N-desmethylclozapine, an allosteric agonist at muscarinic 1 receptor, potentiates *N*-methyl-D-aspartate receptor activity

Cyrille Sur*†, Pierre J. Mallorga*, Marion Wittmann*, Marlene A. Jacobson*, Danette Pascarella*, Jacinta B. Williams*, Philip E. Brandish‡, Douglas J. Pettibone*, Edward M. Scolnick‡, and P. Jeffrey Conn*§

Departments of *Neuroscience and ‡Neurobiology, Merck & Co. Inc., West Point, PA 19486

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The molecular and neuronal substrates conferring on clozapine its unique and superior efficacy in the treatment of schizophrenia remain elusive. The interaction of clozapine with many G protein-coupled receptors is well documented but less is known about its biologically active metabolite, *N*-desmethylclozapine. Recent clinical and preclinical evidences of the antipsychotic activity of the muscarinic agonist xanomeline prompted us to investigate the effects of *N*-desmethylclozapine on cloned human M1–M5 muscarinic receptors. *N*-desmethylclozapine preferentially bound to M1 muscarinic receptors with an IC_{50} of 55 nM and was a more potent partial agonist (EC_{50}, 115 nM and 50% of acetylcholine response) at this receptor than clozapine. Furthermore, pharmacological and site-directed mutagenesis studies suggested that *N*-desmethylclozapine preferentially activated M1 receptors by interacting with a site that does not fully overlap with the acetylcholine orthosteric site. As hypofunction of *N*-methyl-D-aspartate (NMDA) receptor-driven neuronal ensembles has been implicated in psychotic disorders, the neuronal activity of *N*-desmethylclozapine was electrophysiologically investigated in hippocampal rat brain slices. *N*-desmethylclozapine was shown to dose-dependently potentiate NMDA receptor currents in CA1 pyramidal cells by 53% at 100 nM, an effect largely mediated by activation of muscarinic receptors. Altogether, our observations provide direct evidence that the brain penetrant metabolite *N*-desmethylclozapine is a potent, allosteric agonist at human M1 receptors and is able to potentiate hippocampal NMDA receptor currents through M1 receptor activation. These observations raise the possibility that *N*-desmethylclozapine contributes to clozapine's clinical activity in schizophrenics through modulation of both muscarinic and glutamatergic neurotransmission.

Despite the risk of agranulocytosis, the atypical antipsychotic clozapine is still commonly used because of its proven efficacy in treatment-refractory schizophrenic patients (1). Furthermore, in addition to its efficacy in reducing positive and negative symptoms while causing very few extrapyramidal side effects (2), clozapine has been reported to favorably impact most impaired cognitive functions (3) and to reduce the risk of suicide (4) in schizophrenia patients. Although effective, atypical antipsychotics like risperidone and olanzapine do not appear to have a therapeutic spectrum that is as broad as clozapine (2, 3, 5). The unique profile of clozapine cannot be fully explained either by its $5HT_{2A}/D_2$ receptor blockade ratio (6) nor its affinity for D_4 receptors (7) as these characteristics are shared by other drugs (8). Clozapine has a promiscuous pharmacology, interacts with many G protein-coupled receptors, and has nanomolar affinity for all five cloned muscarinic receptors (M1–M5) (8, 9). Clinical observation of hypersalivation in clozapine-treated patients and the ability of scopolamine to block clozapine-induced dopamine efflux in rat striatum supported muscarinic agonist activity for this drug (10, 11). Indeed, studies with recombinant human muscarinic receptors have revealed that clozapine is a partial agonist at M4 receptors and induces a modest activation of M1 and M3 receptors (12–15). Besides helping explain some side effects of clozapine, its partial muscarinic agonist activities may contribute to its unique therapeutic profile. Notably, antimuscarinics have been reported to induce psychosis, confusion, and cognitive deficits in humans (16, 17), and double-blind, placebo-controlled clinical study with M1/M4 agonist xanomeline in Alzheimer's disease patients revealed significant improvements in psychotic behavior and cognitive abilities (18) in agreement with preclinical studies supporting xanomeline's antipsychotic profile (19, 20). Interestingly, these effects of xanomeline are consistent with the reported potentiation of *N*-methyl-D-aspartate (NMDA) receptor activity by muscarinic agonist in rat forebrain (21). Indeed, NMDA receptor-mediated neurotransmission plays a critical role in memnomic and cognitive processes, and its disruption by antagonists such as ketamine has been shown to trigger schizophrenia-like psychosis in humans and exacerbate existing symptoms in schizophrenics (22).

In schizophrenia patients clozapine is metabolized in the liver to yield two major metabolites, *N*-desmethylclozapine and clozapine-*N*-oxide, and unlike clozapine-*N*-oxide, *N*-desmethylclozapine is a major circulating metabolite with levels in patient plasma ranging from 10% to 100% of parent compound (23–25). Rodent studies confirmed that circulating *N*-desmethylclozapine approaches the concentration of clozapine in rat serum, crosses the brain–blood barrier, and induces a specific pattern of c-*fos* expression in rat forebrain that is similar to the one observed after clozapine treatment (24, 26–27). These observations raised the possibility that *N*-desmethylclozapine contributes to the therapeutic efficacy of clozapine. *N*-desmethylclozapine has been reported to have a higher affinity for 5HT1C and 5HT2 receptors than clozapine and a comparable affinity for D2 receptor (28). However, *N*-desmethylclozapine's overall pharmacology remains largely unknown, although its structure suggests a promiscuous pharmacology like clozapine. Therefore the following pharmacological and electrophysiological studies investigated the relationships between *N*-desmethylclozapine and human muscarinic receptors and the effect of *N*-desmethylclozapine muscarinic agonist activity on NMDA receptor-mediated currents in pyramidal neurons of the hippocampus.

