

Different pharmacology of *N*-desmethylozapine at human and rat M_2 and M_4 mAChRs in neocortex

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Abstract Cholinergic transmission plays a pivotal role in learning, memory and cognition, and disturbances of cholinergic transmission have been implicated in neurological disorders including Alzheimer's disease, epilepsy and schizophrenia. Pharmacological alleviation of these diseases by drugs including *N*-desmethylozapine (NDMC), promising in animal models, often fails in patients. We therefore compared the effects of NDMC on glutamatergic and GABAergic transmission in slices from rat and human neocortex. We used carbachol (CCh; an established agonist at metabotropic muscarinic acetylcholine (ACh) receptors (mAChRs)) as a reference. Standard electrophysiological methods including intracellular and field potential recordings were used. In the rat neocortex, NDMC prevented the CCh-induced decrease of GABA_A and GABA_B receptor-mediated responses but not the CCh-induced increase of the paired-pulse depression. NDMC reduced neither the amplitude of the excitatory postsynaptic potentials (EPSP) nor antagonized the CCh-induced depression of EPSP. In the human neocortex, however, NDMC failed to prevent CCh-induced decrease of the GABA_B responses and directly reduced the amplitude of EPSP. These data suggest distinct effects of NDMC in rat and human at M_2 and M_4 mAChRs underlying presynaptic modulation of GABA and glutamate release, respectively. In particular, NDMC might be a M_2 mAChR antagonist in the rat but has no activity at this receptor in human neocortex. However, NDMC has an agonistic effect at M_4 mAChR in the

human but no such effect in the rat neocortex. The present study confirms that pharmacology at mAChRs can differ between species and emphasizes the need of studies in human tissue.

Keywords Carbachol · NDMC · Muscarinic acetylcholine receptors · Temporal lobe epilepsy · Paired-pulse depression

Abbreviations

ACh	Acetylcholine
CCh	Carbachol
EPSP	Excitatory postsynaptic potential
IPSP	Inhibitory postsynaptic potential
ISI	Interstimulus intervals
ITRC	Interface-type recording chamber
mAChR	Muscarinic acetylcholine receptor
NDMC	<i>N</i> -Desmethylozapine
PPD	Paired-pulse depression
PPR	Paired-pulse ratio
STRC	Submerged-type recording chamber

Introduction

Acetylcholine (ACh) is an important neurotransmitter in the central and peripheral nervous system. Dysfunction of muscarinic ACh receptors (mAChRs) contributes to cognitive deficits and neurological diseases including schizophrenia (Dean et al. 2003; Langmead et al. 2008; Scarr et al. 2012). In unmedicated schizophrenia patients, in vivo imaging revealed a regionally confined reduction of mAChRs (Raedler et al. 2003). In addition, decreased numbers of M_1 mAChR were demonstrated in prefrontal cortex (Dean et al. 2002) of M_4 mAChR in hippocampus (Scarr et al. 2007) and of M_1/M_4

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mAChRs in the superior temporal gyrus (Deng and Huang 2005) of schizophrenia patients.

Therapeutic alleviation of schizophrenia relies on few compounds, including clozapine. The mechanism of action of this first atypical antipsychotic is poorly understood (Joober and Boksa 2010). Clozapine interacts with dopamine, serotonin and histamine receptors (Lameh et al. 2007) and mAChRs (Miller and Hiley 1974) with high affinity for all five (M_1 to M_5) human mAChRs (Bolden et al. 1992). However, clozapine may act as either partial agonist (Zorn et al. 1994; Olianas et al. 1999) or antagonist (Bolden et al. 1992; Zorn et al. 1994; Olianas et al. 1999), depending upon the mAChR subtype and experimental conditions. The partial agonistic actions of clozapine and its metabolite *N*-desmethylclozapine (NDMC), at M_1 mAChR in human (Weiner et al. 2004) and in rats, (Sur et al. 2003) are intriguing. It was postulated that clozapine's unique actions are solely mediated by M_1 mAChR (Weiner et al. 2004).

However, some authors have challenged the concept that M_1 agonism is a prerequisite for mimicking clozapine's action (Davies et al. 2005). In addition, we have recently shown that NDMC behaves as an M_1 mAChR antagonist in the human neocortex (Thomas et al. 2010). It was suggested that M_4 mAChRs could play a role in schizophrenia and that M_4 mAChRs agonists might have therapeutic benefits (Chan et al. 2008). Interestingly, NDMC increases the ACh release in rat prefrontal cortex and nucleus accumbens, tentatively attributed to agonism at M_1 mAChR (Li et al. 2008). However, the receptors limiting ACh release seem to be of the M_2 mAChR type in several structures (Stoll et al. 2003; Grilli et al. 2010).

Recently important facets of G protein-coupled receptors emerged, including functional differences by additional factors (e.g. accessory protein interaction, oligomerization, phosphorylation) (May et al. 2007; Schwenk et al. 2010). Hence, the controversial results obtained with NDMC on dopamine D_2 receptors acting either as competitive antagonist (Weiner et al. 2004), partial agonist (Burstein et al. 2005) or inverse agonist (Masri et al. 2008) may relate to functional differences in the assays employed. These ambiguities necessitate the evaluation of NDMC in a native system.

