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DOPAMINE PREVENTS MUSCARINIC-INDUCED DECREASE OF GLUTAMATE RELEASE IN THE AUDITORY CORTEX

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Abstract—Acetylcholine and dopamine are simultaneously released in the cortex at the occurrence of novel stimuli. In addition to a series of excitatory effects, acetylcholine decreases the release of glutamate acting on presynaptic muscarinic receptors. By recording evoked excitatory postsynaptic currents in layers II/III neurons of the auditory cortex, we found that activation of muscarinic receptors by oxotremorine reduces the amplitude of glutamatergic current ($A_{oxo}/A_{ctr}=0.53\pm0.17$) in the absence but not in the presence of dopamine ($A_{oxo}/A_{ctr}=0.89\pm0.12$ in 20 μM dopamine). These data suggested that an excessive sensitivity to dopamine, such as postulated in schizophrenia, could prevent the decrease of glutamate release associated with the activation of cholinergic corticopetal nuclei. Thus, a possible mechanism of action of antipsychotic drugs could be through a depression of the glutamatergic signal in the auditory cortex. We tested the capability of haloperidol, clozapine and lamotrigine to affect glutamatergic synaptic currents and their muscarinic modulation. We found that antipsychotics not only work as dopamine receptor antagonists in re-establishing muscarinic modulation, but also directly depress glutamatergic currents. These results suggest that presynaptic modulation of glutamate release can account for a dual route of action of antipsychotic drugs.

Key words: acetylcholine, patch-clamp, presynaptic, second-messenger, schizophrenia, neocortex.

In most mammalian species, the appearance of salient stimuli into the sensory field elicits the activation of several subcortical nuclei, which, in turn, supply cortex with a modulatory feedback. Among these corticopetal nuclei are the cholinergic nucleus of Meynert in the basal forebrain, which is activated during selective attention and presentation of novel stimuli (Richardson and DeLong, 1991; Voytko et al., 1994; Voytko, 1996; Bentley et al., 2003a,b; Arnold et al., 2002; Passetti et al., 2000; Sarter and Bruno, 2000), and the dopaminergic ventral tegmental area (VTA), also activated by novel stimuli such as unexpected reward (Montague et al., 1996; Fiorillo et al., 2003; Waelti et al., 2001; Schultz, 1998). Acetylcholine (ACh) and dopamine (DA) reach their targets with a diffuse, non-synaptic network of axon terminals, and exert their effect(s) by volume transmission (Descarries and Mechawar, 2000). The co-activation of cholinergic and the dopaminergic nuclei suggests the possibility that these two neurotransmitters may affect cortical function in an interactive manner.

Cholinergic fibers and receptors have been identified across all cortical layers, at all ages and in virtually all mammalian species (Mash and Potter, 1986; Campbell et al., 1987; Van Huizen et al., 1994). Cholinergic modulation is particularly relevant in the temporal cortex (Aramakis et al., 1997; Hsieh et al., 2000; Metherate and Ashe, 1991; Kilgard and Merzenich, 1998), which processes incoming auditory signal and acts as an important associative area. Almost all types of DA receptors (DARs) are present in the temporal cortex of juvenile rodents despite a modest dopaminergic innervation (Berger-Sweeney, 2003; Laplante et al., 2004; Verney et al., 1982; Berger et al., 1991; Wedzony et al., 2000; Meador-Woodruff et al., 1991; Ciliax et al., 2000). On the other hand, a more developed dopaminergic innervation is present in the temporal cortex of higher order mammals like cetacea (Hof et al., 1995), primates (Campbell et al., 1987; Lewis et al., 1986, 1987) and humans (Goldsmith et al., 1997; Goldsmith and Joyce, 1996; Joyce et al., 1998). In these species, dopaminergic fibers travel along layer 1, containing the apical dendrites of layers 2/3 pyramidal neurons and represents thus a potential anatomical substrate for a functional ACh-DA interactions. ACh muscarinic receptors (MRs) and DARs

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Abbreviations: ACh, acetylcholine; ACSF, artificial cerebrospinal fluid; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; APV, D-2-amino-5-phosphonopentanoic acid; cAMP, cyclic AMP; DA, dopamine; DAR, dopamine receptor; DMSO, dimethylsulphoxide; eEPSC, evoked excitatory postsynaptic current; EPSC, excitatory postsynaptic current; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid); mEPSCs, miniature evoked excitatory postsynaptic currents; MR, muscarinic receptor; NMDAR, *N*-methyl-D-aspartate receptor; oxo, oxotremorine; PE, phorbole ester; PKA, protein kinase A; PKC, protein kinase C; PLC, phospholipase C; PPR, paired pulse ratio; PT TX, pertussis toxin; TTX, tetrodotoxin; 4-DAMP, 4-diphenylacetoxy-*N*-(2-chloroethyl)piperidine methiodide.

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are coupled by G-proteins to signaling cascades that have the potential for directly interacting with each other.

Recent clinical and preclinical evidence points to the emergence of hyperglutamatergic states in the temporal cortex of schizophrenic patients as the main physiopathological substrate of auditory hallucinations (Anand et al., 2000; Hoffman et al., 2003; Moghaddam, 2003). Since ACh elicits a marked depression of glutamate release mediated by the activation MRs in the neocortex (Kimura and Baughman, 1997; Metherate and Ashe, 1991), and since most drugs effective in the treatment of schizophrenic psychosis are antidopaminergic, we hypothesized that DA may reduce the muscarinic-induced depression of glutamatergic synaptic transmission in the temporal cortex. We tested this hypothesis using a rat slice preparation and recording excitatory postsynaptic currents (EPSCs) from auditory cortex layers 2/3 neurons.

EXPERIMENTAL PROCEDURES

Preparation

We used an auditory cortex slice preparation similar to one previously described (Atzori et al., 2001). Three to 4-week-old Wistar rats (Charles River, Wilmington, MA, USA) were anesthetized with forane (Baxter, Round Lake, IL, USA), killed 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, 24 $NaHCO_3$, 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 cerebrospinal fluid, ACSF). The recording solution contained also bicuculline methachloride (10 μM) and D-2-amino-5-phosphonopentanoic acid (APV, 100 μM) for blocking, respectively, GABA A receptor ($GABA_AR$)- and *N*-methyl-D-aspartate receptor (NMDAR)-mediated currents. 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 sylvian sulcus corresponding to the auditory cortex (Rutkowski et al., 2003). The recording area in the prefrontal cortex was selected in the medial aspect of coronal slices of the frontal lobes (see for instance Gonzalez-Burgos and Barrionuevo, 2001).

Drugs and solutions

All drugs were purchased from Sigma (St. Louis, MO, USA) or from Tocris (Ellisville, MO, USA). In recordings using DA or DA analogues, 1.5 mM ascorbate was added as antioxidant. The presence of ascorbate induced a slight change in the pH of the extracellular solution (<0.1 pH units). Since the comparison of recordings with or without pH correction after ascorbate showed similar results the corresponding data were pooled together. Stock solutions of DA, SKF38393, quinpirole, SCH23390 and spiperone were prepared on the same day of the experiment, protected from light with aluminum foil and oxygenated only minutes before and during delivery. In order to assess a possible postsynaptic modulation, in some experiments, pulses of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA, 100 μM), were applied at 100–200 μM from the recording areas, once every 30 s. AMPA was dissolved in dimethylsulphoxide (DMSO) and diluted 10-fold in ACSF before being backfilled to a glass pipette similar to the one used for recording. AMPA application was performed with a pressure system (picospritzer, General Valve Corporation, Fairfield, NJ, USA) through a glass pipette (p≅25 p.s.i., 3–12 ms). Stock solutions of all drugs were prepared in water except for tropicamide, U73122, the ester of phorbol phorbol-12-myristate-13-acetate, KT5720 and clozapine, whose stock solutions were prepared in DMSO, and haloperidol, which was dissolved in ethanol. For nonaqueous solutions the final concentration of the solvent was added to the recording control solution. Drugs were bath-applied into the recording chamber except for U73122, rp-cAMP-S and KT 5720, which were added to the incubation chamber as a pre-treatment as specified in the text.

