylonitrile/sodium methalylsulfonate polymer, 310 μm o.d., 220 μm i.d., 40 000 Da cutoff, AN69HF, Hospal; CGH Medical, Lakewood, CO) was fitted over the glass capillary and into the end of the 25 G stainless tube. This junction (0.5 mm) was glued with epoxy (5-Min Epoxy; Devkon, Danverse, MA, USA) after the length of the hollow dialysis fiber was cut to 3 mm and the tip of the membrane (0.5 mm) was plugged with epoxy. The length of exposed nonglued surface for dialyzing was 3 mm.

At 3–5 days after cannulation, a dialysis probe was implanted into the mPFC and NAC under slight anesthesia with isoflurane (Metofane, Pitman-Moore, Mundelein, IL). Rats were then housed individually overnight in a dialysis cage. After the overnight perfusion at 0.4 μl/min of the probe, the flow was increased to 1.5 μl/min. After 1 h, the dialysate samples were collected every 30 min. The perfusion medium was Dulbecco's phosphate-buffered saline solution (Sigma, St Louis, MO), including Ca^{2+} (138 mM NaCl, 8.1 mM Na_2HPO_4, 2.7 mM KCl, 1.5 mM KH_2PO_4, 0.5 mM MgCl, 1.2 mM $CaCl_2$, pH 7.4). No AChesterase inhibitor in the dialysate is required with this procedure (Ichikawa *et al*, 2002b). After stable baseline values in the dialysates were obtained, each rat received two injections, vehicle/NDMC, WAY100635/NDMC, telenzepine/NDMC, or clozapine/NDMC. The locations of the dialysis probes were verified at the end of each experiment by brain dissection. The procedures applied in these experiments were approved by the Institutional Animal Care and Use Committee of Vanderbilt University in Nashville, TN, where the present studies were completed.

Biochemical Assays

Determination of DA. Dialysate samples were directly applied onto a high-performance liquid chromatography (HPLC) with electrochemical detection, and analyzed with a Millennium chromatogram manager (Waters, Milford, MA). DA was separated (BDS Hypersil 3 μm C18, $1.0 \times 100\ mm^2$; Keystone Scientific, Bellefonte, PA) at 35°C maintained by column heater (LC-22C Temperature Controller; BAS, West Lafayette, IN). The mobile phase consisted of 48 mM anhydrous citric acid and 24 mM sodium acetate trihydrate containing 0.5 mM EDTA-Na_2, 10 mM NaCl, 2 mM dodecyl sulfate sodium salt, and 17 % (v/v) acetonitrile, adjusted to pH 4.8 with concentrated NaOH, and was pumped (0.05 ml/min) by LC-10AD (Shimadzu, Kyoto, Japan). A Unijet working electrode (MF-1003, BAS) was set at +0.58 V (LC-4C, BAS) *vs* an Ag/AgCl reference electrode. Reagents used were analytical or HPLC grade.

Determination of ACh. The method has been described previously (Ichikawa *et al*, 2002a). In brief, dialysate samples are directly injected onto the liquid chromatography/electrochemistry (LCEC) system assisted by a chromatography manager (Millennium; Waters, Milford, MA), and analyzed for ACh. ACh is separated on a coiled cation exchanger ACh column (analytical column) (Sepstik 10 nm ID 530°C 1.0 nm; BAS, West Lafayette, IN), followed by the post-IMER (immobilized enzyme reactor) (BAS), which consists of choline oxidase (ChO)/AChesterase. ACh is hydrolyzed by AChesterase to form acetate and choline in the post-IMER, and then choline is oxidized by ChO to produce betaine and hydrogen peroxide (H_2O_2). H_2O_2 is detected and reduced to H_2O on a Unijet amperometric detector cell with a peroxidase-redox-coated glassy carbon electrode (MF-9080; BAS), set at +100 mV (LC-4C; BAS) *vs* Ag/AgCl reference electrode. This reduction is analyzed with the detector (LC-4C; BAS) as signal indicating ACh in the chromatogram.

Drugs. NDMC (ACADIA Pharmaceutical Inc.) and clozapine (Sandoz, East Hanover, NJ) was dissolved in a small amount of 0.1 M tartaric acid and the pH was adjusted to 6–7 with 0.1 N NaOH. WAY100635 (Wyeth Laboratories, Philadelphia, PA) and telenzepine (Research Chemical Inc.) were dissolved in deionized water. Vehicle or drugs in a volume of 1.0 ml/kg were administered subcutaneously to randomly assigned rats.