Materials and Methods

Chemicals. [^{3}H]*N*-methylscopolamine (NMS) (81 Ci/mmmol) was obtained from Perkin–Elmer Life Sciences. Clozapine, *N*-desmethylclozapine, and QX 314 [*N*-(2,6-dimethylphenylcarbamoylmethyl)triethylammonium bromide] were purchased from Tocris Cookson (Ballwin, MO), and clozapine *N*-oxide was

Abbreviations: NMDA, *N*-methyl-D-aspartate; NMS, [^{3}H]*N*-methylscopolamine; AC42, 4-n-butyl-1-[4-(2-methylphenyl)-4-oxo-1-butyl]-piperidine hydrogen chloride; FLIPR, fluorometric imaging plate reader; PI, phosphatidyl inositol.

†To whom correspondence should be addressed. E-mail: cyrille_sur@merck.com.

§Present address: Department of Pharmacology, Vanderbilt University Medical Center, Nashville, TN 37232-6600.

purchased from Biomol (Plymouth Meeting, PA). Haloperidol, chlorpromazine, and thioridazine were obtained from RDI (Flanders, NJ). Olanzapine, risperidone, and AC42 (4-n-butyl-1-[4-(2-methylphenyl)-4-oxo-1-butyl]-piperidine hydrogen chloride) were synthesized in Merck Research Laboratories. Brucine and tetrodotoxin were purchased from Sigma. All other reagents were of the highest purity commercially available.

Cell Lines. Stable cell lines expressing human M1, M3, and M1Y381A mutated receptors were generated in CHONFAT cells by Lipofecatmine 2000 transfection with cDNAs subcloned into pcDNA3 (Invitrogen) for M1 and M3 and pIRESneo2 (Clontech) for M1Y381A. Cells were cultured in DMEM containing 10% FBS, 25 mM Hepes, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 50 units/ml Pen/Strep, 2 mM glutamine, 250 μg/ml Zeocin, and G418 at 1 mg/ml and grown in 5% CO_2, 37°C.

Radioligand Binding Studies. Membranes from CHOK1 M1–M5 cell lines expressing from 0.8 to 3 pmol/mg protein of receptor were obtained from Perkin–Elmer. Membrane were prepared in 20 mM Hepes, pH 7.4 containing 5 mM $MgCl_2$ (CHO buffer) at a final protein concentration of 1–2 mg/ml. Competition binding studies were performed by incubating membranes with 200 pM ^{3}H-NMS, for 90 min at room temperature in a final volume of 500 μl of CHO buffer in the presence of the various concentrations of the compound to be studied. Nonspecific binding was determined by using 1 μM atropine. The assay was terminated by rapid filtration on Filtermat A (Wallac 1204–401, GF/C filter), followed by three washes with ice-cold CHO buffer by using a Skatron Micro 96 Harvester (Molecular Devices). Dried filters were counted in a Wallac Betaplate 1205. When brucine was used it was preincubated with the membranes for 5 min.

Fluorometric Imaging Plate Reader (FLIPR). CHONFAT cells expressing M1, M3, and M1-Y381A mutated receptors were plated (20,000 cells per well) in clear-bottomed, poly-D-lysine-coated 384-well plates in glutamate/glutamine-free medium by using a Labsystems (Chicago) Multidrop. The plated cells were grown overnight at 37°C in the presence of 6% CO_2. The next day, the cells were washed with 3 $\times$ 100 μl assay buffer (Hanks' balanced salt solution containing 20 mM Hepes, 2.5 mM probenecid, and 0.1% BSA). The cells were incubated with 1 μM Fluo-4AM (Molecular Probes) for 1 h at 37°C and 6% CO_2. The extracellular dye was removed by washing as described above. Ca^{2+} flux was measured by using a Molecular Devices $FLIPR_{384}$. For potency determination, the cells were incubated with various concentrations of compound for 5 min, and for potentiation experiments brucine (100 μM) was added 4 min before the agonist.

GTPγS Binding Assay. The GTPγS binding assay was conducted on membranes from CHOK1 cells expressing the human M2 and M4 receptors by using the SPA G protein-coupled receptor assay kit from Amersham Pharmacia Biotech. Final concentrations of 20 mM Hepes, 100 mM NaCl, 10 mM MgCl2, 1 mM EDTA, 0.17 mg/ml DTT, 10 μM GDP, 20 μg membrane protein, 200 pM ^{35}S-GTPγS (1100 Ci/mmol, Amersham Pharmacia), and 7.5 mg/ml SPA beads were used for the assay in a final volume of 0.2 ml in 96-well microplates. Agonists were added for 30 min at room temperature, and plates were centrifuged at 1,000 $\times$ *g* for 10 min and counted in a TopCount counter (Packard). Acetylcholine (1 mM) stimulated basal activity by a factor of 2- to 3-fold with EC_{50} values of 1 μM and 0.8 μM at M2 and M4, respectively.

Phosphatidyl Inositol (PI) Turnover Assay. The PI turnover assay was conducted on CHONFAT cells expressing the M1 and M1-Y381A mutated receptor plated on 96-well tissue culture plates according to the SPA-based method of Brandish *et al.* (29) except that DMEM was used instead of inositol-free DMEM.