Therefore we characterized NDMC at native mAChRs in both rat and human cortical slices using the protocols described previously (Gigout et al. 2012a, b). We compared the effect of NDMC on glutamatergic and GABAergic synaptic transmission, the former being modulated by M_4 mAChRs and the latter by M_2 mAChRs (Gigout et al. 2012a, b).

Materials and methods

Tissue handling and preparation

Human neocortical tissues were obtained from patients with pharmaco-resistant temporal ($n=13$) or frontal ($n=2$) lobe

epilepsy. The patients (11 male, 4 female) were 30.6 ± 4.1 years old. Informed consent of each patient was obtained according to the Declaration of Helsinki. All experiments were approved by the Ethics Committee of the Charité (Berlin, Germany).

The methods have previously been described (Deisz 1999; Teichgraber et al. 2009; Deisz et al. 2011; Gigout et al. 2012b). In brief, the tissues were collected in the operating theatre and transported to the laboratory in cold modified artificial cerebrospinal fluid (mACSF). The tissues were cut into slices of 400- μ m thickness with a vibratome (HM650V, MICROM International, Germany). The slices were stored submerged at room temperature in ACSF, continuously gassed with carbogen (95 % O_2 /5 % CO_2) until individually transferred to the recording chamber.

Coronal slices comprising the sensorimotor cortex were made from male Wistar rats (age: 30–42 days). These slices were made with the same methods and maintained in identical conditions as human tissue slices, except for transport (Teichgraber et al. 2009; Deisz et al. 2011; Gigout et al. 2012a).

Solutions and substances

The normal ACSF contained (in mM) 124 NaCl, 5 KCl, 2 $MgSO_4$, 2 $CaCl_2$, 1.25 NaH_2PO_4 , 26 $NaHCO_3$ and 10 glucose (equilibrated with carbogen, pH 7.4). The mACSF contained (in mM) 70 NaCl, 2.5 KCl, 7 $MgSO_4$, 0.5 $CaCl_2$, 1.25 NaH_2PO_4 , 26 $NaHCO_3$, 25 glucose and 75 sucrose (equilibrated with carbogen, pH 7.4).

All compounds were applied by bath. To this end, aliquots of concentrated stock solutions were added to the ACSF to obtain the desired concentration. Stock solutions of carbachol (gift from GlaxoSmithKline) and oxotremorine-M (Tocris Bioscience, UK) were made of water, and *N*-desmethylclozapine (Tocris) was dissolved in DMSO. These stock solutions were stored in aliquots at $-20^\circ C$ until used.

Electrophysiological recordings

Recording chambers

Experiments were carried out either in an interface-type recording chamber (ITRC) or in a submerged-type recording chamber (STRC). In the ITRC, slices were perfused with ACSF (1.5–2 ml/min, $34\pm 1^\circ C$), and the atmosphere was maintained by a continuous flow of prewarmed and humidified carbogen. In the STRC experiments, slices were held between two nylon grids and perfused with ACSF at 4–5 ml/min ($31\pm 1^\circ C$).

The ITRC enables maximal oxygen supply and improves recordings of evoked field potentials and was therefore chosen for the paired-pulse protocol. The STRC provides more stable conditions and allows a fast equilibration of drugs in the slices

(Reid et al. 1988; Deisz 1999; Deisz et al. 2011; Gigout et al. 2012a, b). STRC was chosen for intracellular recordings.

All measurements were made at least 30 min (ITRC) or 15 min (STRC) after drug application to ascertain steady state of applied drugs.

Electrodes

For extracellular recordings, filamented borosilicate capillaries (Hilgenberg, Germany) were pulled to resistances between 2 and 8 M Ω when filled with ACSF. For intracellular recordings, pipettes were pulled to resistances near 100 M Ω , when filled with 1 M potassium acetate+1 mM potassium chloride.

Extra- and intracellular recordings

The recording electrodes were positioned in neocortical layers II/III. Extracellular signals were fed via a high-impedance preamplifier (EXT-01C) to a second-stage amplifier (DPA 2F; both npi electronic, Tamm, Germany). The signals obtained with intracellular recordings were fed to appropriate amplifier (SEC05L, npi electronic). Both types of recordings were digitized on-line with a PC-based acquisition system (see below). Families of current injections (−0.5 to +1.5 nA, 0.05-nA increment) allowed to estimate membrane resistance (R_m) and firing behaviour. Synaptic responses were elicited by electrical stimuli (100- μ s duration at 0.1 Hz, stimulus intensity 0–20 V, 2-V increment; ISO-Flex isolation unit, AMPI Israel) via bipolar tungsten electrodes placed in deep cortical layers (layers V/VI).

The peak amplitude of the initial component of synaptic responses represents the excitatory postsynaptic potential (EPSP). Input–output curves of EPSP were fitted by the Boltzmann equation, yielding the maximal EPSP amplitude (EPSP_{max}), the stimulus yielding half-maximal responses (I_{50}) and the slope factor (dx).

To evaluate the effects of mAChR agonists/antagonists on neurotransmitter release presumably via presynaptic sites, we used a paired-pulse protocol in the ITRC. The paired-pulse ratio (PPR) was defined as the amplitude of the second synaptic response divided by the amplitude of the first synaptic response. Here we focus on the results obtained at interstimulus intervals (ISI) of 20 ms and of 200 ms to investigate GABA_A (Thompson et al. 1988) and GABA_B (Deisz and Prince 1989) receptor-mediated inhibition, respectively. An intensity of 1 mA was chosen since it elicited a maximal response with pronounced paired-pulse depression (PPD) in healthy neocortex (Gigout et al. 2012a).

