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Prolonged nicotine exposure down-regulates presynaptic NMDA receptors in dopaminergic terminals of the rat nucleus accumbens

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ABSTRACT

The presynaptic control of dopamine release in the nucleus accumbens (NAc) by glutamate and acetylcholine has a profound impact on reward signaling. Here we provide immunocytochemical and neurochemical evidence supporting the co-localization and functional interaction between nicotinic acetylcholine receptors (nAChRs) and N-methyl-D-aspartic acid (NMDA) receptors in dopaminergic terminals of the NAc. Most NAc dopaminergic terminals possessed the nAChR α4 subunit and the pre-exposure of synaptosomes to nicotine (30 μM) or to the α4β2-containing nAChR agonist 5IA85380 (10 nM) selectively inhibited the NMDA (100 μM)-evoked, but not the 4-aminopyridine (10 μM)-evoked, [^{3}H] dopamine outflow; this inhibition was blunted by mecamylamine (10 μM). Nicotine and 5IA85380 pretreatment also inhibited the NMDA (100 μM)-evoked increase of calcium levels in single nerve terminals, an effect prevented by dihydro-β-erythroidine (1 μM). This supports a functional interaction between α4β2-containing nAChR and NMDA receptors within the same terminal, as supported by the immunocytochemical co-localization of α4 and GluN1 subunits in individual NAc dopaminergic terminals. The NMDA-evoked [^{3}H]dopamine outflow was blocked by MK801 (1 μM) and inhibited by the selective GluN2B-selective antagonists ifenprodil (1 μM) and RO 25-6981 (1 μM), but not by the GluN2A-preferring antagonists CPP-19755 (1 μM) and $ZnCl_2$ (1 nM). Notably, nicotine pretreatment significantly decreased the density of biotin-tagged GluN2B proteins in NAc synaptosomes. These results show that nAChRs dynamically and negatively regulate NMDA receptors in NAc dopaminergic terminals through the internalization of GluN2B receptors.

1. Introduction

Dopamine (DA) plays a key signaling role in the nucleus accumbens (NAc) to control reward (Ikemoto and Panksepp, 1999; Di Chiara, 2002). Incoming glutamatergic and cholinergic inputs have also been shown to control information processing in the NAc (Kalivas, 2009; Mark et al., 2011). Since both ionotropic glutamate receptors, namely N-methyl-D-aspartate receptors (NMDAR), as well as nicotinic acetylcholine receptors (nAChR) can control the release of DA through a direct presynaptic action in DAergic terminals (Pittaluga et al., 2001), the understanding of this presynaptic neuromodulation is essential to understand the glutamatergic and cholinergic regulation of reward processes.

Abbreviations: ACh, acetylcholine; AMPA, α-Amino-3-hydroxy-5-methyl-4-isoxazolone propionate; DA, dopamine; DAT, dopamine transporter; ECL, enhanced chemiluminescence; NAc, nucleus accumbens; nAChRs, nicotinic acetylcholine receptors; NMDA, N-methyl-D-aspartic acid; NSSP, non-synaptic synaptosomal protein; PBS, phosphate-buffered saline; BSA, bovine serum albumin; Post, postsynaptic component of the synaptic active zone; Pre, presynaptic component of the synaptic active zone; Stx-1A, syntaxin-1A; Syn, synaptosomes; t-TBS, Tween-containing Tris-buffered saline; 5IA85380, 5-iodo-A-85380; FURA-2AM, Fura-2-acetoxymethyl ester; DHβE, dihydro-β-erythroidine.

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It is particularly important to consider the glutamatergic and cholinergic neuromodulation system simultaneously since there is evidence for a tight interplay between both receptor systems. In fact, the functional impact of nicotine is dependent on ionotropic glutamatergic receptors (Liechti and Markou, 2008; Reissner and Kalivas, 2010; D'Souza and Markou, 2011; Timofeeva and Levin, 2011). Conversely, the acute nicotine administration alters the functional responses of ionotropic glutamate receptors in different brain areas (Risso et al., 2004a; Yamazaki et al., 2006; Vieyra-Reyes et al., 2008; Lin et al., 2010) and chronic nicotine administration affects NMDAR subunit composition (Delibas et al., 2005; Levin et al., 2005; Wang et al., 2007; Rezvani et al., 2008; Kenny et al., 2009). Importantly, previous studies have provided initial evidence that nAChR and NMDAR might also interact to control the release of DA in the NAc (Risso et al., 2004a), whereby the co-activation of nAChR with NMDAR provides a sufficient membrane depolarization to engage NMDAR (Desce et al., 1992; Raiteri et al., 1992; Pittaluga et al., 2005; Tebano et al., 2005). In contrast, the chronic exposure to nicotine was shown to differently modify the ability of ionotropic glutamate receptors to trigger the release of biogenic amines in different brain regions (Risso et al., 2004b; Grilli et al., 2009b). Notably, this effect involved the control of AMPAR trafficking (Grilli et al., 2012). Since the functional impact of NMDAR is also controlled by their trafficking (Yang et al., 2008; Bard and Groc, 2011), we now explored if the prolonged activation of nAChR might affect the ability of NMDARs to control DA release in the NAc and if this effect might involve a nAChR-mediated regulation of NMDAR trafficking.