Electrophysiology. All patch-clamp experiments were performed on slices from 20- to 30-day old Sprague–Dawley rats (Taconic Farms). After decapitation, brains were rapidly removed and submerged in an ice-cold choline chloride buffer (126 mM choline chloride/2.5 mM KCl/8 mM $MgSO_4$/1.3 mM $MgCl_2$/1.2 mM NaH_2PO_4/10 mM glucose/26 mM $NaHCO_3$), equilibrated with 95% O_2/5% CO_2. Parasagittal slices (300 μm thick) obtained by using a Vibraslicer were transferred to a holding chamber containing normal artificial cerebrospinal fluid (ACSF): 124 mM NaCl, 2.5 mM KCl, 1.3 mM $MgSO_4$, 1.0 mM NaH_2PO_4, 2.0 mM $CaCl_2$, 20 mM glucose, 26 mM $NaHCO_3$, equilibrated with 95% O_2/5% CO_2 at room temperature. In all experiments, 5 μM glutathione, 500 μM pyruvate, and 250 μM kynurenic acid were included in the choline chloride buffer and holding chamber to increase slice viability. Whole-cell patch-clamp recordings from CA1 pyramidal neurons were obtained by using the "blind" patch technique. During recordings slices were maintained fully submerged on the stage of a brain slice chamber at 34°C and perfused continuously with equilibrated ACSF (2–3 ml/min). Patch electrodes were pulled from borosilicate glass on a two-stage vertical patch pipette puller and filled with 140 mM cesium methane sulfonate, 16 mM Hepes, 10 mM NaCl, 2 mM EGTA, 0.2 mM NaGTP, 2 mM MgATP, pH adjusted to 7.5 with 1 M CsOH. In all experiments, 1 mM QX 314 [*N*-(2,6-dimethylphenylcarbamoylmethyl)triethylammonium bromide] was added to the internal pipette solution to block voltage-activated Na^+ channels. Addition of cesium and QX 314 to the internal solution was necessary to maintain voltage control during NMDA application. However, this makes it difficult to determine the cell type of the recorded neurons by evaluating membrane and firing properties. It is widely accepted that the CA1 pyramidal neuron layer of the hippocampus contains mainly pyramidal cells. Because we recorded only from neurons in this layer we can be confident that the majority of recorded cells were pyramidal neurons. Electrode resistance was 5–8 MΩ. All recordings were performed by using a HEKA EPC10 patch clamp amplifier (HEKA Electronics, Lambrecht/Pfalz, Germany). For measurement of NMDA-evoked currents 100 μM NMDA was applied directly to the postsynaptic cell with a modification of the u-tube fast application system. NMDA-evoked currents were recorded at a holding potential of -60 mV,

Table 1. Affinity of clozapine and its metabolites to NMS binding site

Compound	hM1	hM2	hM3	hM4	hM5
Acetylcholine	14 $\pm$ 1.8 μM	1.8 $\pm$ 0.2 μM	9.5 $\pm$ 0.8 μM	12 $\pm$ 1.9 μM	15 $\pm$ 0.9 μM
Clozapine	13 $\pm$ 2	51 $\pm$ 4	25 $\pm$ 2	33 $\pm$ 4	26 $\pm$ 3
N-desmethylclozapine	55 $\pm$ 5	1,650 $\pm$ 101	223 $\pm$ 30	344 $\pm$ 25	105 $\pm$ 8
Clozapine-*N*-oxide	2.5 $\pm$ 0.3 μM	33 $\pm$ 3 μM	8 $\pm$ 0.7 μM	4 $\pm$ 0.3 μM	2.5 $\pm$ 0.2 μM

IC_{50} values are the mean $\pm$ SEM of three experiments performed in quadruplicate. IC_{50} are expressed in nM except otherwise indicated. [^{3}H] NMS concentration was 200 pM.

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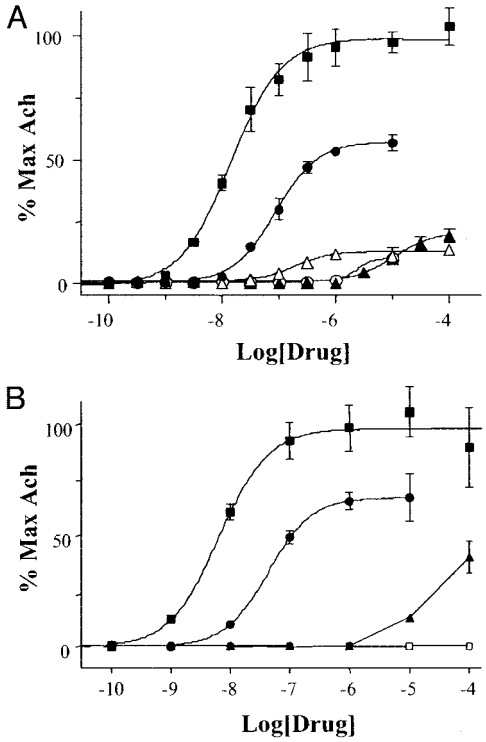

Fig. 1. M1 agonist activity of *N*-desmethylclozapine. (*A*) FLIPR experiments on M1-CHONFAT cells revealed that *N*-desmethylclozapine (●) is a partial M1 agonist compared with acetylcholine (Ach) (■). Clozapine (△) and its metabolite clozapine-*N*-oxide (▲) weakly stimulated intracellular calcium mobilization. Note that the activity of *N*-desmethylclozapine was blocked by 1 μM atropine (○). (*B*) Haloperidol (▲) and olanzapine (□) did not potently activate M1 receptor compared with acetylcholine (■) and *N*-desmethylclozapine (●). Results shown are from a representative experiment (points represent the mean ± SEM of quadruplicate determinations) that was repeated at least three times with comparable results (mean ± SEM given in Table 2).

and 1 μM tetrodotoxin was present in the bath to block synaptic transmission. All drugs were bath-applied to the slice. Atropine was applied at least 10 min before application of *N*-desmethylclozapine. Percent potentiation of NMDA receptor-mediated currents was defined by using the ratio of maximum current during *N*-desmethylclozapine application (average of three trials during maximal drug effect) to average current amplitude of three trials immediately before drug application. All data are expressed as mean ± SEM.

Data Analysis. Binding and functional data represent the means of at least three experiments performed in triplicate unless otherwise indicated and were analyzed by nonlinear regression analysis using PRISM software (GraphPad, San Diego).