Synaptic conductances mediated by GABA_A and GABA_B receptors were estimated similar to previous methods (Deisz and Prince 1989). In human tissue, we focussed on changes of IPSP_B amplitudes before and during application of the drugs,

since IPSP_A in human epileptogenic tissues is often depolarizing (Deisz et al. 2011).

Data acquisition and analysis

Recorded signals were digitized on-line (10 kHz) with a PC-based system (Digidata 1440A and Clampex 10.1 or the preceding hard- and software, Molecular Devices, Sunnyvale CA, USA) and analysed off-line (Clampfit 10.1). The intracellular recordings were from “regular firing” neurons (Connors and Gutnick 1990).

All values given in the text and in the figures represent the mean \pm s.e.m., and n indicates the number of observations. For comparisons, we used paired and unpaired Student's t test and differences were considered significant if $p < 0.05$.

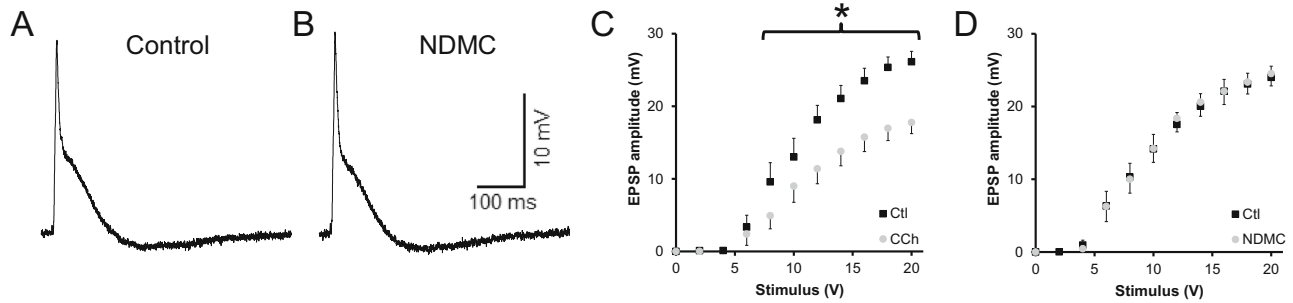
Results

Effects of CCh and NDMC on evoked excitatory postsynaptic potentials (EPSP)

Rat neocortical slices

Application of the non-selective mAChR agonist CCh (10 μ M) decreased the amplitude of EPSP at stimulus intensities larger than 8 V ($n=14$, $p < 0.05$, Fig. 1c). The EPSP_{max} (see “Materials and methods”) decreased from 26.0 ± 1.2 to 18.1 ± 1.6 mV in CCh ($n=14$, $p < 0.05$). Addition of another non-selective mAChR agonist oxotremorine-M (Oxo-M; 2 μ M) yielded similar reductions of EPSP amplitudes (control 34.8 ± 2.4 mV, Oxo-M 27.6 ± 4.4 mV; $n=5$; $p < 0.05$), although the amplitudes were unusually large during this series. Application of NDMC (10 μ M) alone had no significant effect on the amplitude of EPSP at all intensities tested (Fig. 1a, b, d), and the EPSP_{max} was unaltered (control 23.3 ± 1.4 mV, NDMC 23.6 ± 1.6 mV; $n=19$; $p > 0.05$). While CCh decreased EPSP_{max} (control 24.6 ± 1.5 mV, CCh 16.7 ± 3.8 mV; $n=5$; $p < 0.05$), addition of NDMC in the presence of CCh did not alter EPSP_{max} modulation by CCh (CCh 16.7 ± 3.8 mV, CCh + NDMC 19.1 ± 3.5 mV; $n=5$; $p > 0.05$). Conversely, application of NDMC before CCh did not prevent the decrease of EPSP_{max} (control 21.6 ± 2.3 mV, NDMC 21.8 ± 2.3 mV, NDMC + CCh 18.10 ± 2.3 mV; $n=7$; $p < 0.05$). Considering the involvement of M₄ mAChR in the depression of glutamatergic transmission (Gigout et al. 2012b), these data suggest that in rat neocortical tissues, NDMC is not acting at M₄ mAChR or might be a very weak M₄ mAChR antagonist.