Electrophysiology

Slices were placed in an immersion chamber, where cells with a prominent apical dendrite, suggestive of pyramidal morphology, were visually selected using an Axioskop 2 (Zeiss, Oberkochen, Germany) with Nomarski optics and an infrared camera system (DAGE-MTI, Michigan City, IN, USA). EPSCs were recorded in the whole-cell configuration, in voltage clamp mode, holding the membrane potential at $V_h=-60$ mV, with 3–5 MΩ electrodes filled with a solution containing (mM) 100 CsOH, 100 gluconic acid, 5 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid K (BAPTA-K), 1 lidocaine *N*-ethyl bromide (QX314), 1 $MgCl_2$, 10 HEPES, 4 glutathione, 1.5 $ATPMg_2$, 0.3 $GTPNa_2$, 8 biocytin. The holding voltage was not corrected for the junction potential (<4 mV). Holding current (I_h) and input resistance (Ω_{in}) were monitored before and during drug application with a 5 mV negative pulse delivered before the paired pulse protocol. Electrically evoked EPSC were measured by delivering two electric stimuli (90–180 μs, 10–50 μA) 50 ms-apart every 6 s with an isolation unit, through a glass stimulation monopolar electrode filled with ACSF, and placed 150–200 μm from the recording electrode. EPSCs were blocked by 6,7-dinitroquinoxaline-2,3-dione (DNQX), indicative of their glutamatergic origin. Paired pulse ratio (PPR) was defined as the ratio between the mean of the second to the mean of the first response. Miniature evoked excitatory postsynaptic currents (mEPSCs) were measured in the presence of 0.5μM tetrodotoxin (TTX). 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 (typically 3–5 min) along which the EPSC mean amplitude measured during any 1-min assessment did not vary according to an unpaired Student *t*-test. After recording an initial baseline for 3–10 min, drugs were bath-applied for 5 min or longer, until reaching a stable condition (as defined above). Drug effects were assessed by measuring and comparing the different parameters (mean, PPR, and others) between baseline (control) after treatment, with paired Student *t*-tests. Unpaired Student *t*-tests were used for comparisons between different groups of cells. Data were reported as different only if $P<0.05\%$. We indicated as $A_{treatment}$ the mean amplitude of the glutamatergic currents under the described treatment.

RESULTS

DA prevents the muscarinic depression of glutamatergic currents

We first determined the effect of the broad-spectrum muscarinic agonist oxotremorine (oxo, 10 μM) on the evoked excitatory postsynaptic current (eEPSCs) recorded at $V_h=-60$ mV from layers 2/3 neurons after stimulation of the infragranular layers. Application of oxo reversibly decreased the eEPSCs amplitude, as shown in Fig. 1A. Representative eEPSC traces (Fig. 1A, left) and amplitude time courses (Fig. 1A, right) are displayed before and after the application of oxo.

We then tested the possibility that DA affects muscarinic depression of the glutamatergic signal by repeating

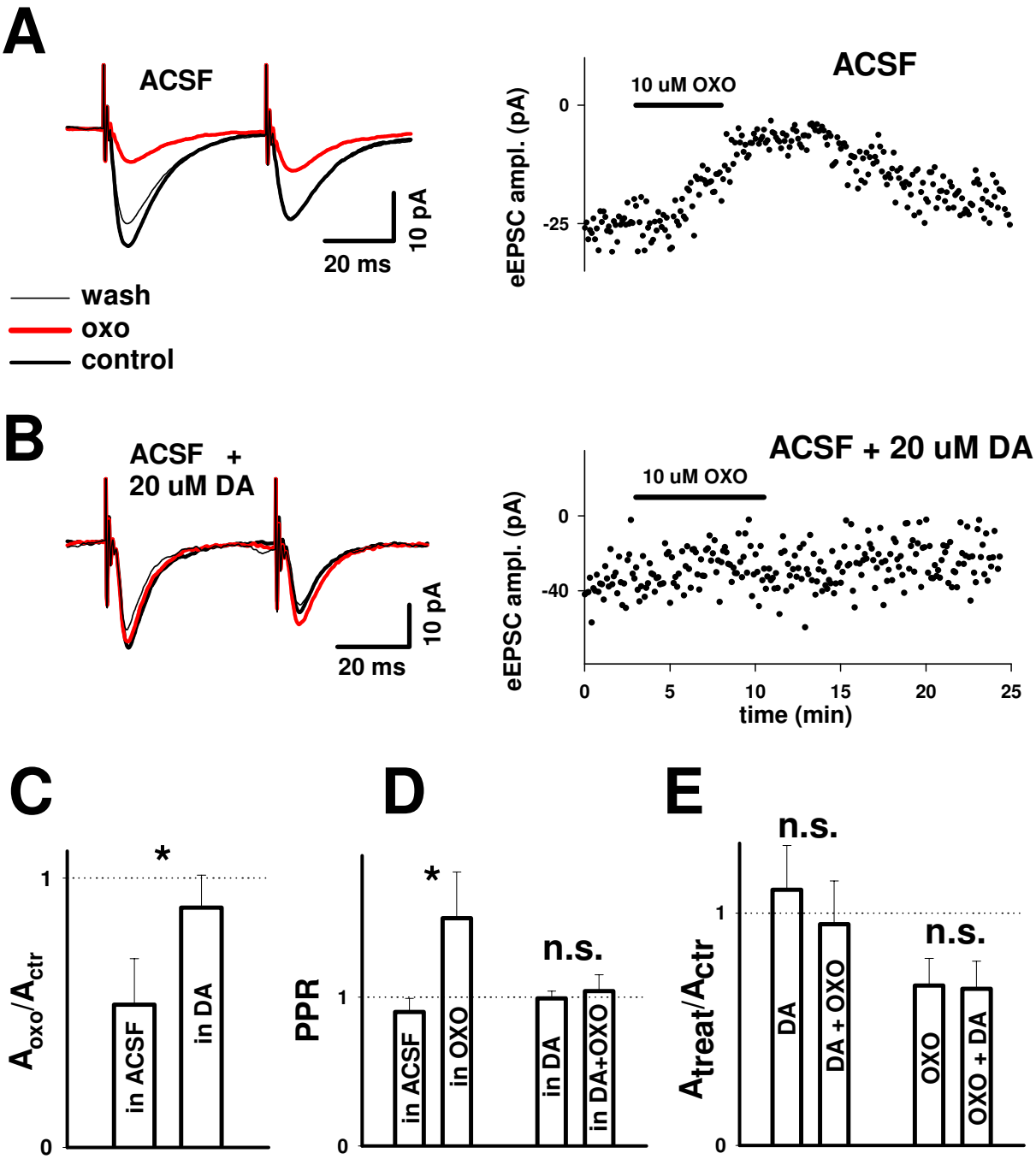

Fig. 1. DA prevents the depression of the AMPA signal induced by oxotremorine. (A, B) Representative traces (left) and time courses (right) showing that application of the muscarinic agonist oxotremorine (oxo) reversibly decreased the amplitude of the eEPSC in ACSF alone (A) but not in the presence of 20 μM DA (B). As above, traces are the average of 20 responses to a pair of stimuli distant 50 ms at 0.166 Hz. (C) Fraction of oxo-induced block in eEPSC amplitude in ACSF ($n=18$) and DA ($n=16$, $P<0.05$, unpaired Student t-test). (D) Change in PPR after oxo in ACSF (left bars, $P<0.05$, paired Student t-test, $n=18$) or in the presence of DA (right bars, n.s., paired Student t-test, $n=12$). (E) DA prevented the depressant effect of oxo (left bars, n.s., paired Student t-test, $n=8$) but did not recover the same effect after its induction (right bars, n.s., paired Student t-test, $n=7$).