Results

***N*-Desmethylclozapine Is a Partial Agonist at M1 Receptor.** The well-established promiscuous pharmacology of clozapine including its high affinity for muscarinic receptors (8, 9) prompted us to first determine the selectivity profile of *N*-desmethylclozapine and clozapine *N*-oxide against the five muscarinic receptor subtypes. Clozapine-*N*-oxide was a weak inhibitor at all muscarinic receptors, whereas *N*-desmethylclozapine antagonized [^{3}H]NMS binding to M1 with an IC_{50} of 55 ± 5 nM (mean ± SEM, $n = 3$) (Table 1). *N*-desmethylclozapine was also relatively potent at M3–M5 receptors, but 60-fold weaker at M2 than M1.

To investigate the potential agonist activity of clozapine and its metabolites, their potency to stimulate calcium mobilization in CHONFAT cells expressing human M1 receptors (B_{max}: 475 ± 25 fmol/mg of protein) was studied. As shown in Fig. 1*A* and Table 2, *N*-desmethylclozapine behaved as a partial agonist activating M1 receptors to 50 ± 5% of the maximal acetylcholine response with an EC_{50} of 115 ± 28 nM ($n = 10$). This response was fully prevented by coincubation with atropine, confirming the muscarinic nature of the effect (Fig. 1*A*), and the agonist activity was selective for the M1 receptor (Fig. 1*A* and Table 2). In contrast, clozapine and clozapine-*N*-oxide were very weak agonists, stimulating M1 receptors to 11 ± 1% and 23 ± 4% of acetylcholine response, respectively (Fig. 1*A* and Table 2). Although weaker, this activity also was selective for M1 subtype as in the GTPγS assay clozapine, and clozapine-*N*-oxide did not stimulate M2–M4 receptors at concentrations up to 100 μM (Table 2). The specificity of *N*-demesthylclozapine M1 agonist activity was further evidenced by the lack of effect of haloperidol and olanzapine at M1 receptors in FLIPR assay (Fig. 1*B*).

***N*-desmethylclozapine Is an Allosteric Agonist at M1 Receptor.** To better understand the interaction of *N*-desmethylclozapine with M1 receptor, and in particular its orthosteric site, we studied the effect of the selective M1 allosteric potentiator brucine (30) on *N*-desmethylclozapine-induced response. As shown in Fig. 2, brucine potentiated acetylcholine response by increasing its EC_{50} 3.9- ± 0.4-fold ($n = 9$) without affecting the maximal response. In contrast, brucine exhibited a negative allosteric interaction with *N*-desmethylclozapine (Fig. 2) and shifted its efficacy ratio by 0.23 ± 0.04 ($n = 3$). In addition, brucine reduced the *N*-desmethylclozapine response from 50 ± 5% to 40 ± 5% ($n = 3$) of acetylcholine maximal response. Mutation of Tyr-381 to alanine in the M1 receptor has been shown to drastically affect the orthosteric site and acetylcholine affinity (31), but not the pharmacology of an agonist acting at an ectopic site on the M1 receptor, AC42 (32). Unexpectedly, this Y381A M1 mutation increased *N*-desmethylclozapine potency 8-fold and doubled its efficacy (96% of acetylcholine) so that the clozapine metabolite became a full M1 receptor agonist (Fig. 3 and Table 3). Similarly, clozapine behaved as a potent, almost full agonist at Y381A mutant, whereas the pharmacology of the ectopic agonist AC42 was almost unchanged (Table 3). The potencies of the orthosteric site agonists, acetylcholine and carbachol, were

Table 2. Potency and efficacy (as % of acetylcholine response) of clozapine and its metabolites at recombinant human muscarinic receptors

Compound	hM1	hM2	hM3	hM4
Acetylcholine	21 ± 6 (100)	1,003 ± 235 (100)	5.2 ± 1.8 (100)	837 ± 192 (100)
Clozapine	293 ± 87 (11 ± 1)	0% at 100 μM	5 ± 3% at 100 μM	0% at 100 μM
N-desmethylclozapine	115 ± 28 (50 ± 5)	857 ± 598 (38 ± 9)	2 ± 1.6 μM (15 ± 4)	0% at 100 μM
Clozapine-*N*-oxide	8 ± 1.5 μM (23 ± 4)	0% at 100 μM	0% at 100 μM	0% at 100 μM

EC_{50} values are mean ± SEM of 3–10 determinations and are expressed in nM unless otherwise specified. Data for hM1 and hM3 and for hM2 and hM4 are from FLIPR experiments and GTPγS assay, respectively. Numbers in parentheses are percentages of acetylcholine response.

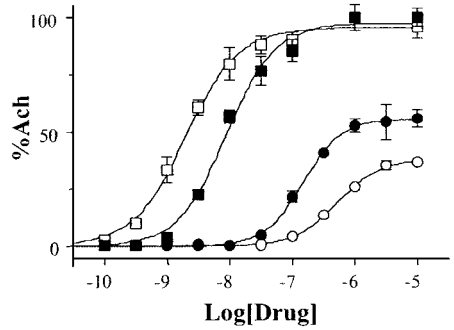

Fig. 2. Effect of the M1 allosteric potentiator brucine (100 μM) on *N*-desmethylclozapine. Dose–response curves for *N*-desmethylclozapine without (●) and with (○) brucine and for acetylcholine (Ach) without (■) and with (□) brucine showed that brucine shifted the acetylcholine concentration–response curve to the left by a factor of 4.5 but shifted the *N*-desmethylclozapine concentration–response curve to the right by a factor of 3.5. Results shown are from a representative experiment (points represent the mean ± SEM of quadruplicate determinations) that was repeated at least three times with comparable results (mean ± SEM given in the text).

reduced >150-fold while both compounds retained full efficacy (Table 3).

