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Cholinergic direct inhibition of N-methyl-D aspartate receptor-mediated currents in the rat neocortex

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Abstract

Acetylcholine (ACh) and N-methyl-D aspartate receptors (NMDARs) interact in the regulation of multiple important brain functions. NMDAR activation is indirectly modulated by ACh through the activation of muscarinic or nicotinic receptors. Scant information is available on whether ACh directly interacts with the NMDAR. By using a cortical brain slice preparation we found that the application of acetylcholine as well of other drugs acting on muscarinic or nicotinic receptors induces an acute and reversible reduction of NMDAR-mediated currents (I_{NMDA}), ranging from 20% to 90% of the control amplitude. The reduction displayed similar features in synaptic I_{NMDA} in brain slices, as well as in currents evoked by NMDA application in brain slices or from acutely dissociated cortical cells, demonstrating its postsynaptic nature. The cholinergic inhibition of I_{NMDA} displayed an onset-offset rate in the order of a second, was resistant to the presence of the muscarinic antagonist atropine (10 μM) in the extracellular solution, and of G-protein blocker $\text{GDP}\beta\text{S}$ (500 μM) and activator $\text{GTP}\gamma\text{S}$ (400 μM) in the intracellular solution, indicating that it was not G-protein dependent. Recording at depolarized or hyperpolarized holding voltages reduced NMDAR-mediated currents to similar extents, suggesting that the inhibition was voltage-independent, while the reduction was markedly more pronounced in the presence of glycine (20 μM). A detailed analysis of the effects of tubocurarine suggested that at least this drug interfered with glycine-dependent NMDAR-activity.

We conclude that NMDAR-mediated currents can be inhibited directly by cholinergic drugs, possibly by direct interaction within one or more subunits of the NMDAR. Our results could supply a new interpretation to previous studies on the role of ACh at the glutamatergic synapse.

Keywords

acetylcholine; NMDA; patch-clamp; auditory cortex; muscarinic; nicotine

Introduction

N-methyl-D-aspartate receptors (NMDARs) mediate excitatory transmission and are involved in synaptic function associated with long-term synaptic changes, oscillations,

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J.F.H. performed some of the slice recording. H.S.B. performed most of the slice recording, analyzed all the data, prepared the statistics and the figures, and contributed to the discussion of the results; J.F.H., V.D.R., T.A.R., O.T.R. and G.L.L. performed the experiments on acutely dissociated cells, M.A., H.S.B. and J.F.H. developed the original idea. M.A. wrote the manuscript.

bursting behavior, learning, and memory (Burgos-Robles et al., 2007; Castellano et al., 2001; Chub et al., 1998; Nicoll and Malenka, 1999). Acetylcholine (ACh) released from terminals of neurons whose cell body are located in the Nucleus Basalis (Voytko, 1996; Voytko et al., 1994) modulates the behavior of many cortical areas by activation of muscarinic receptors (Mash and Potter, 1986) and nicotinic receptors (Alkondon and Albuquerque, 2004). Excitatory synaptic transmission in the primary auditory neocortex is subject to a particularly intense cholinergic modulation (Cox et al., 1992; Metherate and Ashe, 1991; Metherate and Ashe, 1993a; Metherate and Ashe, 1993b; Metherate and Ashe, 1994; Metherate et al., 1990; Metherate and Weinberger, 1989; Metherate and Weinberger, 1990). Cholinomimetics appear to have multiple effects on NMDAR-mediated currents (I_{NMDA}): ACh increases I_{NMDA} by acting on muscarinic and nicotinic receptors located in presynaptic terminals during the early postnatal period (P8-16) (Aramakis et al., 1997; Aramakis et al., 1999; Aramakis and Metherate, 1998), but decreases I_{NMDA} in the retina (O'Dell and Christensen, 1988) and in primary neocortical neuronal cultures (Aizenman et al., 1991).

Less information is available on the cholinergic regulation of I_{NMDA} after early development. In particular, it is not clear whether cholinomimetics interact directly with the NMDAR channel or rather the cholinergic inhibition is mediated by second messengers. By studying the cholinergic modulation of I_{NMDA} in juvenile rats (P23-45) we found that ACh and other cholinergic, muscarinic and nicotinic agonists and antagonists acutely and reversibly inhibited I_{NMDA} . The mechanism of the inhibition appeared to be postsynaptic, resulting directly from the interaction of the cholinergic drug or neurotransmitter with the NMDAR, and was not blocked by a G-protein blocker nor by the presence of the intracellular Ca^{2+} -chelator 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid K (BAPTA), suggestive of a direct interaction between cholinergic drugs and the NMDAR.