the previous experiment in the presence of 20 μM DA. The presence of DA prevented the depressant effect of oxo as shown in the representative traces (Fig. 1B, left) and amplitude time courses (Fig. 1B, right). Panel 1C summarizes the changes in eEPSCs amplitude following application of oxo, alone, or in the presence of DA. DA also prevented the oxo-induced PPR enhancement (Fig. 1D). Since ascorbate was used as antioxidant in all solutions containing DA analogues, we separately tested the effect of ascorbate on the glutamatergic signal. Ascorbate slightly decreased eEPSC amplitude ($A_{ascorbate}/A_{ctrl}=0.85\pm0.10$, $n=8$) with minimal changes in pH and osmolarity of the extracellular

solution. Furthermore, its presence did not change the extent of the oxo-induced eEPSC depression (A_{oxo}/A_{ctrl}=0.54±0.13, in ACSF, n=18, vs. A_{oxo}/A_{ctrl}=0.67±0.12 in ascorbate, n=7, n.s.d.). The effect of oxo was prevented by the presence of the muscarinic blocker atropine (A_{oxo}/A_{ctrl}=1.15±0.21 in 1.5 μM atropine, n=8). In a series of long-lasting experiments the eEPSC amplitude recovered consistently after oxo-induced depression. Nonetheless, we could never fully recover the eEPSC amplitude, possibly due to partial rundown, to a long-term synaptic plasticity already described in the visual cortex (Kirkwood et al., 1999), or to a combination of the two.

Despite its continuous release, a very effective re-uptake process can quickly reduce the concentration of DA in the extracellular space. We tested whether the presence of endogenously released DA could be unmasked by blocking its re-uptake with bath-application of GBR12909 (50 nM), a selective blocker of DA re-uptake. Application of GBR12909 did not significantly affect eEPSC amplitude, but prevented the depressant effect of oxo (A_{GBR}/A_{ctrl}=1.06±0.24, n.s.d., $A_{GBR+oxo}/A_{GBR}$=1.13±0.36, n.s., n=9), suggesting that dopaminergic fibers are present in the auditory cortex, and that an effective re-uptake process limits the concentration of DA. We further tested whether DA could reverse the depression after its induction. Application of DA *following* oxo failed to prevent eEPSCs inhibition. In fact sequential application of DA and DA+oxo left eEPSC amplitude substantially unchanged (Fig. 1E left), whereas application of DA+oxo *following* oxo alone did *not* recover the eEPSC amplitude depression (Fig. 1E right). This result suggested that DA interacts with the muscarinic metabolic cascade at an early stage of the pathway.

Effect of antipsychotics on the depression of the glutamatergic signal

The previous results show that the dopaminergic system can control the cholinergic modulation of the glutamatergic signal. As a consequence, an increase in dopaminergic sensitivity would disrupt MR-mediated depression of glutamate release, leading to a hyperglutamatergic state in the auditory cortex. Since a similar impairment of the DA system and glutamatergic transmission is seen in patients with schizophrenia (Keshavan, 1999), most often associated with auditory hallucinations, we wanted to investigate whether antipsychotic drugs, effective in the treatment of acute schizophrenic episodes, also affect the DA-dependent block of muscarinic modulation of eEPSCs.

We studied the effect of the typical antipsychotic haloperidol, along with that of clozapine, representative of "atypical" antipsychotics, and of lamotrigine, whose use as antiepileptic has recently been extended as a co-adjuvant in the treatment of psychotic episodes (Tiihonen et al., 2003). We first tested whether each of the three antipsychotics might *directly* affect EPSCs in the auditory cortex, finding that *all* of them depressed eEPSC amplitude, to an extent comparable to the depression induced by oxo (Fig. 2A, white bars). Importantly, the presence of DA did *not* affect the eEPSC depression induced by any of the antipsychotic drugs used as shown in another series of similar experiments (Fig. 2A, gray bars). We then tested whether the presence of the antispychotics affected the inhibition of the muscarinic blockade by DA. We found that haloperidol or clozapine, but not lamotrigine effectively "antagonized" the inhibition of the muscarinic blockade by DA, possibly due to their antidopaminergic profile (Fig. 2B, n=7, 7 and 6 respectively).

In order to understand the details of the DA-ACh interaction in the modulation of the glutamatergic signal, we performed a series of experiments for determining the locus, receptor types, and signaling pathways associated with this process.

Locus of modulation of the glutamatergic signal

Application of oxo or DA did not significantly change the mean holding current, I_h (I_h(oxo)−I_h(control)=−2±24 pA, n=8; I_h(DA)−I_h(control)=−3±4 pA, n=8) or input resistance Ω_{in} (Ω_{in}(control)=310±37 MΩ vs. Ω_{in}(oxo)=317±40 MΩ, n=8; Ω_{in}(control)=235±37 MΩ vs. Ω_{in}(DA)=263±48 MΩ, same two samples as above). The change in PPR following application of oxo suggested a presynaptic involvement in the muscarinic eEPSC depression, and, consequently, in its block following DA application. We directly determined the effect of oxo on spike-independent glutamate release by measuring frequency and amplitude of mEPSCS at V_h=−60 mV in the presence of the Na^+ channel blocker TTX (0.5 μM), in addition to bicuculline and APV. mEPSC frequency was decreased after oxo application (2.8±0.7 Hz in control vs. 1.5±0.4 Hz in oxo, P<0.05, n=14), whereas mEPSC amplitude was not changed (9.6±1.5 pA in control vs. 8.5±1.4 in oxo, n.s., same sample). In contrast, in the presence of 20 μM DA, oxo was unable to affect either the mEPSC amplitude or their frequency (n=11, Fig. 3A).

We also tested the effect of oxo and DA on the postsynaptic current evoked by short pressure pulses of the glutamate channel agonist AMPA in a responsive area surrounding the recorded cells (100–200 μm radius). The responses to exogenously-applied AMPA were not changed after oxo application, as shown in the example and insets (Fig. 3B, left). The mean amplitude of the response is reported in Fig. 3B, right (n=10). Likewise, application of DA failed to change the amplitude of response to AMPA (78±19 pA in control vs. 76±17 pA in DA, n.s., n=7).

Altogether, these data confirm the hypothesis that presynaptic MRs decrease excitatory currents by depressing glutamate release, and suggest that DARs interact with MRs at the presynaptic level.