The partial agonist activity of *N*-desmethylclozapine was confirmed in another cell-based assay through determination of PI turnover. In M1 CHONFAT cells, *N*-desmethylclozapine induced production of PI to 22 ± 6% ($n = 3$) of acetylcholine maximal response with a potency similar to acetylcholine (1.1 ± 0.1 μM, $n = 3$ versus 1.1 ± 0.4 μM, $n = 4$), whereas clozapine was inactive in this assay (0% at 100 μM; $n = 2$). The efficacy of *N*-desmethylclozapine to stimulate PI production was increased to 79 ± 5% ($n = 3$) of acetylcholine response at the Y381A M1 mutant, whereas its potency was not significantly changed (1.8 ± 0.9 μM; $n = 3$). As expected the potency of acetylcholine at Y381A mutant in this assay was also significantly diminished from 1.1 ± 0.4 μM ($n = 4$) to 259 ± 39 μM ($n = 3$).

***N*-desmethylclozapine Potentiates NMDA Receptor Currents in CA1 Pyramidal Cells.** The finding that *N*-desmethylclozapine is a partial agonist at recombinant M1 receptors is intriguing. However, this does not necessarily imply that this compound will act as an agonist at native M1 receptors. Indeed, high levels of receptor reserve that can be present in recombinant systems can dramatically shift the apparent efficacy of weak partial agonists. Previous studies have shown that bath application of the muscarinic agonist carbachol potentiates NMDA receptor currents in hippocampal CA1 pyramidal cells, and that this potentiation is mediated by activation of the M1 muscarinic acetylcholine receptor subtype (22). Fast application of NMDA (0.5–1.5 s, 100 μM) in 1-min intervals directly to the recording site produced a stable inward current (30–80 pA) in CA1 pyramidal cells. Bath application of a low dose of *N*-desmethylclozapine (100 nM, 10 min) induced a significant potentiation of NMDA-evoked currents (53 ± 18%, $n = 5$, $P < 0.05$, Student's t test, paired, two-tailed, Fig. 4 A_1 and D). The effect of 100 nM *N*-desmethylclozapine on NMDA-evoked currents slowly increased during the 10-min bath application and showed no clear washout for at least 15 min after the drug was turned off (Fig. 4 A_1 and B). The concentration–response curve for *N*-desmethylclozapine was U-shaped with a maximal effect at 100 nM to increase NMDA-evoked currents (Fig. 4C). Prior bath application of 1 μM atropine blocked the effect of 100 nM *N*-desmethylclozapine (13 ± 16%, $n = 4$, $P > 0.5$, Student's t test, paired, one-tailed, Fig. 4 A_2 and D), indicating that the effect of *N*-desmethylclozapine at low concentrations is mediated by activation of muscarinic receptors. It is well known that *N*-desmethylclozapine has affinities for a variety of other G protein-coupled receptor like serotonin or dopamine (28) that could account for the reversal of the effect at higher concentrations.

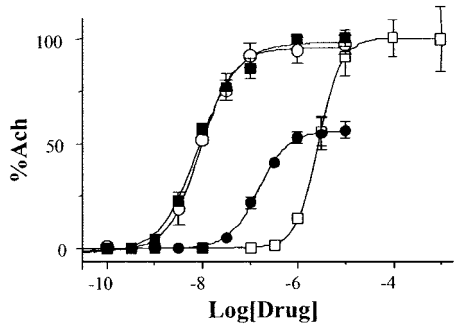

Fig. 3. Effect of mutation at M1 orthosteric site on *N*-desmethylclozapine and acetylcholine (Ach)-induced functional response measured by using FLIPR. The Y381A mutation (□ and ○) caused a shift of acetylcholine (■) and *N*-desmethylclozapine (●) concentration–response curves to the right by a factor of 315 and to the left by a factor of 16, respectively. Results shown are from a representative experiment (points represent the mean ± SEM of quadruplicate determinations) that was repeated at least three times with comparable results (mean ± SEM given in the text).

Table 3. Potency and efficacy (as % of acetylcholine response) of muscarinic agonists at WT and mutated M1 receptors

Compound	hM1-WT	hM1-Y381A
Acetylcholine	21 ± 6 (100)	3,837 ± 677 (100)
Carbachol	76 ± 18 (96 ± 1)	12,538 ± 2,496 (93 ± 3)
Clozapine	293 ± 87 (11 ± 1)	15 ± 2 (90 ± 1)
N-desmethylclozapine	115 ± 28 (50 ± 5)	14 ± 2 (96 ± 2)
AC42	805 ± 251 (71 ± 2)	220 ± 16 (83 ± 3)

C_{50} values (nM) are mean ± SEM of three determinations. Numbers in parentheses are percentage of acetylcholine response.

Discussion

In this study we investigated the interaction of clozapine and its metabolites with recombinantly expressed human muscarinic receptors. Consistent with previous reports (8, 12), we found that clozapine has a high affinity for M1–M5 receptor NMS binding sites. Functionally, clozapine showed a weak agonist activity at M1 receptors similar to its reported partial agonistic effect on the production of inositol phosphate in Chinese hamster ovary cells expressing high levels of M1 receptors (15). The lack of clozapine activity at muscarinic M4 receptors in our assay contrasted with several positive studies in recombinant systems (12–14) but is consistent with clozapine antagonism at striatal M4 receptors (13, 14). *N*-desmethylclozapine interacted with the NMS binding site of all muscarinic receptor subtypes albeit with lower affinity than its parent compound. However, the agonist profile of *N*-desmethyclozapine was different from clozapine by exhibiting a consistent partial agonistic effect at M1 receptors. This metabolite also displayed a weak potency and efficacy at M2 and M3 receptors.