Methods

Preparation

We used an auditory cortex slice preparation similar to ones previously described (Atzori et al., 2005; Atzori et al., 2001). In some recordings we used a medial prefrontal cortex slice preparation for a series of control experiments, as described previously (Atzori et al., 2005; Atzori et al., 2001). Twenty three-fourty five day-old Sprague Dawley rats (Charles River, Wilmington, MA) were anesthetized with forane (Baxter, Round Lake, IL), sacrificed according to the National Institutes of Health guidelines, and their brains sliced with a vibratome in a refrigerated solution (0° - 4°C) containing (mM): 130 NaCl, 3.5 KCl, 10 Glucose, 25 NaHCO_3 , 1.25 NaH_2PO_4 , 1.5 CaCl_2 and 1.5 MgCl_2 , saturated with a mixture of 95% O_2 and 5% CO_2 (artificial cerebro-spinal fluid, ACSF). The recording solution contained also picrotoxin (100 μM) or bicuculline (10 μM) for blocking γ -aminobutyric acid A receptor (GABA_{A} R), dinitroquinoxaline (DNQX, 10 μM) and atropine (10 μM), for blocking alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPA) - mediated currents, and muscarinic receptors, respectively, throughout the whole study. Coronal slices from the most caudal fourth of the brain were retained after removing the occipital convexity, and subsequently incubated in ACSF at 32°C before being placed in the recording chamber. The recording area was selected dorsally to the rhinal fissure corresponding to the auditory cortex (Rutkowski et al., 2003).

Neuron dissociation

Brain slices were placed in an oxygenated cell-stir chamber containing papain (0.5 mg/ml, Sigma) at 35°C bubbled with O_2 , titrated at $\text{pH} = 7.4$ with NaOH, 300-305 mOsm/l. After 20 min of enzyme digestion, tissue was rinsed three times with the low- Ca^{2+} -isethionate

solution and mechanically dissociated with a graded series of fire-polished Pasteur pipettes. The cell suspension was then plated into a 35-mm Lux Petri dish mounted on the stage of an upright fixed-stage microscope containing (N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid) (HEPES) -buffered ACSF.

Drugs and solutions

All drugs were purchased from Sigma (St. Louis, MO) or from TOCRIS (Ellisville, MO). In order to assess a possible postsynaptic modulation, in some experiments, pulses of N-methyl-D aspartate (NMDA, 100 μ M), were applied at 100-200 μ M from the recording areas, once every 30 s. NMDA was dissolved in distilled H₂O and diluted 10-fold in ACSF before being back-filled to a glass pipette similar to the one used for recording. NMDA application was performed with a pressure system (picospritzer, General Valve Corp. Fairfield, NJ) through a glass pipette ($\approx$ 25 psi, 3-12 ms). Stock solutions of all drugs were prepared in water and drugs were either bath-applied or dispensed with a solenoid valve system with manifold application system with a dead-time of < 200 ms as measured in dissociated cells.

Electrophysiology

Slice recording—Slices were placed in an immersion chamber, where cells with a prominent apical dendrite, suggestive of pyramidal morphology, were visually selected using an Olympus BX51 (Olympus, Tokyo, Japan) with Nomarski optics and an infrared camera system (DAGE-MTI, Michigan City, IN). Excitatory postsynaptic currents (EPSCs) were recorded in the whole-cell configuration, in voltage clamp mode, with 3-5 M Ω electrodes filled with a solution containing (mM): 90 CsCl, 90 gluconic acid, 1 lidocaine N-ethyl bromide (QX314), 1 MgCl₂, 10 HEPES, 4 glutathione, 1.5 ATPMg₂, 0.3 GTPNa₂, 8 biocytin, 5 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid K (BAPTA-K), pH 7.2-7.3 with NaOH and 275 mOsm/l. BAPTA blocks second messenger cascades caused by raises in intracellular calcium concentration (Beech, 1993; Jones et al., 1988; Vulpius et al., 1998). The holding voltage was not corrected for the junction potential (< 4 mV). Electrically evoked EPSC were measured by delivering two electric stimuli (90-180 μ s, 10-50 μ A) 100 ms-apart every 20 seconds with an isolation unit, through a glass stimulation monopolar electrode filled with artificial cerebrospinal fluid (ACSF) and placed in layer II/III, 150-200 μ m from the recording electrode. EPSCs were blocked by aminophosphonovaleric acid (APV, 100 μ M), confirming that they were mediated by NMDARs. Paired Pulse Ratio (PPR) was defined as the ratio between the mean of the second to the mean of the first response. I_{NMDA} were induced by applications of NMDA itself (chemically-evoked EPSCs (cEPSCs) in slices, in the presence of the Na⁺-channel blocker tetrodotoxin (TTX, 1 μ M), or from dissociated cell.

