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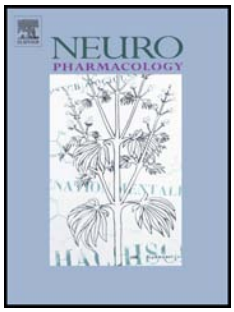

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Prolonged nicotine exposure down-regulates presynaptic NMDA receptors in dopaminergic terminals of the rat nucleus accumbens

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Running title: Nicotine-induced NMDA receptor trafficking

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*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.

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.

Key words: nicotinic receptors, NMDA receptors, calcium levels, neurotransmitter release, isolated nerve endings, nucleus accumbens.

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.

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 AM (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 sec (equivalent to 12 cycles at the 2 emission wavelengths) before superfusion for 60 sec 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 ºC 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 Facility, Toronto Western Research Institute, Canada) in the ImageJ 1.46b software (Wayne Rasband, NIH, MD, USA).

2.5 *Biotinylation and immunoblot assay*

The cell surface levels of the NR2B subunit were evaluated by performing surface biotinylation of synaptosomes from the NAc and subsequent immunoblot analysis (Jayanthi et al., 2004; Johnson et al., 2005). Briefly, crude NAc synaptosomes were prepared from 2 rats, re-suspended in HBM medium at 4°C, divided into 2 aliquots (500 μg protein each) and were incubated for 10 min at 37 °C with mild shacking; then one aliquot was treated for 10 min with 30 μM nicotine (T) while the other one was kept as control (C). The reaction was stopped with a cold (4 ºC) washing buffer composed by (mM): 150 NaCl, 1 EDTA, 0.2% BSA, 20 Tris, pH 8.6. The synaptosomes were then washed twice in ice-cold washing buffer and labeled with 2 mg/mL of sulfo-NHS-SS-biotin in PBS/Ca-Mg of the following composition (mM): 138 NaCl, 2.7 KCl, 1.8 KH_2PO_4, 10 Na_2HPO_4, 1.5 $MgCl_2$, 0.2 $CaCl_2$, pH 7.4 for 1 h at 4 °C. The biotinylation reaction was stopped by incubating the biotinylated

synaptosomes with 1 M NH_4Cl for 15 min at 4 °C, followed by two washes with ice cold PBS/Ca-Mg with 100 mM NH_4Cl to quench biotin. Biotinylated synaptosomes were then lysed in a RIPA buffer (500 µL) (10 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.1% SDS, 1% Triton X-100, 1% sodium deoxycholate, 1 mM orthovanadate, containing protease inhibitors). This lysate was centrifuged at 20,000 × g for 10 min at 4 °C, and samples (100 µg) were incubated with streptavidin magnetic beads (40 µL) for 1 h at room temperature under shaking. Streptavidin magnetic beads were added to the biotinylated synaptosomes (nicotine treated, T, and nicotine untreated, C, synaptosomes) to pull down the biotinylated proteins, as well as to the non-biotinylated synaptosomes to evaluate the specificity of streptavidin pull-down (B). After extensive washes, 1x SDS-PAGE buffer was added and samples were boiled for 5 min at 95 °C before loading on 10% SDS-PAGE gels and then transferred onto PVDF membranes. Non-specific binding sites were blocked with Tween containing Tris-buffered saline (t-TBS, with 20 mM Tris, 150 mM NaCl, and 0.1% Tween 20, containing 5% non-fat dried milk) for 1 h at room temperature and the protein of interest were detected using the primary antibodies, rabbit anti-NR2B (1:2,000) or mouse anti-actin (1:10,000). After washing, the membranes were incubated for 1 h at room temperature with the appropriate horseradish peroxidase-linked secondary antibody (1:20,000), and the immunoblots were visualized with an ECL (enhanced chemiluminescence) Plus Western blotting detection system. The immunoreactivity of NR2B subunits was monitored in the total synaptosomes (Syn), in control and nicotine pretreated biotinylated synaptosomes (respectively C and T) and in the streptavidin pull-down of the non-biotinylated synaptosomal lysate (B). GluA2 and β-actin bands from sub-fractionated samples as well as GluA2 bands from biotinylation experiments were quantified by using the ImageJ 1.46b software. The intensity of the protein bands was within linear range of the standard curves that were obtained by loading increasing amounts of samples.

2.6. Data analysis

Statistical comparison of the results was carried out using a Student's *t*-test for independent means (for single pairs comparison) while multiple comparisons were performed with one- or two-way ANOVA followed by Tukey-Kramer *post-hoc* test. Values are expressed as mean±S.E.M and were considered significant for $p < 0.05$.