In contrast to its reported allosteric potentiation of acetylcholine activity (30, 33), brucine significantly reduced *N*-desmethylclozapine apparent potency at M1 receptor. This effect suggested to us that *N*-desmethylclozapine most likely does not interact in the same way as acetylcholine with all anchoring amino acid residues within M1 orthosteric domain. Such an incomplete sharing of residues and overlap have been documented for acetylcholine and NMS (34), and brucine has been shown to have different allosteric relationships with these

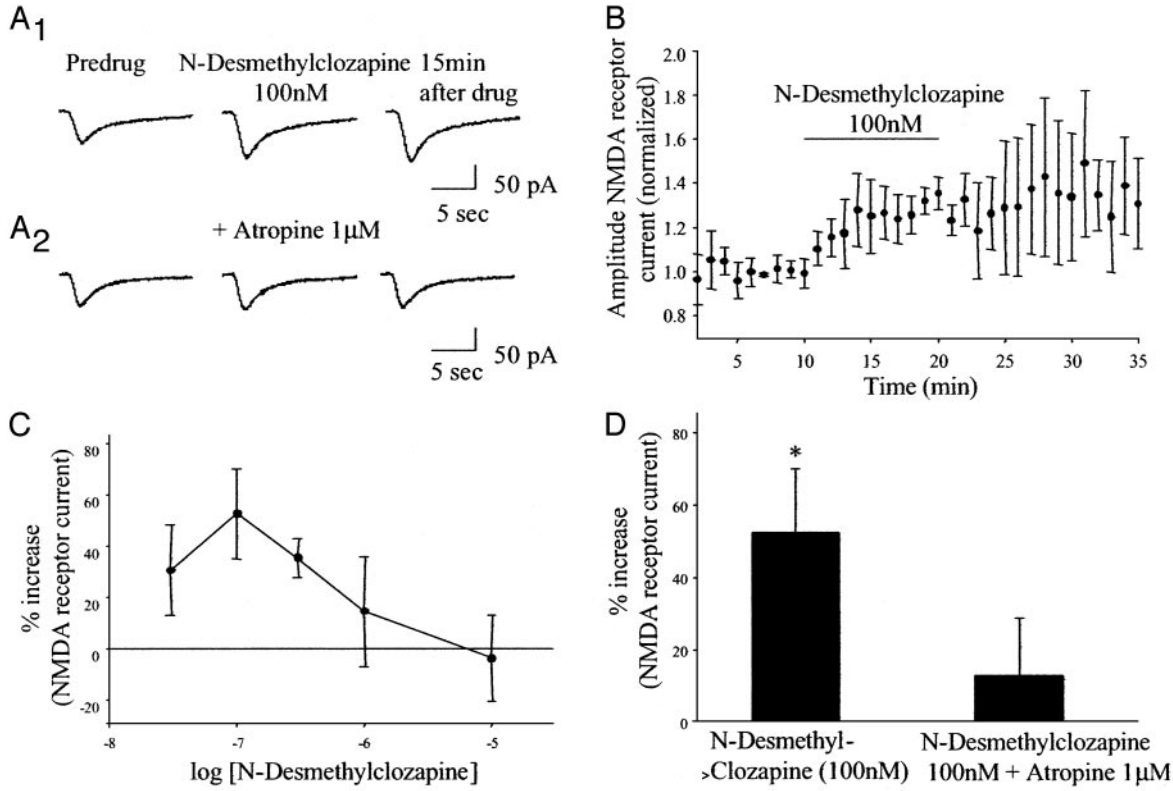

Fig. 4. *N*-desmethylclozapine potentiates NMDA-evoke currents in CA1 pyramidal cells by activation of muscarinic receptors. (A_1 and A_2) Representative traces of NMDA-evoked currents before (predrug), during, and 15 min after application of 100 nM *N*-desmethylclozapine in the absence (A_1) and presence (A_2) of 1 μM atropine. (*B*) Average time course of the effect of 100 nM *N*-desmethylclozapine on NMDA-evoked currents; each point represents the mean ± SEM of data from four cells. (*C*) Dose–response relationship of the *N*-desmethylclozapine-induced potentiation of NMDA-evoked currents; each point represents the mean ± SEM of data from three to six cells. (*D*) Bar graph showing the average maximum effect of 100 nM *N*-desmethylclozapine on NMDA-evoked currents in the absence and presence of 1 μM atropine. Each bar represents the mean ± SEM of data from five and four cells, respectively. *, $P < 0.05$, Student's paired *t* test.

muscarinic receptor ligands (30). Transmembrane domain 6 is important for M1 receptor function, and mutation of its tyrosine residue 381 has been shown to decrease acetylcholine binding affinity ≈30-fold and drastically reduced its potency by >2,700-fold without affecting its efficacy (30). The critical contribution of residue Y381 to the M1 receptor orthosteric site makes Y381A mutant a useful tool to detect agonists acting through another site as demonstrated for the ectopic agonist AC42 (32). Functional experiments confirmed a robust reduction in acetylcholine potency by the Y381A mutation, and its lack of effect on AC42 agonistic activity. In contrast, *N*-desmethylclozapine potency at Y381A mutant M1 receptor was increased 7- to 8-fold, and its efficacy was equivalent to acetylcholine. Even more surprising, clozapine was 30-fold more potent and was almost a full agonist at this mutated receptor. The structurally related antipsychotic olanzapine was completely inactive at the WT M1 receptor, as illustrated in Fig. 1*B*, but a weak partial agonistic effect at mutated M1 receptor was noted (data not shown).