Recordings started after a stabilization period of a few minutes during which the pipette solution dialyzed into the recorded cell. We defined as statistically stable period a time interval along which the EPSC mean amplitude measured during any two-minute assessment did not vary according to an unpaired t-Student test. After recording an initial baseline for more than 5 minutes, drugs were bath-applied for 5 minutes or longer, until reaching a stable condition (as defined above). In the case of recordings with a pipette solution containing the G-protein blocker GDP β S or activator GTP γ S we waited at least 20 min (on average $\sim$ 30 min) before starting electrophysiological recording, in order to allow sufficient time to dialyze the cytosol with the intracellular solution. Drug effects were assessed by measuring and comparing changes between baseline (control) and treatment, with paired t-Student tests (PPR, Wilcoxon's t-test). Analysis of variance (ANOVA) or unpaired t-Student test were used for comparisons between different groups of cells. Data were reported as different only if $P < 0.05$. We indicated as A_{contr} and A_{treat(ment)} the mean amplitude of the glutamatergic

currents in control or under the described treatment. The reduction was defined as $R \equiv 100 * (1 - A_{\text{treat}} / A_{\text{contr}})$.

Dissociated cell recordings

Whole-cell recordings employed standard techniques (Flores-Hernandez et al., 2002). Electrodes were pulled from Corning 8250 glass (A-M Systems, Inc, Carlsborg, WA) and heat polished prior to use. The internal solution consisted of (mM): 175 N-methyl-D-glucamine (NMDG), 40 HEPES, 2 MgCl_2 , 10 Ethylene glycol-bis(γ -aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), 12 phosphocreatine, 2 Na_2ATP , 0.2 Na_2GTP , 0.1 leupeptin, pH = 7.2-7.3 with H_2SO_4 , 265-270 mOsm/L. The external solution consisted of (in mM): 127 NaCl, 20 CsCl, 5 BaCl_2 , 2 CaCl_2 , 12 glucose, 10 HEPES, 0.001 TTX, 0.02 glycine, pH = 7.3 with NaOH, 300-305 mOsm/L.

Recordings were obtained with an Axon Instruments 200A patch clamp amplifier and controlled with a Pentium clone running pClamp (v. 8.01) with a DigiData 1200 series interface (Axon Instruments, Foster City, CA). Electrode resistance was typically 2-4 $\text{M}\Omega$ in the bath. After seal rupture, series resistance ($<20 \text{ M}\Omega$) was compensated (70-90%) and periodically monitored. Recordings were made from pyramidal cells that had short ($<75 \mu\text{m}$) apical dendrites (Fig. 1B). Unless otherwise noted, the cell membrane potential was held at -80 mV .

Drug application

Drugs were applied with a gravity-fed “two-pipe” system. The array of application capillaries -glued at approximately $800 \mu\text{m}$ from each other- was positioned a few hundred μm from the cell under study. Solution were applied by changing the position of the array with a DC drive system controlled by a SF-77B perfusion system (Warner Instruments, Hamden, CT) synchronized by pClamp. The solution changes were complete within less than 100 msec. Generally, NMDA ($1\text{-}1000 \mu\text{M}$) was applied for 3 sec every 20 sec. Drugs were prepared as concentrated stocks in deoxygenated water.

Results

Acetylcholine inhibits I_{NMDA} postsynaptically

In order to study the effect of cholinergic drugs on synaptic I_{NMDA} we recorded electrically-evoked NMDA currents (eEPSCs) in the presence of DNQX ($10 \mu\text{M}$), bicuculline ($10 \mu\text{M}$) or picrotoxin ($100 \mu\text{M}$), and atropine ($10 \mu\text{M}$) in the control solution, at a holding potential of -40 mV , in order to remove Mg^{2+} block. The NMDAR-mediated nature of the response was confirmed by complete block following the application of the NMDAR blocker amino-phosphonovaleric acid (APV, $100 \mu\text{M}$, fig. 1A, $n = 3$). Neurons were injected with biocytin for post-hoc identification. A cortical layer II biocytin-injected reconstructed neuron is shown in fig. 1B.