Data analysis. Mean predrug baseline levels (time −60, time −30, and time 0) were designated as 100%. Following a significant overall repeated measures ANOVA (treatment × time), Fisher's protected least significant difference *post hoc* pairwise comparison and one-way ANOVA (StatView® 4.5 for the Macintosh) were used to determine group differences. A probability $p<0.05$ was considered significant in this study. All results are given as mean ± SEM.

RESULTS

Basal extracellular DA levels in the dialysates obtained from all the rats used in this study were 1.93 ± 0.11 (mean ± SEM fmol/10 μl; $N=51$) for the mPFC, 2.28 ± 0.09 (mean ± SEM fmol/10 μl; $N=45$) for the HIP, and 15.26 ± 0.52 (mean ± SEM fmol/20 μl; $N=42$) for the NAC, respectively. Basal extracellular ACh levels in the dialysates obtained from all the rats used in this study were 7.85 ± 0.22 (mean ± SEM fmol/10 μl; $N=40$) for the mPFC, 6.15 ± 0.37 (mean ± SEM fmol/10 μl; $N=38$) for the HIP, and 4.28 ± 0.65 (mean ± SEM fmol/20 μl; $N=45$) for the NAC, respectively. The ACh concentration in the mPFC or HIP was significantly higher than that in the NAC. There were no significant differences in basal extracellular DA or ACh levels between treatment groups within each region.

As shown in Figure 1, NDMC, at doses of 10 and 20 mg/kg, but not 5 mg/kg, dose-dependently increased extracellular DA concentrations in the mPFC ($F(1,12)=14.77$, $p=0.0002$; $F(1,11)=32.49$, $p<0.0001$, and $F(1,10)=1.27$, $p=0.26$, respectively). NDMC, at 10 and 20 mg/kg, but not 5 mg/kg, also significantly increased cortical ACh release, but in a nondose-dependent manner ($F(1,10)=4.18$, $p=0.04$; $F(1,9)=6.8$, $p=0.01$; and $F(1,10)=2.02$, $p=0.16$, respectively). High doses of NDMC and clozapine produced a similar effect on DA release (~250% over the baseline) (Kuroki *et al*, 1999). However, at a low dose, 5 mg/kg, clozapine had a greater effect in cortical DA release than NDMC since at 5 mg/kg NDMC had no effect on DA release but clozapine produced a significant increase in DA release in the mPFC (Kuroki *et al*, 1999). Clozapine produced a much greater increase in ACh release than NDMC since both low (5 mg/kg) and high (20 mg/kg) doses of clozapine

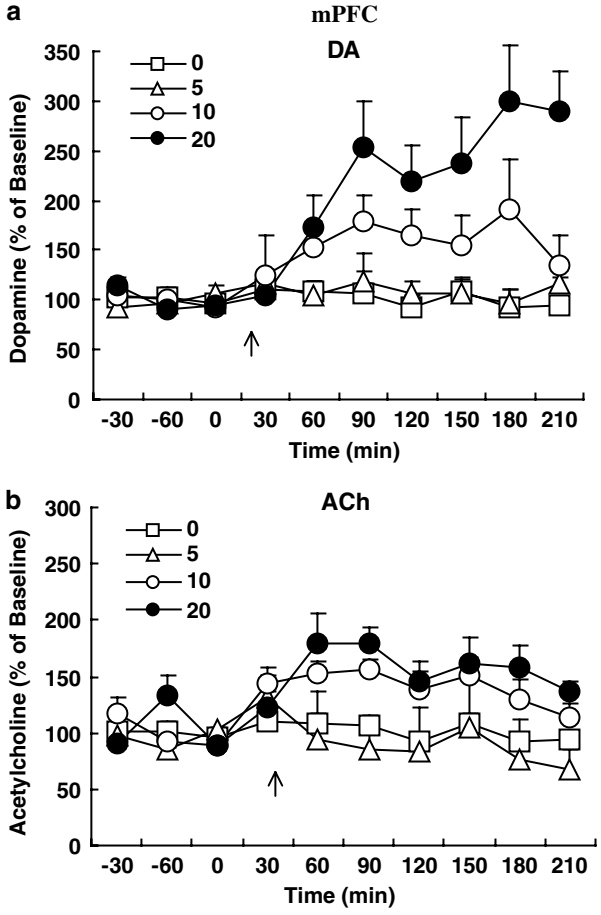

Figure 1 Time course effects of *N*-desmethylclozapine on extracellular dopamine (a) and acetylcholine (b) levels in the medial prefrontal cortex. The arrows indicate drug injection times. Data are means ± SEM ($N = 4$–7) of the dialysate dopamine or acetylcholine levels, expressed as a percentage of each predrug baseline dopamine or acetylcholine value.