The opposite effects of mutation Y381 on the response of a standard orthosteric ligand like acetylcholine versus clozapine and *N*-desmethylclozapine suggested that the latter compounds do not interact in the same way with M1 orthosteric site as does acetylcholine and thus can be considered allosteric ligands as defined by Christopoulos (35). However, the simple fact that Y381A mutation affected clozapine and *N*-desmethylclozapine activity also suggested that these drugs interact with a domain close to or even partially overlapping with the orthosteric site. In contrast, a different site of interaction is implicated for AC42 because its activity was unaltered by the Y381 mutation. Additional mutagenesis and pharmacological analyses will be critical to define more precisely the molecular determinants implicated in *N*-desmethylclozapine and M1 receptor interaction.

Independent of *N*-desmethylcozapine's site of interaction with M1, it was important to determine whether this agonist activity had any physiological relevance. Indeed, cholinergic neurotransmission is well known to modulate cortical and hippocampal activities and thus to be important for mnemonic and cognitive functions (36). M1 receptors are colocalized with NMDA receptors in rat hippocampus, and this specific topographical arrangement allows activated M1 receptors to potentiate NMDA-receptor currents in CA1 pyramidal cells (21). *N*-desmethylclozapine potentiated NMDA-evoked currents in CA1 pyramidal cells via activation of muscarinic receptors at a concentration consistent with its *in vitro* potency for M1 receptors. Moreover, the well-established relationship between M1 and NMDA receptors at this synapse (21) supports the view that the basic mechanism underlying this effect of *N*-desmethylclozapine is M1 mediated. A consistent observation has been the persistence of *N*-desmethylclozapine enhancement of NMDA receptor current amplitude, contrasting with the reported transient effect of the nonselective and full muscarinic agonist carbachol (21). More systematic studies are warranted to elucidate this phenomenon given the reported implication of muscarinic M1 receptors in the expression of long-term potentiation (36). The potentiation of NMDA receptor function by *N*-desmethylclozapine is reminiscent of the reported facilitation of NMDA receptor-mediated transmission in rat prefrontal cortex by clozapine (37). As this proglutamatergic activity of clozapine has been proposed to contribute to its unique therapeutic profile, an important issue is whether *N*-desmethylclozapine

participates to the overall clozapine clinical action through its M1 agonistic activity.

Transgenic mice deficient for M1 receptors displayed phenotypic alterations consistent with M1 playing a role in neuronal functions known to be disrupted in psychiatric disorders such as schizophrenia (36, 38, 39). Indeed, M1 knockout mice have been shown to be specifically impaired in matching-to-sample working memory, a process requiring cortico-hippocampus communication (36). *In vivo* microdialysis studies in M1 receptor-deficient mice have also revealed an increase in striatal dopamine levels (39), providing evidence for a regulation of dopaminergic transmission by M1 receptors. Moreover the muscarinic agonist xanomeline has been shown to be effective in treating psychosis in Alzheimer's disease patients (18).

In vivo experiments in rodents have demonstrated that *N*-desmethylclozapine crosses the brain–blood barrier (24, 27) and stimulates neuronal activity as indicated by its ability to induce c-*fos* expression in rat forebrain (26). Moreover, measurements of clozapine and *N*-desmethylclozapine brain levels in chronically treated rats (27) have revealed a clozapine/*N*-desmethylclozapine ratio of ≈3. In schizophrenia patients treated with clozapine, plasma concentrations of *N*-desmethylclozapine have been reported to be equal to clozapine and to reach micromolar levels (23–25). Furthermore, absolute plasma level of *N*-desmethylclozapine and clinical response in schizophrenics can be modified by cytochrome P450 polymorphism or concomitant treatment with antidepressant, for instance (40, 41). However, an activation of M1 receptors by *N*-desmethylclozapine could be curtailed by the concomitant presence of clozapine; being a partial agonist, clozapine can antagonize *N*-desmethylclozapine-induced M1 responses in cell lines recombinantly expressing M1 receptors (P.J.M. and C.S., unpublished observations). Therefore, whether the M1 agonist activity of *N*-desmethylclozapine contributes in a meaningful way to the beneficial effects of clozapine in schizophrenia could depend on whether suitably high levels are achieved in the CNS relative to clozapine itself at therapeutic doses. Yet, if the M1 agonist activity of *N*-desmethylclozapine contributes to the unique antipsychotic activity of clozapine, then selective M1 agonists could be a novel therapeutic approach to antipsychotic treatment.