Application of ACh ($1 \mu\text{M}$) or of the muscarinic agonist oxotremorine (oxo, $10 \mu\text{M}$) decreased eEPSCs amplitude ($R \equiv 100 * (1 - A_{\text{treat}} / A_{\text{contr}}) = 36.4 \pm 6 \%$ and $37.8 \pm 6.3 \%$, respectively, $n = 8$ each) without changing the pair pulse ratio (PPR) (PPR in control was 1.34 ± 0.13 vs. 1.43 ± 0.1 in ACh, $n = 8$, n.s., Wilcoxon's t-test; PPR = 1.22 ± 0.07 in control vs 1.25 ± 0.09 in oxo, $n = 8$, n.s., Wilcoxon's t-test). In fig. 1C, D, E and F, we show representative traces and PPR values illustrating the effect of ACh (C and D) and oxo (E and F). The invariance of the PPR following the application of ACh or oxo suggests, but does not prove, that the reduction of I_{NMDA} is due to a postsynaptic effect.

In order to test whether the cholinergic-induced I_{NMDA} inhibition was due to postsynaptic modulation we used a fast drug-application system to apply the prototypical agonist NMDA (100 μM , chemically-evoked or cEPSC) and/or other drugs to acutely-dissociated auditory cortical neurons. Application of NMDA produced large inward currents whose profile was unchanged during the application of ACSF (example in fig. 2A). On the contrary, the application of either ACh or oxo on top of the NMDA application resulted in a reversible decrease of the I_{NMDA} ($R = 42.3 \pm 2\%$, $n = 11$, and $R = 22.9 \pm 1.4\%$, $n = 8$, respectively; fig. 2B and C) whose onset and offset kinetics were only slightly slower than the drug dispensing speed of the system ($\tau = 1089 \pm 28$ ms, $n = 11$, example in the time expansion of fig. 2B). Mean $\pm$ s.e.m. of R , the reduction induced by ACh or oxo is shown in fig 2D.

The cholinergic inhibition of I_{NMDA} is not G-protein mediated

The resistance of cholinergic inhibition of I_{NMDA} to atropine suggested that muscarinic receptors were not responsible for the effect. In order to determine whether the activation of other GTP-dependent protein (G-protein) receptors was responsible for the I_{NMDA} inhibition we measured the effect of ACh on currents evoked by pressure application of NMDA using an intracellular solution containing the non-hydrolyzable analogue of guanosine-diphosphate, $\text{GDP}_{\beta}\text{S}$ (500 μM) to lock the α -subunit of trimeric G-protein complexes in a permanently inactive state. In the presence of $\text{GDP}_{\beta}\text{S}$ in the pipette solution the application of a low concentration ACh (10 nM) or oxo (10 nM) still induced a reversible decrease in the cEPSC I_{NMDA} (examples in fig. 3A and B). In order to test the possible effect of endogenous ACh we determined the action of the ACh-esterase inhibitor physostigmine on the cEPSC. In the presence of $\text{GDP}_{\beta}\text{S}$ in the recording pipette, physostigmine reversibly decreased cEPSC amplitude, ($10.6 \pm 4\%$ in 10 μM , $n = 4$, and $28.4 \pm 5\%$ in 50 μM , $n = 4$, $P < 0.05$, t-test, examples in fig. 3C), raising the possibility that endogenous ACh reduces I_{NMDA} in a G-protein-independent fashion, similar to exogenously-applied ACh. Mean $\pm$ s.e.m. for the effects of ACh, oxo, or physostigmine on the cEPSC I_{NMDA} amplitudes are shown in fig. 3D.

Nicotinic agonists and antagonists inhibit I_{NMDA}

To determine whether muscarinic drugs selectively inhibited I_{NMDA} , we tested the effect of the nicotine and tubocurarine - prototype nicotinic agonist and antagonist, respectively - on the amplitude of the cEPSC. Application of nicotine or of tubocurarine consistently inhibited I_{NMDA} in brain slices as well as in dissociated cells (fig. 4). In the presence of $\text{GDP}_{\beta}\text{S}$, nicotine (10 μM) reversibly inhibited NMDAR-mediated cEPSC in brain slices ($R = 58 \pm 3\%$, $n = 7$) as well as in dissociated cells ($R = 35.5 \pm 8\%$, $n = 5$), as shown in the examples in fig. 4A and B. Similarly, tubocurarine, also in the presence of $\text{GDP}_{\beta}\text{S}$, reversibly decreased cEPSC amplitude in brain slices (example in fig. 4C) as well as in dissociated cells (example in fig. 4D). Tubocurarine also inhibited electrically-evoked I_{NMDA} in brain slices ($38.2 \pm 6.6\%$, $n = 6$, example in fig. 4E).