We also wanted to determine whether the depression of the glutamatergic signal directly induced by neuroleptic application had a pre- or a postsynaptic origin. Neuroleptic treatment did not change eEPSCs PPR (Fig. 3C, left), leaving open the possibility of a postsynaptic effect. However, bath application of haloperidol, clozapine or lamotrigine failed to affect the amplitude of the currents evoked by exogenous application of AMPA

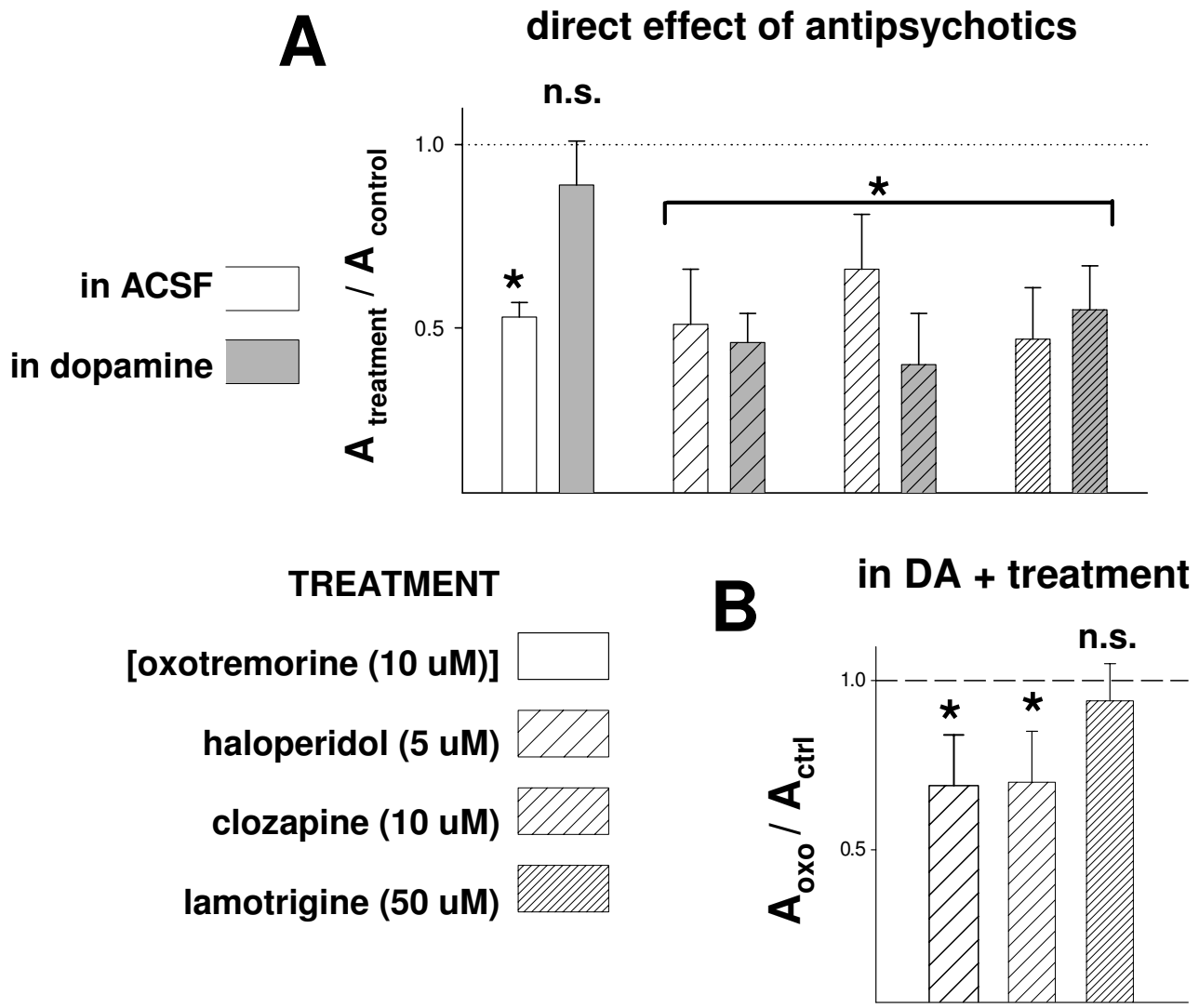

Fig. 2. Effect of antipsychotics on glutamatergic signaling. (A) DA-induced blockage of muscarinic depression: haloperidol and clozapine, but not lamotrigine, prevent the dopaminergic block of the muscarinic depression (n=7, 7 and 6 respectively). (B) Bath-application of any of the three drugs decreases eEPSC amplitude in control (left columns of each pair, n=11, 15 and 8), as well as in the presence of DA (right columns, n=6 each). The extent of the reduction is similar to the decrease induced by oxo in control but not in DA, shown for comparison in the first group of columns. The asterisks indicate statistical differences (P<0.05) assessed with a paired Student t-test.

(Fig. 3C, right), allowing to discard the hypothesis of a postsynaptic locus of action.

D_1- and D_2-like receptor co-activation is necessary for blocking the muscarinic eEPSC depression

In order to determine which class of DARs is responsible for the suppression of the muscarinic eEPSC inhibition, we performed a series of experiments using agonists and antagonists of either D_1- or D_2-like receptors. In the first series we applied the D_1-like agonist SKF 38393 (50 μM, n=11) or the D_2-like agonist quinpirole (10 μM, n=9) prior to the application of oxo. In none of these experiments could we fully reproduce the antagonistic effect of DA with respect to the cholinergic suppression of the eEPSC (Fig. 4A, bars 3 and 4, compare with bars 1 and 2). Surprisingly, simultaneous application of both agonists did mimic the DA suppressive effect (Fig. 4A, bar 5, n=8). We then sequentially applied DA and DA+oxo in the presence of either the D_1-like or the D_2-like antagonists SCH 23390 (10 μM, n=4) or spiperone (10 μM, n=6). In both cases the presence of either substance alone prevented the suppressive effect of DA (Fig. 4A, bars 6 and 7). These data suggest that a cooperative effect of the two classes of DARs blocked the cellular cascade activated by MRs.

MRs involved in the eEPSC depression

For understanding the molecular nature of the interaction between DA and the MR(s) involved in eEPSCs depression, we further investigated the nature of the oxo-induced eEPSC inhibition, utilizing a series of muscarinic antagonists. Two broad classes of MRs are known: one class is composed of the M_1R, M_3R and M_5R, and is associated