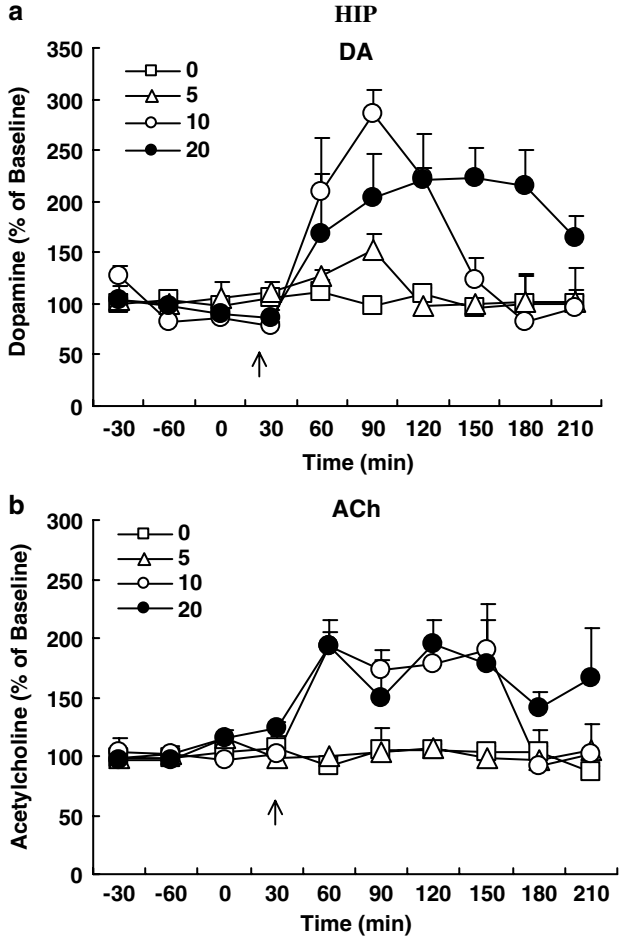

Figure 2 Time course effects of *N*-desmethylclozapine on extracellular dopamine (a) and acetylcholine (b) levels in the hippocampus. The arrows indicate drug injection times. Data are means ± SEM ($N = 5$–6) of the dialysate dopamine or acetylcholine levels, expressed as a percentage of each predrug baseline dopamine or acetylcholine value.

produced a great increase in ACh release in the mPFC (Ichikawa *et al*, 2002b).

In the HIP, NDMC, 10 and 20 mg/kg, significantly but nondose-dependently increased DA release ($F(1,8) = 13.54$, $p = 0.0004$ and $F(1,10) = 13.18$, $p = 0.004$, respectively) as well as ACh release ($F(1,10) = 19.48$, $p < 0.0001$ and $F(1,9) = 32.83$, $p < 0.0001$, respectively) (Figure 2). However, 5 mg/kg of NDMC did not increase either DA or ACh release in this region ($F(1,7) = 3.025$, $p = 0.756$ and $F(1,7) = 3.339$, $p = 0.705$, respectively) (Figure 2). In the NAC, neither 10 or 20 mg/kg of NDMC had any effect on DA ($F(1,10) = 0.64$, $p = 0.43$ and $F(1,11) = 0.6$, $p = 0.44$, respectively) or ACh release ($F(1,10) = 0.56$, $p = 0.49$) (Figure 3).

Telenzepine, 3 mg/kg, completely blocked 10 mg/kg NDMC-induced DA (Figure 4; $F(1,12) = 5.71$, $p = 0.018$) and ACh (Figure 4; $F(1,9) = 38.29$, $p < 0.0001$) release in the mPFC. Clozapine, 1.25 mg/kg, which itself had no effect on mPFC DA or ACh release (Figure 5), blocked NDMC (10 mg/kg)-induced ACh (Figure 5; $F(1,9) = 9.63$, $p = 0.003$) but not DA (Figure 5; $F(1,10) = 0.0003$, $p = 0.99$) release. WAY100635 partially and significantly blocked the increased mPFC DA release produced by NDMC, 20 mg/kg (Figure 6; $F(1,8) = 4.73$, $p = 0.03$), but had no effect on ACh release produced by the same dose of NDMC (Figure 6; $F(1,8) = 4.73$, $p = 0.03$).