For ascertaining whether the inhibiting effect of physostigmine was due to its block of cholinesterase as suggested by the experiment in fig. 3C and D, or was rather a direct block of I_{NMDA} we also measured the effect of physostigmine bath-applications on cEPSC I_{NMDA} in dissociated cells. Unexpectedly, physostigmine significantly decreased I_{NMDA} (example in fig. 4F), indicative of a direct postsynaptic effect. Mean $\pm$ s.e.m. for the tubocurarine, nicotine and physostigmine inhibition of chemically-induced I_{NMDA} are summarized in the bar graphs in fig. 4G and H for brain slices and dissociated cells, respectively.

In order to verify the effectiveness of intracellular dialysis, we used an intracellular solution with $\text{GTP}_{\gamma}\text{S}$ (400 μM) - which like or $\text{GDP}_{\beta}\text{S}$ locks the α -subunit of trimeric G-protein complex, this time in a permanently active state - to test the effect of serotonin on the

chemically evoked I_{NMDA} in recordings from cells of the medial prefrontal cortex, where 5-HT postsynaptically decreases NMDAR-mediated current through activation of the G-protein mediated 5-HT_{1A} receptor (Yuen et al., 2005). As expected, we found that application of 5-HT (20 μM) reduced I_{NMDA} in control pipette solution (example in fig. 5A) but not in the presence of $\text{GTP}\gamma\text{S}$ (example in fig. 5B and 5C). On the contrary, tubocurarine (50 μM) still depressed I_{NMDA} even in the presence of $\text{GTP}\gamma\text{S}$ in the pipette solution (example in fig. 5C). The presence of $\text{GTP}\gamma\text{S}$ in the recording pipette produced similar results also in the auditory cortex, blocking any effect of 5-HT ($R = 3 \pm 2\%$, n.s., $n = 4$, data not shown), without changing the extent of the I_{NMDA} inhibition induced by tubocurarine (50 μM , $R = 50 \pm 4\%$, $n = 8$, $P < 0.05$, paired t-Student).

Mechanism of the cholinergic inhibition of I_{NMDA}

NMDARs receptors are subject to voltage-dependent block by neurotransmitters and modulators which carry multiple positive electric charges, and may block the channel pore in an activity- and voltage-dependent fashion (Cui et al., 2006). Furthermore, the NMDAR is subject to a block by Mg^{2+} ions which is relieved at depolarized potential (Mayer et al., 1984). We tested the possibility that the cholinergic inhibition was voltage-dependent by recording cEPSC I_{NMDA} at a depolarized holding potential (V_h) = +40 mV. At this holding potential, cEPSC were recorded as outward currents, consistent with a reversal voltage for I_{NMDA} around 0 mV. Application of ACh, nicotine or tubocurarine inhibited I_{NMDA} to a similar extent measured at $V_h = -40$ mV ($n = 4$), suggesting that the reduction was not voltage-dependent (representative examples in fig. 6A, B, and C, respectively). In particular, ACh produced a dose-dependent response whose maximal effect was similar to that produced by either nicotine (10 nM - 10 μM , $n = 6$ each) or tubocurarine (50 μM , $n = 4$). Summary of these results are reported as mean $\pm$ s.e.m. in the graph bar in fig. 6D).

Since all cholinergic drugs that we tested effectively inhibited I_{NMDA} we decided to test the effect of atropine (10 μM) on the amplitude of cEPSC in the presence of $\text{GTP}\gamma\text{S}$ (400 μM). Atropine applications did not change cEPSC amplitude (example in fig. 7A), while in the same recording conditions ($\text{GTP}\gamma\text{S}$ in the pipette solution) tubocurarine reliably induced a reversible reduction of I_{NMDA} , as shown in the example in fig. 7B.