RAT



HUMAN

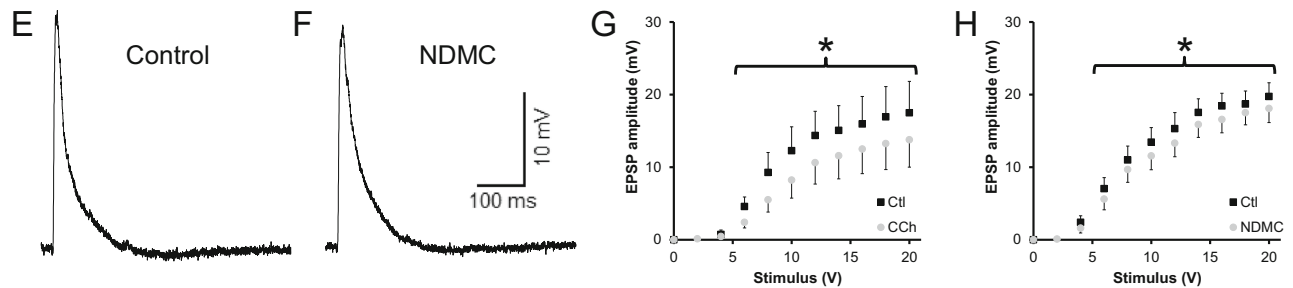


Fig. 1 Effect of NDMC (10 μ M) on glutamatergic transmission in rat and human cortex. **a, b** Voltage traces of synaptic responses (stimulus intensity 18 V) of a rat neocortical neuron in control (**a**) and in the presence of NDMC (**b**). **c, d** Plot of the average EPSP amplitudes vs. stimulus intensity in control, in the presence of 10 μ M CCh ($n=14$,

* $p<0.05$) and in the presence of NDMC as indicated ($n=19$). **e, f** Synaptic responses (stimulus intensity 18 V) of a human neocortical neuron in control (**e**) and in the presence of NDMC (**f**). **g, h** Plot of the average EPSP amplitudes vs. stimulus intensity in control and in the presence of CCh ($n=8$) or NDMC ($n=19$, * $p<0.05$)

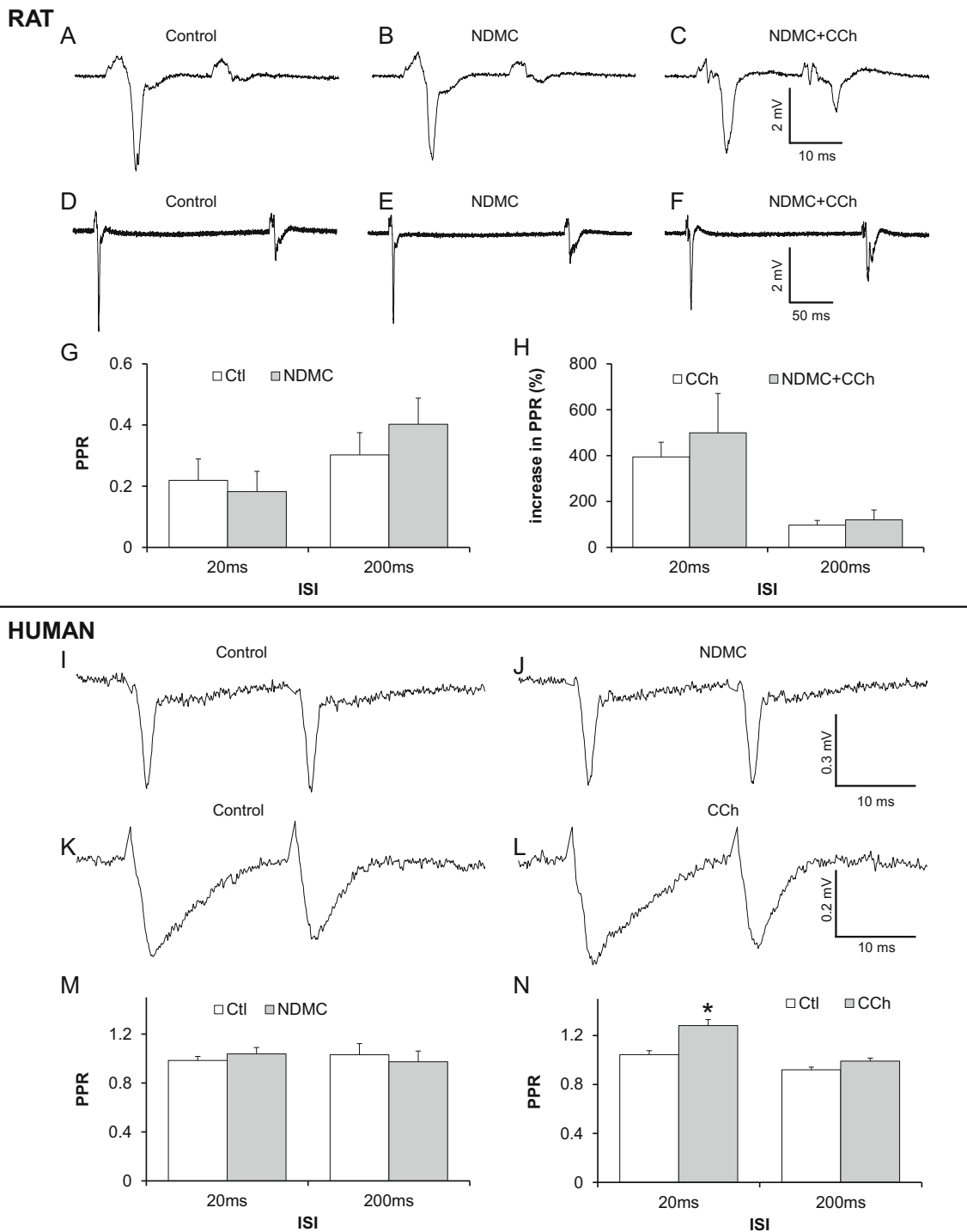
Human neocortical slices

Similar to rat cortical slices, CCh decreased the EPSP amplitudes also in human neurons (at stimulus intensities >6 V; $n=8$, $p<0.05$, Fig. 1g). EPSP_{max} decreased in the presence of CCh from 16.5 ± 4.0 to 13.4 ± 3.7 mV ($n=8$, $p<0.05$). This percentage change was similar in rat (33.3 ± 4.2) and human (27.3 ± 10.4). Again, Oxo-M mimicked the effect of CCh (control 18.1 ± 12.1 mV, Oxo-M 11.4 ± 2.5 mV; $n=2$).