DISCUSSION

The main findings of the present study are that (1) NDMC, the major active metabolite of clozapine, significantly increased DA and ACh release in the mPFC and HIP, but not the NAC; (2) the M_1-preferring antagonist telenzepine completely blocked DA and ACh release in the mPFC produced by NDMC; (3) NDMC (10 mg/kg)-induced ACh release was completely blocked by clozapine (1.25 mg/kg), consistent with previous reports that NDMC is a potent M_1 agonist, while clozapine has M_1 antagonist properties *in vivo*; (4) clozapine pretreatment did not block NDMC-induced cortical DA release, indicating M_1 agonism did not contribute to this effect of NDMC; and (5) the increases in DA, but not ACh, release in the mPFC produced by NDMC was partially blocked by the 5-HT_{1A} antagonist WAY100635, indicating that cortical DA release is partially dependent upon 5-HT_{1A}-receptor stimulation.

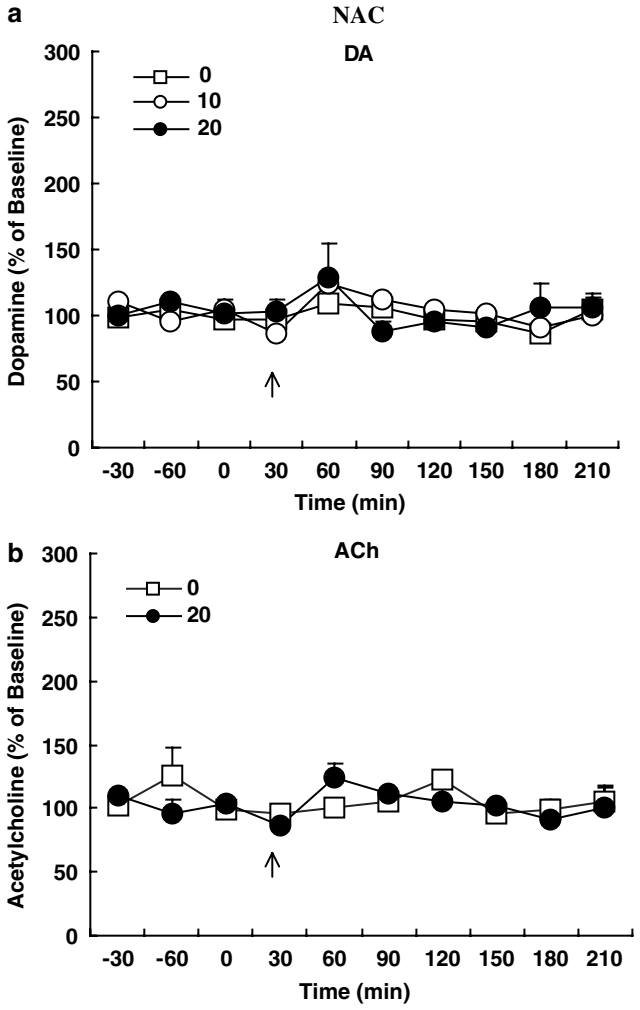

Figure 3 Time course effects of *N*-desmethylclozapine on extracellular dopamine and acetylcholine levels in the nucleus accumbens. The arrows indicate drug injection times. Data are means ± SEM ($N = 4$–7) of the dialysate dopamine levels, expressed as a percentage of each predrug baseline dopamine or acetylcholine value.