The NR1 subunit of the NMDAR has a binding site for the aminoacid glycine and serine, whose presence is required for opening the channel following glutamate binding (Parsons et al., 1998). We considered the possibility that cholinergic inhibition of I_{NMDA} operated by modifying glycine or serine binding. To test this hypothesis we measured cEPSC I_{NMDA} in ACSF or after application of glycine (20 μM) to the extracellular solution, with a pipette solution containing $\text{GDP}\beta\text{S}$ (500 μM). In the presence of glycine, the tubocurarine-induced decrease in cEPSC I_{NMDA} amplitude more than doubled (mean $\pm$ s.e.m. in the bar graph in fig. 7C). An example of time course of the effects of tubocurarine on the cEPSC I_{NMDA} amplitude in the presence or in the absence of glycine is shown in the example in fig. 8A. For investigating in more depth tubocurarine-induced inhibition we obtained a dose-response of the inhibition by tubocurarine in the presence or in the absence of 20 μM glycine. We found that while in the nominal absence of glycine tubocurarine induced only a relatively modest decrease of the I_{NMDA} , in the presence of 20 μM glycine tubocurarine induced a reduction that was consistently larger throughout the dose-response curve, leading, for saturating doses of tubocurarine, to approximately the same current regardless of the presence or absence of glycine – around 40% of the maximal current in glycine (fig. 8B) -. A plot of the non normalized I_{NMDA} percentage reduction by tubocurarine (fig. 8C) showed the greater potency of the tubocurarine reduction in the presence of glycine (maximum reduction by tubocurarine was $31 \pm 2.5\%$ in control vs. $56 \pm 7\%$ in 20 μM glycine). The complexity of the interaction between tubocurarine and glycine is illustrated by the change of the normalized dose response curve, showing a glycine-induced right-shift

(fig. 7D, $IC_{50_{\text{tubocurarine}}(\text{control})} = 8 \pm 1 \text{ nM}$ vs. $IC_{50_{\text{tubocurarine}}(\text{control})} = 98 \pm 7 \text{ nM}$), with no change in the Hill coefficient. Small differences between I_{NMDA} reductions obtained with the same concentration of tubocurarine measured in $GTP_{\gamma}S$ vs. $GDP_{\beta}S$ were not statistically significant. These results are compatible with the hypothesis of a non competitive mechanism of inhibition whereby tubocurarine displaces glycine from its binding site.

Discussion

Nicotinic agonists are suggested to inhibit I_{NMDA} through a non-competitive mechanism (O'Dell and Christensen, 1988). Our data confirm that nicotinic agonist inhibit NMDAR-mediated currents, and show for the first time that also the nicotinic antagonists tubocurarine, oxo, the endogenous neurotransmitter ACh, as well as the cholinesterase inhibitor physostigmine, all decrease I_{NMDA} in an atropine-insensitive manner, suggestive of a non-muscarinic mechanism. The unchanged PPR of the synaptic responses suggested a postsynaptic site of the effect, confirmed by the cholinergic reduction of currents evoked by the application of NMDA in brain slices as well as in dissociated neurons. The rapid kinetic of the reduction of I_{NMDA} induced by ACh and oxo in dissociated neurons suggested a direct interaction between the cholinergic drugs and the NMDAR.

Mechanism

Since several drugs and ions operate a voltage-dependent block of NMDAR-dependent currents, we tested whether cholinergics would inhibit I_{NMDA} at depolarized potentials to similar extent of the blockage in a hyperpolarized cell. No substantial differences were detected in the extent of the blockage at $V_h = +40 \text{ mV}$ vs. $V_h = -40 \text{ mV}$, failing to demonstrate any voltage dependence of the inhibition.

Similar to the inhibition induced by ACh and oxo, the reduction in I_{NMDA} following nicotine and tubocurarine applications were not blocked by the presence of drugs preventing a large range of intracellular second-messenger cascades, suggesting that nicotinic and muscarinic substances alike might inhibit NMDAR-mediated currents using similar mechanisms.

We considered the possibility that cholinergic drugs interact at the glycine site by displacing the NMDAR co-agonist glycine. Because of its high effectiveness and reversibility as I_{NMDA} blocker, tubocurarine was selected as prototype inhibitor. While tubocurarine induced a large I_{NMDA} reduction in the presence of the NMDAR co-agonist glycine, the same substance induced only a modest but measurable, dose-dependent inhibition in nominal absence of glycine suggesting that tubocurarine might compete with this aminoacid displacing glycine from its binding site at the NR1 subunit. The specific mechanism of inhibition might differ for other cholinergic drugs.