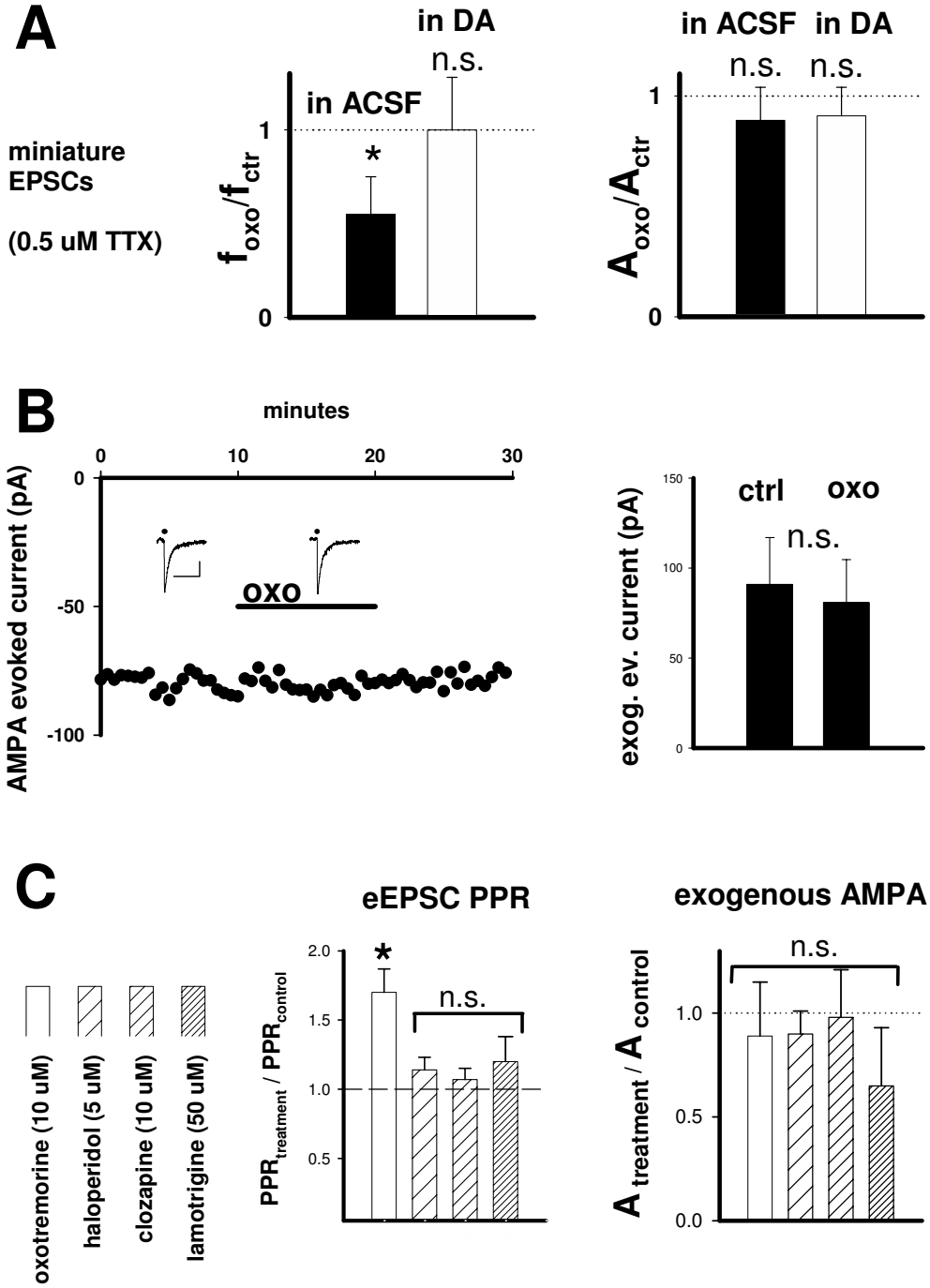

Fig. 3. The muscarinic depression of EPSCs is presynaptic. (A) Effect of oxo on mEPSCs frequency (left) and amplitude (right) in the presence of 0.5 μM TTX. The presence of oxo reduced mEPSCs frequency but not amplitude in control ($n=14$) but not in the presence of 20 μM DA ($n=11$, paired Student *t*-test). (B) Applications of oxo did not affect the response to exogenously applied AMPA as shown in the representative time course (left, 9 ms duration AMPA application indicated by the dot; scaling bar of the insets: 2s, 20 pA). Traces in the inset are the average of five consecutive responses before or after oxo application. Average is shown at the right ($n=10$, n.s., paired Student *t*-test). (C) Oxo but not antipsychotics changed PPR (left panel, $n=18$, 11, 15 and 8 respectively, paired Student *t*-test), compatible with a post-synaptic effect. However none of these drugs affected the amplitude of the response to exogenously applied AMPA (right panel, none statistically different, paired Student *t*-test, $n=10$, 6, 7, and 6 respectively).

with phospholipid metabolism and activation of phospholipase C (PLC); the other class is composed of the M_2 and M_4 receptor subtypes, which negatively modulate the adenylyl cyclase (Loffelholz, 1996). Since both D_1- and D_2-like receptors are associated with adenylyl cyclase activity, we hypothesized an interaction between DARs and M_2 or M_4 MRs. Thus, blockade of M_2 or M_4 receptors should prevent the muscarinic depression of the eEPSC ampli-