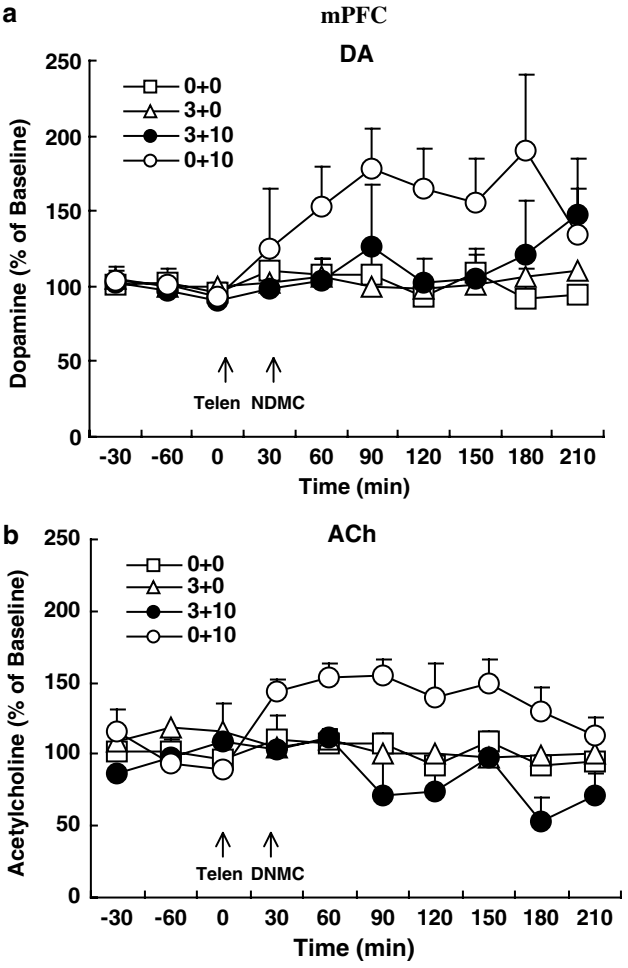

Figure 4 The effect of the M_1-receptor antagonist telenzepine (3 mg/kg, s.c.) on extracellular dopamine (a) and acetylcholine (b) release induced by *N*-desmethylclozapine (10 mg/kg, s.c.) in the medial prefrontal cortex. Rats were pretreated with telenzepine 30 min prior to administration of *N*-desmethylclozapine. The arrows indicate drug injection times. Data are means ± SEM ($N = 5$–7) of the dialysate dopamine or acetylcholine levels, expressed as a percentage of each predrug baseline dopamine or acetylcholine value.

Effect of NDMC on DA Release

Like clozapine and other atypical APDs, NDMC preferentially increased DA release in the mPFC and HIP compared to the NAC. Low-dose NDMC (5 mg/kg) had no effect on DA release in the mPFC, whereas the same dose of clozapine significantly increased mPFC DA release (Kuroki *et al*, 1999). However, NDMC and clozapine, at a dose of 20 mg/kg, produced similar increases in DA release. This suggests that NDMC may contribute to the ability of clozapine to increase cortical DA release in the rodent.

The fact that the increased DA release induced by NDMC in the mPFC was completely blocked by the M_1-receptor antagonist telenzepine indicates that the cortical DA release produced by NDMC is dependent upon activation of M_1 receptors. However, telenzepine also partially or completely blocked the effect of clozapine and risperidone, respectively, to increase DA release in the mPFC (Ichikawa *et al*, 2004). Risperidone, which has very low affinity for any muscarinic receptor subtype, is not an effective agonist at M_1 receptors (Schotte *et al*, 1996; Weiner *et al*, 2004). This suggests that both NDMC and risperidone increase cortical DA release by a mechanism that is not dependent upon direct stimulation of M_1 receptors, but could involve indirect mechanism as well, and so does not prove that NDMC is acting through a direct M_1 mechanism. The same may be true for ACh release. The M_1 receptor is the primary muscarinic receptor in the human frontal, temporal, parietal, and occipital cortical areas (Flynn *et al*, 1995). Cortical M_1 receptors are localized mainly on postsynaptic dendrites and spines associated with both glutamatergic and cholinegic transmission (Mrzljak *et al*, 1993). As the density of M_1 receptors is much greater than M_4 receptors in the cortex (Levey *et al*, 1991; Volpicelli and Levey, 2004), it seems more likely that the effect of atypical APDs to increase ACh release is more likely to be M_1- rather than M_4-mediated. We are currently investigating whether the effect of NDMC and clozapine is cortically mediated through local injection studies.

The ability of NDMC, like clozapine, to increase cortical DA release was also partially blocked by the 5-HT_{1A}-