Our data did not allow to assess whether I_{NMDA} is physiologically inhibited by ACh, as initially suggested by the inhibition of synaptic I_{NMDA} by the cholinesterase blocker physostigmine, since physostigmine itself inhibited I_{NMDA} also in dissociated cells, indicative of its direct interaction with the NMDAR. Our data do not completely rule out the possibility of a protein-protein interaction between the NMDAR and functional or non-functional ionotropic channels like those formed by the nicotinic $\alpha 9$ and/or $\alpha 10$ subunits, which possess a mixed muscarinic-nicotinic pharmacology (Baker et al., 2004; Lustig, 2006).

The cholinergic reduction of NMDARs is less surprising if we consider that NMDAR the “inverse” interaction (the block of nicotinic channels by NMDA antagonists) has already

been reported in several studies (Aracava et al., 2005; Buisson and Bertrand, 1998a; Buisson and Bertrand, 1998b; Plazas et al., 2007) suggesting that at least nicotinic and NMDA ligands somehow overlap at least partly in their ligand affinities, although the specific mechanisms of their interactions might differ.

The present data would suggest an alternative interpretation to the finding that in the sensory neocortex, nicotine, but also carbachol, inhibit I_{NMDA} (Levy et al., 2006). Differences with our work might be due to the different cortical area (somatosensory vs. auditory) or different cortical layer (LV vs. LII/III), or shorter duration of the drug application. Our data also supply a simple explanation to a series of observations reporting that exposure to nicotine protects from NMDAR-mediated glutamate toxicity in a number of preparations (Ferchmin et al., 2003; Prendergast et al., 2001; Rezvani and Levin, 2003)

Physiological significance

Microdialysis studies have shown that the concentration of ACh measured in the presence of cholinesterase blockers is in the order of a fraction of micromolar (McIntyre et al., 2003). Even in the case that actual brain concentration of ACh would be two orders of magnitude lower, the significant reduction of I_{NMDA} observed for a concentration of ACh as low as 10 nM indicates that ACh could significantly inhibit tonically at least extrasynaptic NMDARs at physiological concentrations, while the transient reduction of synaptic NMDAR-mediated conductance would be possible following intense ACh release by cholinergic varicosities located in the neighborhood of glutamatergic synapses.

A cholinergic reduction of NMDARs could be an alternative or complementary mechanism for the induction of neocortical long-term depression which is associated to cholinergic modulation (Kirkwood et al., 1999). Considering that NMDARs and nicotinic receptors have high Ca^{2+} permeabilities, it is tempting to speculate that the effect of the increase in the physiologic concentrations of ACh in the neocortex would shift the distribution of neuronal Ca^{2+} entry from NMDAR-rich to nicotine receptors-rich neuronal regions, potentially introducing a parallel shift in the loci of synaptic plasticity.

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Fig. 1. Acetylcholine and oxotremorine inhibit electrically-evoked NMDAR-mediated synaptic currents

A: NMDAR-mediated synaptic currents were evoked by stimulating afferents in layer II/III with an extracellular electrode and recording them in an ACSF solution containing AMPAR and GABA_AR and muscarinic receptor blockers at $V_h = -40$ mV. A pair pulse protocol was used to measure the PPR. The current was blocked by 100 μ M APV. **B:** representative example of spiny neuron recorded in layer II/III. **C and D:** ACh (1 μ M) inhibits postsynaptic I_{NMDA} without changing PPR. Traces are the average of 10 individual responses recorded every 20 seconds. **E and F:** the muscarinic agonist oxotremorine (10 μ M) produces an effect similar to ACh, also without change in PPR

Fig. 2. Acetylcholine and oxotremorine inhibit chemically evoked NMDAR-mediated currents
A, B and C: Effect of application of ACSF, ACh or oxo on the current evoked by NMDA application in acutely dissociated neocortical neurons. Insert below B shows that the onset of the cholinergic block measured by an exponential curve with $\tau = 1089 \pm 28$ ms ($n = 11$).
D: bars indicate mean $\pm$ s.e.m. of the reduction by ACh or oxo ($n = 11$ and 8, respectively). The asterisk (*) indicates a statistically significant reduction ($P < 0.05$, Student- t test).