2. Materials and methods

2.1. Animals and brain tissue preparation

Adult male rats (Sprague–Dawley, 200–250 g) were housed at constant temperature (22 ± 1 °C) and relative humidity (50%) under a regular light–dark schedule (light 7.00 a.m. – 7.00 p.m.). Food and water were freely available. The animals were killed by decapitation and the brain was rapidly removed at 0–4 °C. Fresh tissue was dissected according to the Atlas of Paxinos and Watson (1986; sections between Bregma 0.7–2.2 mm for NAc). The experimental procedures followed the ARRIVE guidelines and were approved by the Ethics Committees of the Pharmacology and Toxicology Section, Department of Pharmacy (protocol number 124/2003-A) and of the Center for Neurosciences and Cell Biology, Faculty of Medicine, University of Coimbra, in accordance with the European legislation (European Communities Council Directive of 24 November 1986, 86/609/EEC).

2.2. Preparation of synaptosomes and release experiments

Synaptosomes from the NAc were prepared essentially as previously described (Grilli et al., 2008, 2009a). The tissue was homogenized in 40 volumes of 0.32 M sucrose, buffered to pH 7.4 with phosphate (final concentration 0.01 M). The homogenate was centrifuged at 1000 *g* for 5 min, to remove nuclei and cellular debris, and crude synaptosomes were isolated from the supernatant by centrifugation at 12,000 *g* for 20 min. The synaptosomal pellet was then resuspended in Krebs-Ringer solution of the following composition (mM): NaCl 128, KCl 2.4, $CaCl_2$ 3.2, KH_2PO_4 1.2, $MgSO_4$ 1.2, HEPES 25, pH 7.5, glucose 10, pH 7.2–7.4.

For the release experiments, NAc synaptosomes were incubated for 20 min at 37 °C with [^{3}H]dopamine ([^{3}H]DA; final concentration 0.03 μM) in the presence of 6-nitroquipazine (final concentration 0.1 μM) and desipramine (final concentration 0.1 μM) to avoid false labeling of serotonergic and noradrenergic terminals, respectively. Identical portions of the synaptosomal suspension were then layered on microporous filters at the bottom of parallel superfusion chambers kept at 37 °C and the trapped synaptosomes were superfused at 0.5 mL/min with Krebs-Ringer solution. Starting from $t = 36$ min to $t = 48$ min of superfusion, 4 consecutive 3-min fractions (b1–b4) were collected. The synaptosomes were then exposed to agonists or to depolarizing agent (4-aminopyridine) at $t = 39$ min, until the end of superfusion, while antagonists were present from 8 min before agonists. When carrying out a pre-treatment with nicotine, the synaptosomes were exposed for 10 min to nicotine (30 μM) or to nAChR agonists at $t = 29$ min of superfusion in the absence or in the presence of nAChR antagonists. The effluent samples collected and the trapped synaptosomes were then counted for radioactivity. Agonist-induced effects were expressed as the percentage of the total retained radioactivity, and were calculated by subtracting the neurotransmitter content released in the four fractions collected under basal condition (no drug added) from that released in presence of the stimulus.