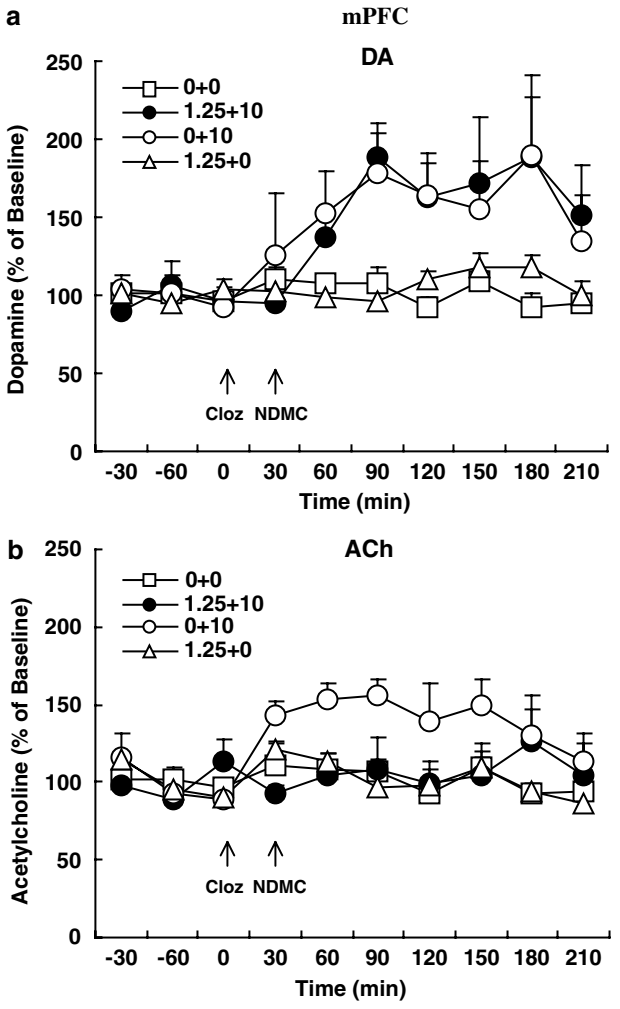

Figure 5 The effect of clozapine (1.25 mg/kg, s.c.) on extracellular dopamine (a) and acetylcholine (b) release induced by *N*-desmethylclozapine (10 mg/kg, s.c.) in the medial prefrontal cortex. Rats were pretreated with clozapine 30 min prior to administration of *N*-desmethylclozapine. The arrows indicate drug injection times. Data are means ± SEM ($N = 5$–6) of the dialysate dopamine or acetylcholine levels, expressed as a percentage of each predrug baseline dopamine or acetylcholine value.

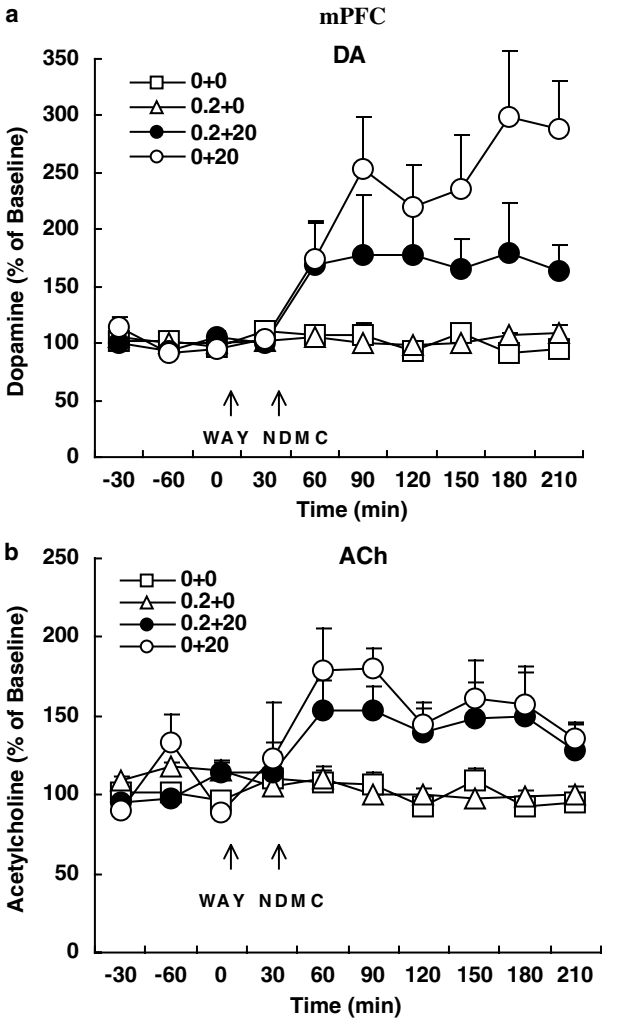

Figure 6 The effect of the 5-HT_{1A}-receptor antagonist WAY100635 (0.2 mg/kg, s.c.) on extracellular dopamine (a) and acetylcholine (b) release induced by *N*-desmethylclozapine (20 mg/kg, s.c.) in the medial prefrontal cortex. Rats were pretreated with WAY100635 30 min prior to administration of *N*-desmethylclozapine. The arrows indicate drug injection times. Data are means ± SEM ($N = 4$–8) of the dialysate dopamine or acetylcholine levels, expressed as a percentage of each predrug baseline dopamine or acetylcholine value.

receptor antagonist, WAY100635. Thus, both NDMC and clozapine increase cortical DA release, in part by a 5-HT_{1A}-dependent mechanism. NDMC has a higher affinity for the 5-HT_{1A} (111 nM) than for the D_2 (265 nM) receptor (P Herrling and P Neumann, personal communication, 1989) and is most likely a 5-HT_{1A} partial agonist, as is clozapine. However, WAY100635 also inhibits the increase in DA release produced by olanzapine and risperidone, neither of which are 5-HT_{1A} partial agonists (Ichikawa *et al*, 2001), suggesting an indirect mechanism that includes 5-HT_{1A} receptor stimulation.