In contrast to rat neurons, application of NDMC reduced slightly but consistently the EPSP amplitude (e.g. at 20 V: control 19.7 ± 1.8 mV, NDMC 18.3 ± 1.8 mV; $n=19$; $p<0.05$) in human neurons (Fig. 1e, f, h). The depression of EPSPs by NDMC was already detectable at low stimulus intensities (e.g. 6 V: control 6.9 ± 1.6 mV, NDMC 5.6 ± 1.5 mV; $n=19$; $p<0.05$) (Fig. 1h). EPSP_{max} was decreased from 19.4 ± 1.8 to 17.7 ± 1.8 mV ($n=19$; $p<0.05$). This NDMC-induced decrease in EPSP_{max} was significantly stronger in human (9.3 ± 2.6 %, $n=19$) than in rat (-1.2 ± 3.5 %, $n=19$, $p<0.05$). In female and male patients, NDMC decreased EPSP_{max} with similar efficacy, on average by 6.8 ± 1.8 % ($N=4$ patients and $n=6$ neurons) and by 10.5 ± 2.9 % ($N=8$ patients and $n=13$ neurons, $p=0.45$), respectively. Correlation analysis revealed no relationships between the age of the patients and the NDMC effect ($R^2=0.1894$, $n=19$ neurons, $N=12$ patients).

Considering the marginal non-significant changes on E_m (control -71.0 ± 1.2 mV, CCh -70.5 ± 1.2 mV; $p>0.05$; see

Fig. 2 Effect of NDMC (10 μ M) on paired-pulse stimulation of field potentials in rat and human cortex. **a–c** Example of field potential recordings using a paired-pulse protocol for an ISI of 20 ms in control (**a**), NDMC (**b**) and during co-application of NDMC+10 μ M CCh (**c**) in rat neocortex. Note that NDMC had no effect on the paired-pulse depression (PPD) compared to control, but co-application of CCh with NDMC attenuated the PPD. **d–f** Example of field potential recordings using a paired-pulse protocol for an ISI of 200 ms in control (**d**), NDMC (**e**) and during NDMC+10 μ M CCh co-application (**f**) in rat neocortex. Note that NDMC had no effect on the PPD compared to control condition. Co-application of CCh with NDMC decreased the PPD. **g** Plots of the paired-pulse ratio (PPR) at two ISI (20 and 200 ms), in control and in the presence of NDMC in rat neocortex ($n=12$). **h** Plots of the increase of PPR at two ISI (20 and 200 ms), during CCh application in control ACSF ($n=49$) and in NDMC-containing ACSF ($n=12$) in rat neocortex. **i, j** Example of field potential recordings using a paired-pulse protocol for an ISI of 20 ms in control (**i**) and during NDMC (**j**) in human neocortex. Note the absence of PPD in control. Note that NDMC had no effect on the PPD compared to control. **k, l** Example of field potential recordings using a paired-pulse protocol for an ISI of 20 ms in control (**k**) and during 10 μ M CCh application (**l**) in human neocortex. Note the relatively weak PPD in control and unaffected by CCh compared to control. **m** Plots of the PPR at two ISI (20 and 200 ms), in control and in the presence of NDMC in human neocortex ($n=4$). **n** Plots of the PPR at two ISI (20 and 200 ms), in control and in the presence of CCh in human neocortex ($n=29$). * $p<0.05$



(Thomas et al. 2010)), the decrease of EPSP_{max} cannot be attributed to membrane depolarization. These data suggest that in human tissue, NDMC interacts with M₄ mAChR as an agonist to reduce EPSP (Gigout et al. 2012b). We then tested whether NDMC does not act as a M₄ mAChR antagonist. While CCh decreased EPSP_{max} (control 14.8±5.4 mV,

CCh 11.2±4.7 mV; n=6; p<0.05), addition of NDMC in the presence of CCh left EPSP_{max} unaltered (CCh 11.2±4.7 mV, CCh + NDMC 12.3±5.6 mV; n=6; p>0.05) and application of NDMC before CCh did not prevent the decrease of EPSP_{max} (NDMC 13.6±2.5 mV, NDMC + CCh 10.1±2.8 mV; n=8; p<0.05).

Effects of CCh and NDMC on paired-pulse ratio

Rat neocortical slices

The paired-pulse depression (PPD) paradigm (Davies et al. 1990) delineates the temporal properties of two consecutive synaptic events, i.e. a crucial component of short- and long-term plasticity. PPD provides an estimate for presynaptic GABA_B receptors, decreasing transmitter release via attenuation of Ca²⁺ currents (Deisz and Lux 1985). To investigate modulation of paired-pulse inhibition, a high stimulus intensity ($I=1$ mA) was used to maximally activate GABA release (Gigout et al. 2012a). The mean amplitude of the second field potential (FP2) was $22\pm 7\%$ of the first (FP1; $n=12$) at an interstimulus interval (ISI) of 20 ms and $30\pm 7\%$ at an ISI of 200 ms ($n=12$; Fig. 2g). NDMC affected the amplitude of neither FP1 nor FP2 ($n=12$, $p>0.05$) for both ISI, leaving the paired-pulse ratio (PPR) unaltered (Fig. 2a, b, d, e, g). In addition, pre-application of NDMC did not prevent the CCh-induced increase of PPR (Fig. 2c, f). This increase in PPR was similar to the increase induced by CCh applied in a control solution without NDMC (Fig. 2h).