Fig. 3. The cholinergic inhibition of (cEPSC) I_{NMDA} does not depend on G-proteins

Recordings performed in the presence of $\text{GDP}_{\beta\text{S}}$ in the intracellular solution. **A** and **B**: ACh (10 nM) or oxo (10 nM) decrease the current evoked by the pressure application of NMDA. **C**: The cholinesterase inhibitor physostigmine (50 μM) also reduces cEPSC. **D**: Effect of different treatments on I_{NMDA} , normalized to control. Compare mean $\pm$ s.e.m. of the effects of cholinomimetics in the presence of $\text{GDP}_{\beta\text{S}}$.

Fig. 4. Nicotine or tubocurarine inhibit I_{NMDA}

A and B: Representative traces showing that nicotine (10 μ M) reduces chemically-evoked I_{NMDA} in brain slices (A) and nicotine (10 nM) in dissociated neurons (B), recorded at $V_h = -40$ mV. **C and D:** Representative trace showing that tubocurarine (TB, 50 μ M) inhibits electrically evoked synaptic I_{NMDA} (eEPSCs) in brain slices or dissociated cells (TB 50 nM), respectively. **E:** tubocurarine also reversibly inhibits eEPSC. **F:** Representative trace illustrating the effect of physostigmine on chemically induced I_{NMDA} in dissociated cells. **G :** Bar graphs report mean $\pm$ s.e.m. of the reduction in slices or dissociated cells, respectively. G: first and second column: reduction of chemically evoked I_{NMDA} by 10 μ M nicotine or 50 μ M tubocurarine, respectively, third column, reduction of synaptic I_{NMDA} by 50 μ M tubocurarine. **H:** inhibition of I_{NMDA} by nicotine (10 nM) or tubocurarine (50 nM) and physostigmine (50 μ M) in dissociated cells.

Fig. 5. G-proteins are activated with GTP γ S and tubocurarine still reduces I_{NMDA}

A: 5-HT (20 μ M) decreases chemically evoked I_{NMDA} in control, but not in the presence of GTP γ S (400 μ M, **B**). **C:** Tubocurarine (50 μ M) but not 5-HT inhibit chemically-induced I_{NMDA} in the presence of GTP γ S. **D:** mean $\pm$ s.e.m. of the reduction by 5-HT in control (n = 5), serotonin in GTP γ S (n = 5), and tubocurarine (50 μ M) in GTP γ S (n = 4).

Fig. 6. The inhibition of I_{NMDA} is not voltage-dependent

A, B, and C: Representative traces showing that ACh (10 nM or 10 μ M), nicotine (10 nM or 10 μ M), or tubocurarine (50 μ M), respectively inhibit cEPSC I_{NMDA} in brain slices at a holding voltage $V_h = +40$ mV. **D:** Mean $\pm$ s.e.m. of the reduction induced by ACh (10 nM, or 10 μ M), nicotine (10 μ M or 50 μ M) or tubocurarine (50 μ M).

Fig. 7. Effect of cholinergic antagonists

Recordings in the presence of $GTP_{\gamma}S$ (400 μ M in the pipette solution **A**, Atropine (10 μ M) was unable to reduce I_{NMDA} , while tubocurarine (50 μ M, **B**) produced a completely reversible reduction in I_{NMDA} in two consecutive applications. **C**, Bar graphs report mean $\pm$ s.e.m. of the I_{NMDA} reduction by atropine, tubocurarine without glycine, or tubocurarine with glycine ($n = 4$).

Fig. 8. Tubocurarine inhibits I_{NMDA} in a glycine dependent manner

Recordings in the presence of GDP β S (500 μ M) in the pipette solution **A:** Example of time course illustrating that tubocurarine (50 μ M) exerts a modest reduction of I_{NMDA} in control. As expected, application of glycine (20 μ M) greatly increases I_{NMDA}. Subsequent application of the same concentration of tubocurarine in the presence of glycine produces a much larger percent of inhibition, bringing I_{NMDA} to the minimum recorded current. All effects were completely reversible. **B, C, and D:** Dose-response of tubocurarine in the presence (red lines and symbols) or in the absence (black lines and symbols) of glycine (20 μ M). **B:** Normalizing currents to the maximum current in the presence of glycine shows that saturating doses of tubocurarine bring I_{NMDA} to a minimal level independent on the presence of glycine (approximately 40% of the maximal current in glycine). **C:** Tubocurarine potency, measured as the maximum percentage reduction of the control current, is greatly increased in the presence of 20 μ M glycine. **D:** the presence of glycine brings about large differences in the dose-response of I_{NMDA} to tubocurarine.