NDMC also has higher affinities for the 5-HT_{2A} and 5-HT_{2C} receptors than the parent compound clozapine (Kuoppamaki *et al*, 1993; Weiner *et al*, 2004). The antagonism of 5-HT_{2A}, 5-HT_{2C} and D_2 receptor by NDMC may contribute to its ability to increase DA release in the mPFC and HIP, as is the case for other atypical APDs (Kuroki *et al*, 1999; Liegeois *et al*, 2002; Meltzer *et al*, 2003).

Effect of NDMC on ACh Release

NDMC, like clozapine and other atypical APDs (Ichikawa *et al*, 2002a), significantly increased ACh release in the mPFC and HIP, but not the NAC. The NDMC-induced ACh release in the mPFC was blocked by telenzepine but not WAY100635, as has been previously reported for clozapine and risperidone (Ichikawa *et al*, 2002a, 2004). This suggests that NDMC-induced cortical ACh release may be mediated by direct or indirect stimulation of M_1 but not 5-HT_{1A} receptors. In the present study, low-dose clozapine attenuated NDMC-induced cortical Ach, but not DA release, suggesting the M_1-receptor antagonism of clozapine blocked the M_1 agonism of NDMC. Therefore, the net effect of clozapine to increase cortical ACh release *in vivo* may be due, in part, to its metabolite NDMC, which would be partially attenuated by the M_1 antagonist actions of

clozapine. NDMC displays high potency interactions with all five human muscarinic receptors, with marked agonist activity at the M_1, M_4, and M_5 receptors (Weiner *et al*, 2004). The M_1 receptors involved in DA and ACh release may be located on DA and ACh postsynaptic nerve terminals in the cortex, HIP, or elsewhere in the forebrain on circuits that regulate the release of these neurotransmitters by 5-HT_{1A} receptors as well as glutamatergic and GABAergic mechanisms. Johnson *et al* (2005) recently reported that intra-hippocampal infusion of 10 μM clozapine and 100 μM olanzapine, but not intra-septal infusion, by reverse dialysis, increased HIP ACh efflux to an extent comparable to that of systemic administration. Cholinergic neurons from the mesopontine cholinergic nuclei (Ch5, Ch6) project to the DA cell bodies in the VTA (Bymaster *et al*, 2002). However, mainly M_5, not M_1, muscarinic receptors are localized on these neurons (Weiner *et al*, 1990). Further studies are required to determine if M_1 receptors located elsewhere, for example, the ventral tegmentum, nucleus basalis Meynert, or the septum, are involved in the effect of clozapine or NDMC in enhancing cortical or HIP DA release.

Previous *in vivo* microdialysis studies suggest that the muscarinic autoreceptor modulating ACh efflux in the mammalian medial pontine reticular formation (Baghdoyan *et al*, 1998), striatum (Billard *et al*, 1995), and cortex (Iannazzo and Majewski, 2000; Douglas *et al*, 2001) is M_2. Therefore, clozapine, NDMC, and olanzapine which are, to varying extents, M_2 antagonists (Bymaster *et al*, 2002; Weiner *et al*, 2004), may also enhance cortical and HIP ACh release via blockade of M_2 autoreceptors (Bymaster *et al*, 1996). Since completion of this study, Johnson *et al* (2005) reported that clozapine and olanzapine, 10 mg/kg, produced a marked increase in extracellular ACh in the HIP while ziprasidone produced a small increase. Based upon correlation of the $ED_{400\%}$ and *in vitro* functional potencies at muscarinic M_2 receptors, these authors concluded that the increase in ACh release produced by these compounds was due to M_2 antagonism. It should be noted that the study of Johnson *et al* (2005) used neostigmine in the dialysate fluid. We have shown elsewhere that this may alter the effect of some but not all psychotropic drugs. As ziprasidone and risperidone, which lack significant M_2 antagonism produce large increases in ACh release in the HIP, which are blocked by telenzepine, as is the case with clozapine (Chung *et al*, 2004; Ichikawa *et al*, 2004), we propose that M_1 agonism, direct or indirect, rather than M_2 antagonism, is primarily responsible for the release of ACh in the HIP.