Human neocortical slices

Similar to rat tissue, NDMC had no effect on the amplitude of FP1 or FP2 ($n=4$, $p>0.05$) in human tissue for ISI of 20 or 200 ms (Fig. 2i–l). However, comparison to rat neurons is hampered by the rather small PPD in human neurons, due to the reduced function of GABA_B receptors (Teichgraber et al. 2009). As a consequence, the PPR was not changed at both ISI (Fig. 2m). CCh produced an increase in PPR (Fig. 2n) albeit much smaller compared to rat.

Effects of CCh and NDMC on GABA_A and GABA_B receptor-mediated responses

Rat neocortical slices

To directly test modulation of GABAergic inhibition by mAChRs, we evaluated synaptic responses. We have previously shown that CCh consistently decreased IPSP_B amplitude in rat neocortex via activation of M₂ mAChR (Gigout et al. 2012a). In the present study, NDMC had no effect on IPSP_B amplitudes ($n=11$, Fig. 3a). However, pre-application of NDMC prevented the CCh-induced decrease of IPSP_B amplitude ($n=7$, Fig. 3b). Considering ambiguities of small amplitudes, we next investigated whether NDMC prevents the CCh-induced decrease of IPSP_A and IPSP_B conductances, mediated by M₂ mAChRs (Gigout et al. 2012a). Following a pre-application of NDMC, CCh failed to decrease both IPSP_A (Fig. 3c–f) and IPSP_B (Fig. 3c–e, g) conductances ($n=5$). This

suggests that NDMC acts as an M₂ mAChR antagonist/modulator in rat neocortex.

We ascertained that in the present set of experiments, CCh alone decreased both IPSP_A (Fig. 3h–j) and IPSP_B (Fig. 3h, i, k) conductances ($n=9$).

Human neocortical slices

As in rat, NDMC had no effect on IPSP_B amplitude ($n=19$, Fig. 3l). In addition, it failed to prevent the CCh-induced decrease of IPSP_B amplitude ($n=15$, Fig. 3m). This suggests that in human neocortical tissues, NDMC is not acting at M₂ mAChR.

Discussion

Methodological considerations

The present data challenge the hitherto used pharmacological distinction of mAChRs in different species. Based on the effects of CCh and established agonists and antagonists for mAChR subtypes, we had proposed that M₁ mAChR is involved in the decrease in M-current, M₄ mAChR in the depression of glutamate release and M₂ mAChR in the depression of GABA release. These effects were similar in rat and human cortical tissues (Gigout et al. 2012a, b), except for some presumably epilepsy-related quantitative differences. For instance, PPD is greatly attenuated in human slices (Fig. 2a vs. 2i) presumably due to the reduced function and density of GABA_B receptors in human tissues (Teichgraber et al. 2009). Since NDMC binds to all five mAChRs with similar affinity (Thomas et al. 2010), a similar modulation of all the above effects by NDMC in both species might be anticipated. However, our data indicate marked discrepancies in the NDMC effects between rat and human neurons.

Effect of NDMC on M₁ mAChR

Before discussing the present data, we would like to briefly reiterate previous data indicating the interaction of NDMC with M₁ mAChRs. A CCh-induced increase in neuronal action potential (AP) firing has been linked to activation of M₁ mAChR because the increase was antagonized by pirenzepine (M₁/M₄ mAChR antagonist) and atropine (mAChR antagonist), unaffected by AFDX (M₂/M₄ mAChR antagonist) and similar to linopirdine (a K_v7 blocker) in both rat and humans (Gigout et al. 2012a, b). Yet, in slices of rat cortex, NDMC increased the slope of AP firing much less than CCh (Thomas et al. 2010). Such altered firing was also observed in rat hippocampal neurons (Thomas et al. 2010) compatible with a partial agonistic effect of NDMC at rat M₁ mAChR. The