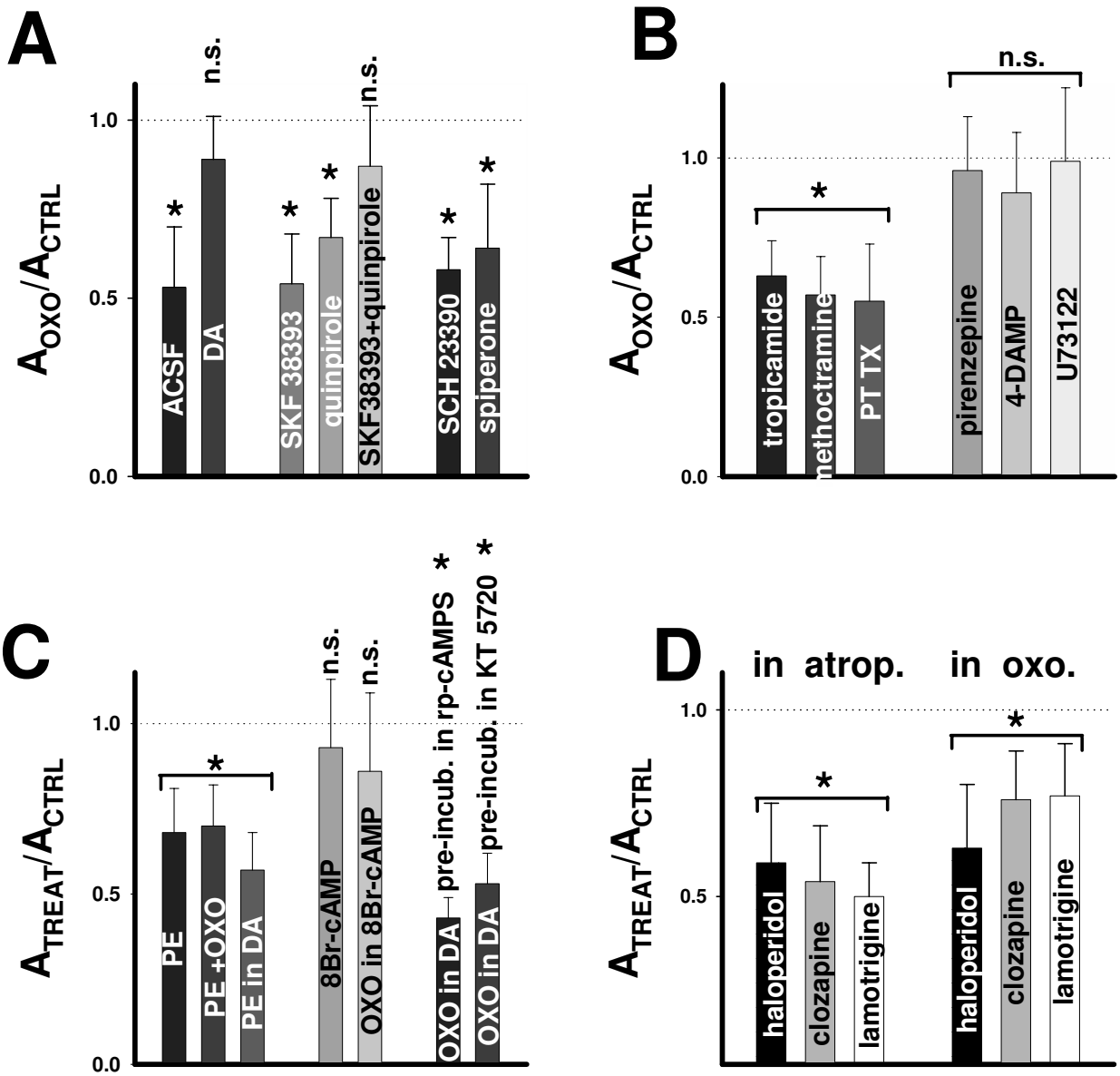

Fig. 4. Pharmacology of muscarinic depression. (A, B) Bars represent the ratio between eEPSC amplitude after application of oxo over the corresponding control amplitude when the ACSF contained the substance indicated in the column during the whole experiment. (A) Dopaminic-muscarinic interaction: D_1- and D_2-like receptors cooperativity. The first group of bars, shown for comparison, represents the effect of oxo in ACSF and in DA. The second group (columns 3, 4 and 5) represents the effect of oxo in the presence of either or both the D_1-like agonist SKF38393 (10 μM), or the D_2-like agonist quinpirole (10 μM). While none of the two substances alone was able to mimic the effect of DA, the combination of the two did. The presence of the D_1- or D_2-like antagonists SCH23390 (10 μM) or spiperone (10 μM), antagonized the dopaminergic effect, suggestive of a cooperative effect of D_1- and D_2-like receptors (col. 6 and 7). (B) Involvement of MRs activating Phospholipase C. Bars 1 and 2 reproduce the effect of the antagonists of the M_4 receptor tropicamide (10 μM), and M_2 receptor methoctramine (10 μM), both associated with adenylyl cyclase activation, while bar 3 represents the effect of 2-h-long pre-incubation in PT TX (100 μg/100 mL), an inhibitor of the G_{α}-protein associated with the M_2 and M_4 receptors. In no case did the treatment prevent the oxo-induced eEPSC depression. Bars 4, 5 and 6 show the effect of pirenzepine (10 μM), and 4-DAMP (10 μM), antagonists of the PLC-associated M_1 and M_3 receptors respectively, or of >2-h-long incubation in U73122 (10 μM), a PLC blocker. In the last three conditions oxo failed to depress eEPSCs, suggesting the involvement of the PLC pathway. (C, D) Bars represent the ratio of the eEPSC amplitude after treatment over the corresponding control amplitude. (C) DA blocks PLC by activating PKA. The non-inactivating analog of the PLC metabolite diacylglycerol, PE, mimicked oxo in depressing the AMPA signal (bar 1), and saturated its effect (bar 2), confirming the involvement of PLC. DA was unable to prevent the PE-induced reduction of the AMPA signal, indicating that the monoamine acted at the level of the PLC or uphill. The membrane-permeable cAMP analog and PKA activator 8Br-cAMP (2 mM) did not depress eEPSC per se (bar 4), but mimicked the effect of DA in preventing the oxo-induced eEPSC depression (bar 5). Bars 6 and 7 show that the application of rp-cAMPS (50 μM) or pre-incubation in KT 5720 (1 μM), which respectively prevented the activation of and specifically blocked PKA, also prevented the DA suppression of the eEPSC by oxo, indicating that PKA activation is required for blocking the muscarinic eEPSC depression. (D) Antipsychotics act through a non-muscarinic mechanism. The presence of the muscarinic blocker atropine did not prevent the eEPSC decrease by antipsychotics (first three columns, n=6, 7, and 6 respectively). In the presence of oxo antipsychotics were still able to reduce eEPSC amplitude, although to a lesser extent than in control (last three columns, n=8, 7, and 6 respectively). Statistical differences were assessed using the paired Student t-test throughout Fig. 4.

tude. However, neither the presence of the selective M_4 receptor antagonist tropicamide (10 μM, $n=10$), nor of the M_2 receptor antagonist methoctramine (10 μM, $n=7$), was able to prevent the oxo-induced eEPSC depression (Fig. 4B, col. 1 and 2). To further test the possibility of an involvement of M_2 or M_4 receptors, we incubated the slices in 2 μg/mL of pertussis toxin (PT TX, $n=9$), an inhibitor of the G-protein-associated with M_2 and M_4 receptors, for 2 h prior to recording. However, PT TX incubation was unable to prevent the depression (Fig. 4B, col. 3).

In contrast, application of the M_1R antagonists pirenzepine dihydrochloride (10 μM, $n=8$) or of the M_3R antagonist 4-diphenylacetoxy-*N*-(2-chloroethyl)piperidine methiodide (4-DAMP, 10 μM, $n=16$) *did* prevent the oxo-induced decrease of eEPSC amplitude (Fig. 4B, col. 4 and 5). Furthermore, pre-incubation of the PLC inhibitor U73122 (10 μM, $n=5$) abolished the oxo-induced inhibition of eEPSCs (Fig. 4B, col. 6), suggesting that oxo inhibited eEPSC by acting on M_1 or M_3 receptors, signaling via activation of PLC and not using a cAMP-dependent mechanism.

Pharmacology of the ACh–DA interaction

The previous data suggested that activation of MRs activates PLC, which in turn decreases the release of glutamate by activating protein kinase C (PKC). This was confirmed by the eEPSCs inhibition following permanent activation of PKC by application of the phorbole ester phorbol-12-myristate-13-acetate (PE, 2 μM, $n=7$, Fig. 4C, bar 1). No further decrease of the eEPSC amplitude followed the subsequent application of oxo ($n=7$), indicating the same cellular target for oxo and PLC (Fig. 4C, bar 2). However, pre-application of DA did not block the suppressive effect of the PE on the eEPSC (Fig. 4C, bar 3, $n=7$), suggesting that DAR activation might impair the muscarinic cascade at an early stages along the metabolic pathway.

In contrast, application of the membrane-permeable cyclic AMP (cAMP) analog 8-Br-cAMP (2 mM) did not affect the eEPSC amplitude by itself, but mimicked DA in preventing the oxo-induced eEPSC depression (Fig. 4C, bars 4 and 5, $n=8$ each). These results suggested a cross-talk between the DA-cAMP cascade and the MR-PLC metabolic pathway, perhaps mediated by the activation of protein kinase A (PKA). We directly tested this possibility by pre-incubating the tissue in rp-cAMPS (50 μM, $n=6$) or KT 5720 (1 μM, $n=7$), both of which are potent and selective inhibitors of PKA (but not PKC). Either treatment successfully prevented the blocking effect of DA on the oxo-induced eEPSCs depression (Fig. 4C, last two bars). Together these data are consistent with the hypothesis that DA activated PKA and, in turn, impaired the ability of ACh to depress glutamate release by inactivating PLC and the following metabolic cascade.

The effect of antipsychotics is not mediated by MRs

We tested whether the depression of eEPSC amplitude induced by direct application of antipsychotics was mediated by MRs. The presence of the muscarinic antagonist atropine did not affect the inhibition of glutamate release by any of the used antipsychotics (Fig. 4D, first three columns). We also tried to occlude the inhibition of glutamate release by pre-application of oxo. Even in this circumstance, application of the three antipsychotic drugs significantly decreased eEPSCs amplitude, although to a lesser extent than in the absence of oxo (Fig. 4D, last three columns). These data suggest that the direct depression of the glutamatergic signal by antipsychotics is mediated, at least in part, by non-MRs.

Since clinical use of lamotrigine indicates its effectiveness after unsuccessful treatment of psychotic episodes with typical or atypical antipsychotics (Tiihonen et al., 2003), we tested the hypothesis that lamotrigine decreases glutamatergic responses using a mechanism independent from the other antipsychotics. We found that application of lamotrigine decreases eEPSCs amplitude even in the presence of either clozapine ($A_{lamot+clozap}/A_{clozap}=0.59\pm0.15$, $P<0.05$, $n=6$), or haloperidol ($A_{lamot+halop}/A_{halop}=0.67\pm0.13$, $P<0.05$, $n=6$), consistent with its therapeutic profile.

These data suggest the possibility that antidopaminergic activity and direct depression of glutamate release associated with antipsychotics are distinct cellular mechanisms converging to limit a hyperglutamatergic state as seen during psychosis.

Comparison between prefrontal and temporal cortices, and variability of the glutamate depression

Because of its involvement in working memory and several neuropsychiatric disorders, the prefrontal cortex has been more intensively studied than the auditory cortex. Assuming that modulatory effects might be region-specific, we compared the effect of the muscarinic agonist oxo and of DA separately in the two cortical areas.

Application of oxo decreased reversibly and to a similar extent eEPSCs amplitude in the auditory cortex and in the in medial prefrontal cortex (example in Fig. 5A, mean in Fig. 5B). However, several differences resulted from a detailed analysis of the results in the two areas. For instance, PPR remained unchanged after oxo application in the prefrontal cortex but not in the auditory cortex (Fig. 5C). Also, DA application depressed the eEPSC amplitude in the prefrontal cortex but not in the auditory cortex (example in Fig. 5D, mean in Fig. 5E, $n=8$). Moreover, prefrontal and auditory cortex differed in the sensitivity of the PPR to DA. In fact DA application increased PPR in the prefrontal but not in the auditory cortex (Fig. 5F).