Clinical Significance: NDMC, M_1-Receptor Agonism and Cognition

As previously mentioned, the M_1 receptor subtype is the most abundant of the muscarinic receptors in the cortex and hippocampus (Levey *et al*, 1991; Wei *et al*, 1994), brain regions crucial to normal cognitive function. M_1 receptors in the hippocampus have been shown to activate extracellular signal-regulated kinases (ERK), which are crucial for many neural functions, including learning, memory, and synaptic plasticity (Berkeley *et al*, 2001). These authors concluded that M_1 receptor-mediated ERK activation provides a mechanism by which M_1 receptors could modulate learning and memory. M_1 receptor agonists have been reported to improve working memory in animals (Aura *et al*, 1997; McDonald *et al*, 1998). Muscarinc antagonists with weak specificity for the M_1 receptor may worsen working memory in patients with schizophrenia (Spohn and Strauss, 1989; King, 1990), while more specific M_1 antagonists do so in laboratory animals (Bymaster *et al*, 1993; Roldan *et al*, 1997). Mice lacking M_1 receptors exhibit deficits in measures of spatial learning and memory, indicative of impaired hippocampal and cortical function (Anagnostaras *et al*, 2003). Learning deficits in the radial arm maze and fear-conditioning paradigm have also been reported in M_1-knockout mice (Miyakawa *et al*, 2001). Moreover, M_1-deficient mice have significantly elevated DA neurotransmission in the striatum (Gerber *et al*, 2001), significantly increased locomotor activity and increased response to the stimulatory effects of amphetamine, evidence of an inhibitory effect of the M_1 receptor on dopaminergic transmission, which suggests a possible basis for an antipsychotic effect of M_1 agonists. As previously mentioned, the $M_{1/4}$ agonist xanomeline has been reported to mimic the effect of D_2 antagonists to produce an antipsychotic-like profile in rats (Stanhope *et al*, 2001). It has been reported that NDMC dose-dependently potentiated NMDA receptor currents in CA1 pyramidal cells by 53% (Sur *et al*, 2003). Decreased glutamatergic activity in pyramidal neurons has been hypothesized to be a major factor in the pathophysiology of schizophrenia (Moghaddam, 2004; Javitt, 2004). Thus, the M_1 agonism of NDMC may, by stimulating glutamatergic activity, be of particular importance to the beneficial effects of NDMC and the parent compound, clozapine, on cortical function. Patients with schizophrenia who are heterozygous for the C267A polymorphism (267C/A) of the M_1 receptor have been reported to produce more correct responses and less perseverative errors on the Wisconsin Card Sort test, which is dependent upon prefrontal cortical function (Morice, 1990; Berman *et al*, 1995), than those who were homozygous for 267 C/C, providing additional genetic evidence suggesting that M_1 receptors have an important effect on prefrontal cortical function (Liao *et al*, 2003).

The effect of clozapine on DA or ACh release is most likely the result of the combined effect of clozapine and NDMC, the agonist/antagonist mixing. Thus, high NDMC levels, and particularly high NDMC/clozapine ratios, would increase M_1 muscarinic receptor stimulation, as predicted by mass action and by agonist/antagonist mixing studies (Brauner-Osborne *et al*, 1996). Brain clozapine concentrations in the rat during chronic treatment have been reported to exceed those of NDMC during chronic treatment by three-fold (Weigmann *et al*, 1999). There is no information on what the relative levels are in man. High concentrations of NDMC are found in plasma samples in some patients treated with clozapine (Hasegawa *et al*, 1993). High NDMC levels, and a high NDMC/clozapine ratio even more so, would increase M_1 muscarinic receptor stimulation. The present data on the blockade of NDMC-induced ACh release by clozapine are consistent with clinical data from our laboratory, which suggest that the NDMC/clozapine ratio is a better predictor of clinical response to clozapine than clozapine levels alone (Frazier *et al*, 2003; Mauri *et al*, 2003; Weiner *et al*, 2004).

In conclusion, NDMC preferentially increased DA and ACh release in the mPFC and HIP but not the NAC, similar to the effect of clozapine and other atypical APDs. The blockade of NDMC-induced ACh release by telezenpine and clozapine indicates that the stimulation of M_1 receptors contributes to the ability of NDMC to increase cortical DA and ACh release, confirming that NDMC has significant M_1 agonistic actions, whereas the parent compound, clozapine, is an antagonist.

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