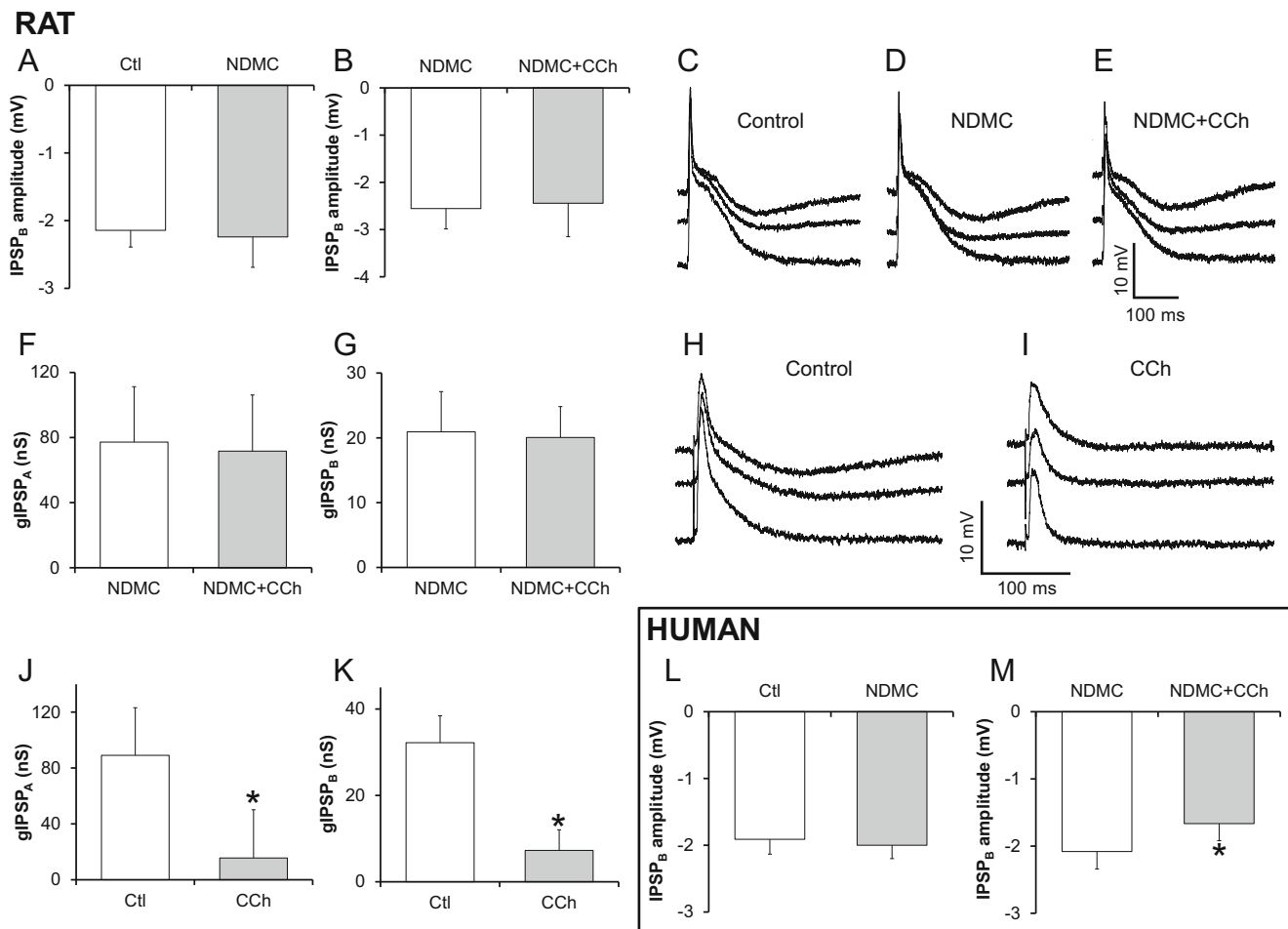


Fig. 3 Effect of NDMC (10 μ M) on synaptic inhibition in rat and human cortex. **a** Plot of the mean IPSP_B amplitude in control and in the presence of NDMC ($n=11$) in rat cortex. **b** Plot of the mean IPSP_B amplitude in the presence of NDMC and during co-application of NDMC+10 μ M CCh ($n=7$) in rat cortex. **c–e** Voltage traces of a rat neuron in control (**c**), in the presence of NDMC (**d**) and during NDMC+CCh co-application (**e**). Orthodromic stimulation elicited compound synaptic responses consisting of an EPSP, IPSP_A and IPSP_B. The two IPSPs are particularly obvious at less-negative membrane potentials. The membrane potential was altered by current injections (+0.3, 0, -0.5 nA, from top to bottom). Note the absence of strong depressant effect of CCh when co-applied with NDMC. **f, g** Plot of the mean gIPSP_A (**f**) and

gIPSP_B (**g**) in NDMC and during NDMC+10 μ M CCh co-application ($n=5$) in rat cortex. **h–i** Voltage traces of a rat neuron in control (**h**) and in the presence of CCh (**i**). The membrane potential was altered by current injections (+0.3, 0, -0.5 nA, from top to bottom). Note the strong depressant effect of CCh. **j, k** Plot of the mean gIPSP_A (**j**) and gIPSP_B (**k**) in control and during 10 μ M CCh application ($n=9$) in rat cortex ($*p<0.05$). **l** Plot of the mean IPSP_B amplitude in control and in the presence of NDMC ($n=19$) in human neocortex. **m** Plot of the mean IPSP_B amplitude in the presence of NDMC and during NDMC+CCh co-application ($n=15$) in human cortex. Note that CCh depresses IPSP_B amplitudes during co-application with NDMC ($*p<0.05$)

agonistic effect of CCh on AP firing in human neurons was much smaller (31 %) compared to that in rat neurons (71 %) (Gigout et al. 2012a, b), perhaps due to epilepsy-related deficits of M₁ mAChRs or K_{v7} channels. In any case, a partial agonistic effect of NDMC observed in rats may therefore be undetectable in human neurons. Addition of NDMC to CCh-containing ACSF did not significantly alter neuronal firing in both species. Conversely, addition of CCh to NDMC-containing ACSF had no effect in rat neurons (Thomas et al. 2010). However, pretreatment with NDMC prevented the CCh-induced increase in AP firing in human neurons (Thomas et al. 2010), suggesting a type of antagonistic effect.