1. Kane, J, Honigfeld, G., Singer, J. & Meltzer, H. (1988) *Arch. Gen. Psychiatry* **45,** 789–796.
2. Volavka, J., Czobor, P., Lindemayer, J.-P., Citrome, L., McEvoy, J. P., Cooper, T. B., Chakos, M. & Lieberman, J. A. (2002) *Am. J. Psychiatry* **159,** 255–262.
3. Meltzer, H. Y. & McGurk, S. R. (1999) *Schizophrenia Bull.* **25,** 233–255.
4. Meltzer, H. Y. & Okayli, G. (1995) *Am. J. Psychiatry* **152,** 183–190.
5. Lindenmayer, J.-P., Iskander, A., Park, M., Apergi, F.-S., Czobor, P., Smith, R. & Allen, D. (1998) *J. Clin. Psychiatry* **59,** 521–527.
6. Meltzer, H. Y., Matsubara, S. & Lee, J. C. (1989) *J. Pharmacol. Exp. Ther.* **251,** 238–246.
7. Seeman, P. (1992) *Neuropsychopharmacology* **7,** 261–284.
8. Miyamoto, S., Duncan, G. E. & Lieberman, J. A. (2001) in *Current Issues in the Psychopharmacology of Schizophrenia*, eds. Breier, A., Tran, P. V., Herrera, J. M., Tollefson, G. D. & Bymaster, F. P. (Lippincott–Williams & Wilkins, Philadelphia), p. 224.
9. Bolden, C., Cusack, B. & Richelson, E. (1992) *J. Pharmacol. Exp. Ther.* **260,** 576–580.
10. Baldessarini, R. J. & Frankenburg, F. R. (1991) *N. Engl. J. Med.* **324,** 746–754.
11. Meltzer, H. Y., Chai, B. L., Thompson, P. A. & Yamamoto, B. K. (1994) *J. Pharmacol. Exp. Ther.* **268,** 1452–1461.
12. Zorn, S. H., Jones, S. B., Ward, K. M. & Liston, D. R. (1994) *Eur. J. Pharmacol.* **269,** R1–R2.
13. Zeng, X. P., Le, F. & Richelson, E. (1997) *Eur. J. Pharmacol.* **321,** 349–354.
14. Olianas, M. C., Maullu, C. & Onali, P. (1997) *Br. J. Pharmacol.* **122,** 401–408.
15. Olianas, M. C., Maullu, C. & Onali, P. (1999) *Neuropsychopharmacology* **20,** 263–270.
16. Gershon, S. & Olariu, J. (1960) *J. Neuropsychiatry* **1,** 282–283.
17. Neubauer, H., Sundland, D. M. & Gershon, S. (1966) *J. Nerv. Ment. Dis.* **142,** 265–277.
18. Bodick, N. C., Offen, W. W., Levey, A. I., Cutler, N. R., Gauthier, S. G., Satlin, A., Shannon, H. E., Tollefson, G. D., Rasmusen, K., Bymaster, F. P., *et al.* (1997) *Arch. Neurol.* **54,** 465–473.
19. Shannon, H. E., Rasmussen, K., Bymaster, F. P., Hart, J. C., Peters, S. C., Swedberg, M. D. B., Jeppesen, L., Sheardown, M. J., Sauerberg, P. & Fink-Jensen, A. (2000) *Schizophrenia Res.* **42,** 249–259.
20. Stanhope, K. J., Mirza, N. R., Bickerdike, M. J., Bright, J. L., Harrington, N. R., Hesselink, M. B., Kennett, G. A., Lightowler, S., Sheardown, M. J., Syed, R., *et al.* (2001) *J. Pharmacol. Exp. Ther.* **299,** 782–792.
21. Marino, M. J., Rouse, S. T., Levey, A. I., Potter, L. T. & Conn, P. J. (1998) *Proc. Natl. Acad. Sci. USA* **95,** 11465–11470.
22. Tsai, G. & Coyle, J. T. (2002) *Annu. Rev. Pharmacol. Toxicol.* **42,** 165–179.
23. Aravagiri, M. & Marder, S. R. (2001) *J. Pharmacol. Biomed. Anal.* **26,** 301–311.
24. Baldessarini, R. J., Centorrino, F., Flood, J. G., Volpicelli, S. A., Huston-Lyons, D. & Cohen, B. M. (1993) *Neuropsychopharmacology* **9,** 117–124.
25. Guitton, C., Kinowski, J.-M., Gomeni, R. & Bressolle, F. (1998) *Clin. Drug Invest.* **16,** 35–43.
26. Young, C. D., Meltzer, H. Y. & Deutch, A. Y. (1998) *Neuropsychopharmacology* **19,** 99–103.
27. Weigmann, H., Hartter, S., Fisher, V., Dahmen, N. & Hiemke, C. (1999) *Eur. Neuropsychopharmacol.* **9,** 253–256.
28. Kuoppamaki, M., Syvalathi, E. & Hietala, J. (1993) *Eur. J. Pharmacol.* **245,** 179–182.
29. Brandish, P. E., Hill, L. A., Zheng, W. & Scolnick, E. M. (2003) *Anal. Biochem.* **313,** 311–318.
30. Lazareno, S., Gharagozloo, P., Kuonen, D., Popham, A. & Birdsall, N. J. M. (1998) *Mol. Pharmacol.* **53,** 573–589.
31. Ward, S. D. C., Curtis, C. A. M. & Hulme, E. C. (1999) *Mol. Pharmacol.* **56,** 1031–1041.
32. Spalding, T. A., Trotter, C., Skjaerbaek, N., Messier, T. L., Currier, E. A., Burstein, E. S., Li, D., Hacksell, U. & Brann, M. R. (2002) *Mol. Pharmacol.* **61,** 1297–1302.
33. Jakubik, J., Bacakova, L., El-Fakahany, E. E. & Tucek, S. (1997) *Mol. Pharmacol.* **52,** 172–179.
34. Lu, Z.-L., Saldanha, J. W. & Hulme, E. C. (2001) *J. Biol. Chem.* **276,** 34098–34104.
35. Christopoulos, A. (2002) *Nat. Drug Discovery* **1,** 198–210.
36. Anagnostaras, S. G., Murphy, G. G., Hamilton, S. E., Mitchell, S. L., Rahnama, N. P., Nathanson, N. M. & Silva, A. J. (2003) *Nat. Neurosci.* **6,** 51–58.
37. Arvanov, V. L., Liang, X., Schwartz, J., Grossman, S. & Wang, R. Y. (1997) *J. Pharmacol. Exp. Ther.* **283,** 226–234.
38. Miyakawa, T., Yamada, M., Duttaroy, A. & Wess, J. (2001) *J. Neurosci.* **21,** 5239–5250.
39. Gerber, D. J., Sotnikova, T. D., Gainetdinov, R. R., Huang, S. Y., Caron, M. G. & Tonegawa, S. (2001) *Proc. Natl. Acad. Sci. USA* **98,** 15312–15317.
40. Centorrino, F., Baldessarinin, R. J., Frankenburg, F. R., Kando, J., Volpicelli, S. A & Flood, J. G. (1996) *Am. J. Psychiatry* **153,** 820–822.
41. Ozdemir, V., Kalow, W., Okey, A. B., Lam, M. S. M., Albers, L. J., Reist, C., Fourie, J., Posner, P., Collins, E. J. & Roy, R. (2001) *J. Clin. Psychopharmacol.* **21,** 603–607.