2.3. Calcium imaging in individual nerve terminals

Purified nerve terminals with less than 2% of postsynaptic or glial contaminations (Rodrigues et al., 2005a) were prepared as previously described using a discontinuous Percoll gradient (Dunkley et al., 1986). The calcium imaging of individual nerve terminals was carried out as previously described (Rodrigues et al., 2005b). Briefly, purified nerve terminals (500 μg of protein) were resuspended in 1 mL of HEPES-buffered Krebs medium [HBM, containing (in mM): 122 NaCl; 3.1 KCl; 0.4 KH_2PO_4; 5 $NaHCO_3$; 1.2 $MgSO_4$; 10 HEPES; 10 glucose, pH 7.4], loaded with FURA-2 a.m. (5 μM) together with 0.02% pluronic acid F-127 and 0.1% fatty-acid free bovine serum albumin (BSA) in HBM in presence of 1.33 mM $CaCl_2$ for 1 h at 25 °C and then adsorbed onto poly-D-lysine-coated cover slips. The cover slips were washed with HBM containing 1.33 mM $CaCl_2$ and mounted in a small superfusion chamber (RC-20; Warner Instruments, Harvard, UK) on the stage of an inverted fluorescence microscope (Axiovert 200; Carl Zeiss, Jena, Germany). The nerve terminals were alternately excited at 340 and 380 nm (split time of 5 ms) using an optical splitter (Lambda DG4; Sutter Instruments, Novato, CA, USA) during a total period of 1360 ms, and the emitted fluorescence at 510 nm (using a 510 nm band-pass filter; Carl Zeiss) was captured through a 40 × oil objective connected to a digital camera (Cool SNAP; Roper Scientific, Trenton, NJ, USA). Acquired images were processed using MetaFluor software (Universal Imaging Corp., Buckinghamshire, UK) and the results were expressed by plotting the time course of the ratio of fluorescence intensity emitted as the average light intensity in a small elliptical region inside each terminal. The data are represented as the normalized fluorescence ratio at 510 nm that increases when $[Ca_2]_i$ increases (Lev-Ram et al., 1992; Castro et al., 1995; Mateo et al., 1998). The basal ratio was measured for 60 s (equivalent to 12 cycles at the 2 emission wavelengths) before superfusion for 60 s with NMDA (100 μM) + glycine (10 μM). To test the impact of the pretreatment with different nAChR agonists and antagonists, nicotine (100 μM), 5IA85380 (10 nM) or DHβE (1 μM) were added 1 min before exposure to NMDA + glycine. A 30 s pulse of KCl (final concentration of 25 mM) was applied at the end of each experiment to confirm the preservation of the viability of the studied nerve terminals. All the tested compounds were prepared in normal HBM medium, or without Mg^{2+} to study the NMDA effect, and were added to the superfused nerve terminals using a rapid-pressurization system (AutoMate Scientific, Berkeley, CA, USA) in 95% O_2 and 5% CO_2.

2.4. Immunocytochemical assays

The immunocytochemical identification of presynaptic receptors in different types of nerve terminals was carried out essentially as described previously (Rodrigues et al., 2005a,b). Briefly, purified nerve terminals (500 μg of protein) were resuspended in 1 mL of phosphate-buffered saline (PBS containing, in mM: 137 NaCl, 2.6 KCl, 1.5 KH_2PO_4, 8.1 Na_2HPO_4, pH 7.4) and adsorbed onto poly-D-lysine-coated cover slips, although in some cases, the immunocytochemical detection was carried out after the analysis of calcium levels in individual nerve terminals described above and, in such cases, we used gridded cover slips for unambiguous identification of each individual nerve terminal. After fixation with 4% (w/v) paraformaldehyde for 15 min followed by washing twice with PBS, the nerve terminals were permeabilized in PBS with 0.2% Triton X-100 for 10 min, blocked for 1 h in PBS with 3% BSA and 5% normal horse serum and washed twice with PBS. Triplicate coverslips from each sample were incubated at 25 °C for 1 h and the following primary antibodies were diluted in PBS with 3% BSA and 5% normal horse serum: rabbit anti-synaptophysin (1:200), mouse anti-NR1 (1:500), rat anti-DAT (1:1000), rabbit anti-α4 nAChR (1:500). After three washes with PBS with 3% BSA and 3% normal horse serum, the nerve terminals were incubated for 1 h at room temperature with AlexaFluor-594 (red)-labeled goat anti-rat IgG secondary antibodies (1:200) together with Alexa Fluor-488 (green)-labeled donkey anti-rabbit and with Alexa Fluor-350 (blue)-labeled donkey anti-mouse IgG secondary antibodies (1:200). We confirmed that the secondary antibodies only yielded a signal in the presence of their targeted primary antibodies and that the individual signals obtained in double-labeled fields were not enhanced over the signals obtained under single-labeling conditions. After washing and mounting onto slides with Prolong Antifade, the preparations were visualized in a Zeiss Axiovert 200 inverted fluorescence microscope equipped with a cooled CCD camera and analyzed with AxioVision software (version 4.6). Each coverslip was analyzed by counting three different fields and in each field a minimum of 500 elements.

The quantitative estimation of co-localized proteins in immunocytochemical studies was performed by calculating the co-localization coefficients from the red and green two color-channel scatter plots (Manders et al., 1992). Co-localization coefficients express the fraction of co-localized molecular species in each component of a dual color image and are based on the Pearson's correlation coefficient, a standard procedure for matching one image with another in pattern recognition (Gonzalez and Wintz, 1987). If two molecular species are co-localized, the overlay of their spatial distributions has a correlation value higher than what would be expected by chance alone. Costes et al. (2004) developed an automated procedure to evaluate correlation between first and the second color channel with a significance level >95%, which also automatically determines an intensity threshold for each color channel based on a linear least-square fit of the two colors intensities in the image's 2D correlation cytofluorogram. This procedure was employed using macro routines integrated as plug-ins (WCIF Co-localization Plugins, Wright Cell Imaging