Effect of NDMC on M₄ mAChR

Also the interaction of NDMC with M₄ mAChR presented here reveals a marked discrepancy. The data indicate that NDMC seems to be a partial M₄ mAChR agonist in human but not in rat neurons. NDMC decreased the amplitude of EPSP in human neurons. The effect was smaller compared to CCh, but NDMC was without any detectable effect on glutamatergic transmission in rat neurons. When co-applied with CCh, NDMC has no additive effect on the depression of EPSP amplitude in human epileptogenic neocortex. This suggests that CCh and NDMC converge onto the same type of receptor,

i.e. M_4 mAChR (Gigout et al. 2012b). NDMC did not antagonize the CCh-induced decrease of EPSP amplitude in the rat neocortex, suggesting that this substance is not an antagonist at M_4 mAChR in rat. Our proposal of NDMC being a M_4 mAChR agonist in human cortex depends only on the similarity of effects of this substance to CCh and existing evidence for NDMC acting at M_4 mAChRs (Weiner et al. 2004), rather than an irrefutable pharmacological characterization of the receptors involved. This latter is beyond the scope of the present study since clinical interest in NDMC faded after failure in phase IIb clinical trial for the treatment of acute psychosis in schizophrenic patients (Thomas et al. 2010).

Interestingly, clozapine was shown to act as an agonist at human M_4 mAChR expressed in Chinese hamster ovary cells (Zorn et al. 1994). Agonism of NDMC was also observed at human M_4 AChR in an expression system (Weiner et al. 2004). Our data suggest that NDMC behaved as an agonist also at native M_4 mAChR in human neocortex. Conversely, the similarity of NDMC effects on M_4 mAChRs in human cortical neurons and in the artificial expression system indicates that the differences to rat neurons are unlikely to be due to epilepsy-related changes of human M_4 mAChRs. Thus, the differences in NDMC effects on rat and human neocortical neurons are rather due to inherent differences of M_4 mAChRs between the two species. Hence, the therapeutic effect of clozapine and NDMC may not be due to agonism at M_1 mAChR (Davies et al. 2005; Thomas et al. 2010), but rather at M_4 mAChR. This needs to be further investigated.

Effect of NDMC on M_2 mAChR

Finally we demonstrate that NDMC applied alone has no effect on GABAergic transmission both in human and rat, excluding the possibility of an agonistic effect at M_2 mAChRs. This is in agreement with findings indicating that clozapine has no depressant effect on GABAergic transmission in rats (Gemperle et al. 2003). However, NDMC prevented the CCh-induced decrease of IPSP in rat (Fig. 3b) but not in human cortex (Fig. 3m). Given the pharmacological evidence delineated above, NDMC might therefore act as an antagonist at M_2 mAChR in rat neurons. In paired recordings of hippocampal neurons, a direct presynaptic effect of NDMC was demonstrated at GABAergic synapses (Ohno-Shosaku et al. 2011). The lack of NDMC effects on IPSP_B observed here indicates that NDMC neither caused detectable effects on GABA release nor alters signalling between GABA_B receptors and Kir3 channels, consistent with the observation that NDMC, unlike clozapine, does not interact with GABA_B receptors (Wu et al. 2011). This suggests that NDMC affects GABAergic transmission in rat via another mechanism, probably M_2 mAChR. NDMC might act as M_2 mAChR antagonist and prevent the action of CCh on this receptor. We had shown

previously that this receptor is involved in modulation of GABA release (Gigout et al. 2012a, b).

Possible reasons for different pharmacology of *N*-desmethylclozapine at mAChR

The human tissue was obtained from patients with frontal or temporal lobe epilepsy. Differences between these two locations were not obvious and therefore we refrained from a statistically evaluation. Altered mAChR function related to the individual history of seizures or medications cannot be evaluated in the relatively small cohort of patients but appears unlikely considering similar pharmacology to human receptors in expression system (Weiner et al. 2004). Also gross changes in the pharmacological properties of mAChRs appear unlikely since several other ligands at mAChRs (CCh, AFDX, atropine, pirenzepine) had a qualitatively similar effect in human and rats (Thomas et al. 2010; Gigout et al. 2012a, b).

Conceivably M_2 and M_4 mAChRs of rat and human neurons exhibit subtle differences in the conformational arrangement of the seven transmembrane helices yielding constraints for the access of compounds to ortho- or allosteric sites. This view stems from the marked differences in amino acid sequences between rat and humans, particularly of M_2 mAChR (19 amino acids difference) and M_4 mAChR (15 amino acids difference).

In conclusion, the present data cast some doubts on extrapolating from rat neurons to human neurons, when going beyond the standard compounds, because the pharmacology of NDMC differs markedly in the two species (Thomas et al. 2010). Thus, the present study emphasizes the need of studies in human tissue, despite the inherent limitations of these tissues.

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We confirm that we have read the journal's position on issues involved in ethical publication and affirm that this report is consistent with those guidelines.

Conflict of interest None of the authors has any conflict of interest to disclose. All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. All applicable international, national and/or institutional guidelines for the care and use of animals were followed.

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