Another difference between the responses of the two areas was in the homogeneity of the responses (Table 1). In particular, application of oxo resulted in a systematic reduction of eEPSCs in the auditory cortex but not in the prefrontal cortex.

The effect of oxo in the temporal cortex was very homogeneous in respect to the prefrontal cortex. In fact, although oxo, similar to the three antipsychotics tested had, on average, a depressant effect on the EPSCs, oxo differed from the antipsychotic drugs in the homogeneity of the effect. On the contrary, application of clozapine had a mixed effect by depressing strongly the synaptic signals in

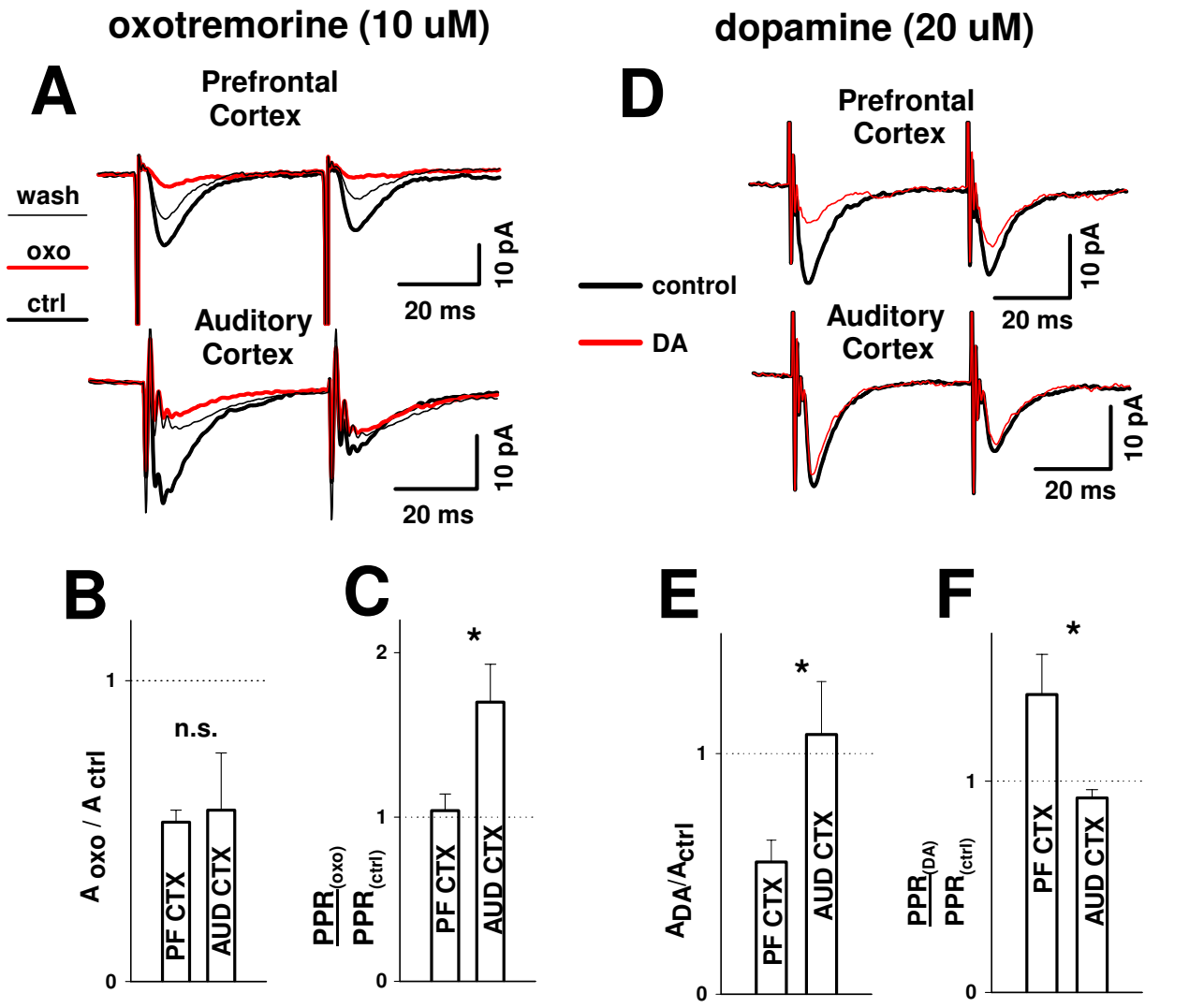

Fig. 5. Cortical modulation of glutamatergic eEPSCs. Application of the muscarinic agonist oxo (10 μM) decreased EPSCs amplitude in the prefrontal as well as in the auditory cortex: (A) representative traces. (B) Average (n=6 and 18, respectively). Traces are the average of 20 responses to a pair of stimuli distant 50 ms at 0.167 Hz. (C) Oxo induces an increase in PPR (defined as A_2/A_1) in the auditory but not in the prefrontal cortex, same sample as in B. (D) DA depressed EPSCs in the prefrontal but not in the auditory cortex: D, representative traces, E, average (n=6 and 8 respectively). (F) DA-induced increase in PPR in the prefrontal but not in the auditory cortex (same sample as E). Asterisks indicate statistically significant differences ($P<0.05$, unpaired Student t-test).

some cells while leaving it unaltered or even increasing it in many other cells, in the auditory as well as in the prefrontal cortex (Table 1). These data indicate that ACh depresses synaptic glutamatergic signal using an area-specific molecular mechanism differing from antipsychotics.

DISCUSSION

Activation of muscarinic agonists depresses AMPAR-mediated currents in many brain areas, including hypoglossal motorneurons (Bellingham and Berger, 1996), mesencephalon (Grillner et al., 1999), the amygdala (Yajeya et al., 2000), basal forebrain (Sim and Griffith, 1996), neostriatum (Hsu et al., 1995), visual cortex (Kimura and Baughman, 1997) and auditory cortex (Metherate and Ashe, 1991), presumably due to a presynaptic effect. We confirmed that the activation of MRs presynaptically depresses the glutamatergic signal in the auditory cortex, and found that this effect is suppressed by the presence of DA. The effectiveness of the specific DA reuptake blocker in mimicking the presence of DA suggests that endogenously-released DA has the potential to modulate the muscarinic depression of glutamate release. DA could pre-

Table 1. Homogeneity of muscarinic modulation in the temporal cortex

	Auditory cortex		Prefrontal cortex	
	Depressed	Non-depr.	Depressed	Non-depr.
Oxotremorine	18/18 (100%)	0/18 (0%)	4/6 (67%)	2/6 (33%)
Clozapine	8/15 (53%)	7/15 (47%)	6/15 (40%)	9/15 (60%)

vent the depression only if applied *before* MRs activation, possibly due to a cross-talk at an early stage of the cascades initiated by the two modulators (Cordeaux and Hill, 2002).

Possible function of cortical ACh and DA in the auditory cortex

Sensory information processing in the cortex is hampered by the spontaneous activation of cortico-cortical connections, which represent more than 95% of the cortical input (Mountcastle, 1998). Despite its excitatory effects, such as block of K^+ channels (Krnjevic, 1993) and decrease of GABA release (Metherate and Ashe, 1995), ACh can contribute to create a background for the emergence of thalamocortical input by (1) transiently decreasing cortico-cortical glutamate release, with the activation of MRs and (2) selectively enhancing thalamocortical transmission by presynaptic nicotinic receptors (Metherate and Ashe, 1993; Hsieh et al., 2000; Kimura, 2000).

The presence of DA could, in turn, open a spatio-temporal window of enhancement of the information flow associated with glutamate release, whose strength is normally limited by the influence of ACh, necessary for information encoding and retrieval (Linster et al., 2003; Patil et al., 1998). The concomitant presence of DA and ACh could also increase NMDAR-mediated currents (Seamans et al., 2001; Wang and O'Donnell, 2001; Aramakis et al., 1997; Chen and Yang, 2002) contributing to auditory cortical plasticity following the activation of corticopetal cholinergic and dopaminergic nuclei coupled with auditory stimulation during a critical period for hearing (Kilgard and Merzenich, 1998; Bao et al., 2001; Zhang et al., 2002).

Further investigation is needed to determine whether the relevance of the ACh–DA interactions that we described extends to the adult period.

Effects of excess DA activity

A corollary of our results is that in case of DA hypersensitivity, due to either a pathological increase of phasically-released DA, decreased DA re-uptake, increased DAR density or enhanced intracellular signaling in response to DA (Keshavan, 1999), cortically released ACh would fail to depress glutamate release. In this situation thalamocortical activity would no longer be able to emerge in a background of uninhibited cortico-cortical connections. A combination of hyperglutamatergic activity in the auditory cortex together with frontal hypofunction has the potential for disturbing cortical processing of information (Weinberger et al., 1992; Weinberger and Berman, 1996).

This interpretation could also give a rationale for the antipsychotic properties of muscarinic agonists (Stanhope et al., 2001) and the psychotomimetic characteristics of muscarinic antagonists (Muller and Wanke, 1998; Andersen et al., 2003), in that muscarinic agonists would decrease glutamate release while muscarinic antagonists would prevent the depressing action of endogenous ACh on cortico-cortical communication. In this scenario, drugs inhibiting the release of glutamate would relieve symptoms originated in the auditory areas and possibly in the whole temporal cortex.

We have shown that: (1) haloperidol and clozapine oppose the DA blockage of the muscarinic depression of glutamate release, representing an indirect mechanism for decreasing cortical excitability, (2) both drugs, as well as lamotrigine, directly depress eEPSC amplitude in a DA-insensitive fashion: these two effects might represent complementary mechanisms of action of antipsychotic drugs.

Mechanisms of action

Several lines of evidence indicate that the muscarinic depression of the glutamate signal is presynaptic: the change in PPR following the application of the muscarinic agonist, the decrease of mEPSC frequency but not of their amplitude, the failure of oxo to alter neuronal electrotonic properties, or the response to exogenously applied AMPA. MRs activating PLC were responsible for the depression of the EPSCs, similar to the muscarinic depression of EPSCs in rat neostriatum (Hsu et al., 1995) and mesencephalon (Grillner et al., 1999) but different from visual cortex (Kimura and Baughman, 1997). Direct activation of PKC with PE mimicked and occluded muscarinic depression of glutamate release, suggesting that PKC is a possible downstream target of PLC. The failure of DA to block the PE-induced depression indicates that DA might block the muscarinic effect at the level of PLC or at earlier steps in the signaling cascade. Activation of MRs could impair the release machinery either directly or at the level of Ca^{+2}-dependent elements such as Ca^{+2} channels involved in neurotransmitter release, or by affecting other voltage-gated conductances at the synaptic terminal. The block of the muscarinic depression of glutamate release was caused by cooperative action of D_1 and D_2 receptors, consistent with the D_1R-D_2R synergism not unusual in the striatum (Murer et al., 1997; Maltais et al., 2000; Hu and White, 1997; Waszczak et al., 2002), and confirmed by the recent finding that simultaneous activation of D1- and D2-like receptors directly activates PLC metabolism (Lee et al., 2004). A similar mechanism has been advocated for drugs used in the treatment of maniac-depressive psychosis (Williams et al., 2002; Bergson et al., 2003), and might in principle compete with the activation of the muscarinic pathway in a cAMP-independent manner. However, application of a cAMP agonist, although incapable of directly affecting eEPSCs, prevented their muscarinic depression, suggesting an interaction between PKA and the PLC cascade. This possibility was, corroborated by the observation that slice incubation with PKA antagonists prevented DA from blocking the muscarinic depression. Similar pathways were previously described outside the CNS (Vogl et al., 2000; Cordeaux and Hill, 2002; Ali et al., 1998; Ding et al., 1997). Possible mechanisms are PKA-induced phosphorylation of the $G_{\beta}\gamma$ subunit which prevents PLC activation (Godwin and Soltoff, 2000), or conformational change of PLC induced by PKA phosphorylation at Ser^{1105} (Dodge and Sanborn, 1998; Yue et al., 1998). Alternatively, the interaction could directly involve a voltage-gated Ca^{+2}-channel whose tonic, PLC-induced, down-regulation may

be prevented by PKA phosphorylation (Wu et al., 2002). The possibility that DA acts as norepinephrine receptor agonist offers a complementary series of interpretation for the present data. Further studies will be necessary to uncover the biochemical details of the ACh–DA interaction.

Antipsychotics and depression of glutamate release

We demonstrated that antispychotics depress eEPSC amplitude using a non-muscarinic mechanism. Furthermore, the capability of lamotrigine for depressing the glutamatergic signal in the presence of either haloperidol or clozapine is a possible mechanism accounting for the reported effectiveness of the drug in patients with haloperidol- or clozapine-resistant psychoses (Tihonen et al., 2003; Kremer et al., 2004; Dursun and Deakin, 2001), and consistent with the hypothesis that lamotrigine exerts a direct effect at the presynaptic terminal (Wang et al., 1996a,b; Von Wegerer et al., 1997; Calabresi et al., 1999; Cunningham and Jones, 2000).

Although oxo and the antipsychotics tested induced similar degrees of eEPSC depression, the effect of the antipsychotics differed from that of the muscarinic agonist not only in their insensitivity to the presence of DA, but also in other aspects of the glutamatergic depression, including the change in PPR and the homogeneity of the glutamate depressant action. All these data suggested the possibility that antipsychotics, most of which possess a complex pharmacological profile (Olianas et al., 1999), exert their glutamate-release depressant properties by acting on presynaptic receptors other than muscarinic M_1 or M_3, impairing glutamate release in a DA-insensitive fashion.

Our data suggest the possibility of a dual route of action of antispychotics: an antidopaminergic action which preserves muscarinic sensitivity of glutamatergic axons, and a direct decrease of glutamate release which substitutes the effect of endogenous ACh in depressing glutamate release. The present results shed new light on the hypothesis that some antipsychotics act primarily as glutamate release inhibitors (Krystal et al., 2003). We speculate that an excess of DA sensitivity might contribute to the production of psychotic symptoms by preventing the reduction of glutamate release in the temporal cortex, following the activation of corticopetal cholinergic nuclei.

Regional specificity of the modulation of glutamate release

Although we confirmed that the activation of MRs presynaptically depresses the glutamatergic signal equally in the prefrontal and in the auditory cortex, we detected a series of regional differences in the modulation of the release of glutamate: while in the prefrontal cortex DA induced a large reduction (−45%) in eEPSC amplitude, DA failed to affect AMPA currents in the auditory cortex, as its presence did not affect eEPSC amplitude or the exogenously-evoked AMPA responses. The increase in PPR induced in the prefrontal cortex by the presence of DA corroborates the hypothesis of the reduction of glutamate release suggested previously (Seamans et al., 2001; Gao et al., 2001). Prefrontal cortex differed from auditory cortex also for the absence of facilitation following the muscarinic depression of glutamate release, and for the presence of a postsynaptically-mediated current enhancement (+16%, Gonzalez-Islas and Hablitz, 2003) which we failed to observe in the auditory cortex. Together, these data indicate that neurotransmitter modulation may be substantially different in different cortical areas, suggesting caution in the extrapolation of pharmacological data derived from different brain regions.

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