2.7. Materials

[7,8-^{3}H]Dopamine (>70 Ci/mmol (2.59 TBq/mmol) was purchased from Perkin Elmer. Nicotine hydrogen tartarate salt, dihydro-β-erythroidine (DHβE), 4-aminopyridine (4AP), N-methyl-D-aspartate (NMDA), fatty-acid free BSA, ifenprodil, $ZnCl_2$, 6-nitroquipazine, desipramine and the protease inhibitor cocktail were from Sigma (Sigma-Aldrich, St Louis, MO, USA); 5-iodo-A-85380, varenicline, cotinine, MK801, CGS-19755, Ro 25-6981, (R)-CPP were from Tocris (Tocris Bioscience, Bristol, UK). FURA-2-AM, pluronic acid and F-127 were from Molecular Probes (Leiden, Netherlands). Sulfo-NHS-SS-biotin, Tris (2-carboxyethyl)phosphine hydrochloride (TCEP) and Streptavidin 14 Magnetic Beads were purchased from Pierce Thermo Scientific (Rockford, IL, USA). Western blotting detection system was from GE-Healthcare (Italy). Goat anti-rat IgG antibodies were obtained from Gene Tex (Irvine, CA, USA), rabbit anti-synaptophysin was from Synaptic System (Goettingen, Germany), rat anti-DAT antibody was from Novus Biologicals (Littleton, CO, USA), mouse anti-β-actin, horseradish peroxidase-conjugated anti-mouse and anti-rabbit secondary antibodies were obtained from Sigma and AlexaFluor-594 (red)-labeled goat anti-mouse IgG, Alexa Fluor-488 (green)-labeled donkey anti-rabbit, Alexa Fluor-350 (green)-labeled donkey anti-mouse and AlexaFluor-594 (red)-labeled goat anti-rat IgG secondary antibodies were from Invitrogen (Carlsbad, CA, USA).

3. Results

3.1. NMDARs are located in dopaminergic terminals of the NAc

In order to determine the existence and estimate the percentage of DA nerve endings equipped with NMDARs, synaptosomes were labeled with antibodies against synaptophysin, a generic marker of presynaptic terminals, DAT, a marker of DA nerve endings, and GluN1 subunits (Fig.1a). The histogram displayed in Fig.1b shows that the 40% of the synaptophysin-positive nerve terminals were also labeled with the anti-GluN1 subunit antibody and with anti-DAT antibody; furthermore, around 20% of dopaminergic nerve terminals were GluN1-immunopositive and 20% of GLuN1-immunopositive terminals were dopaminergic (Fig 1b), thus confirming the co-localization of GluN1 subunits and DAT proteins on a subset of NAc nerve terminals.

3.2. NMDARs trigger DA outflow from nerve terminals in a manner controlled by nAChR

We then tested the functional impact of NMDA on the release of [^{3}H]DA from NAc and their control by nAChRs. Fig 1c shows that nicotine (30 μM) and NMDA (100 μM) caused a marked overflow of [^{3}H]DA from pre-labeled NAc synaptosomes, which was quantitatively similar to that caused by 4AP (10 μM). In *vitro* pre-exposure of the synaptosomes for 10 minutes to nicotine (30 μM) caused a marked reduction of the nicotine-induced [^{3}H]DA overflow, suggesting that nicotine receptors undergo an agonist-induced receptor desensitization (Fig. 1c). Furthermore, the pre-treatment of NAc synaptosomes with nicotine (30 μM) also caused a significant reduction of the NMDA-evoked overflow of [^{3}H]DA, but it failed to modify the 4AP-induced [^{3}H]DA overflow (Fig. 1c); this reduction of NMDA-induced DA release was completely counteracted when the synaptosomes were pretreated with nicotine (30 μM) plus mecamylamine (20 μM) (Fig. 1c). It is worth noting that we already observed a significant inhibitory effect of the NMDA-evoked [^{3}H]DA overflow after pretreatment with nicotine at very low concentrations, similar to these (15 ng/mL) present in

the blood of smokers (Table 1, Hukkanen et al., 2005 and references therein).

This interaction between nAChRs and NMDARs was further confirmed by measuring the evoked elevation of intracellular calcium levels in individual NAc synaptosomes. As shown in Fig. 1d, both NMDA (100 μM) and nicotine (100 μM) triggered an enhancement of intracellular calcium in individual NAc synaptosomes; furthermore, the response to NMDA (100 μM) was curtailed by the previous exposure to nicotine (100 μM) (see also Fig. 1e).

3.3. Pharmacological characterization of nAChR and NMDAR in dopaminergic terminals

Fig 2a shows the stimulatory effects of several nAChR agonists on [^{3}H]DA release. The selective α4β2-containing nAChR agonist 5IA85380 (10 nM) produced a stimulatory effect similar to that produced by nicotine, whereas the partial agonist, varenicline (10 μM), also produced a stimulatory effect with an amplitude lower than that of nicotine. Cotinine, the major metabolite of nicotine, even at an high concentration (30 μM), was inefficacious to elicit [^{3}H]DA release. Choline, an α7-containing nAChR agonist when used at high concentration (1 mM), caused a modest stimulatory effect suggesting that, at this concentration, it may be partially effective also on other nAChRs (Fig. 2a).

We next used an immunocytochemical approach to directly confirm the presence of nAChR□4 subunits in NAc dopaminergic terminals. We found that near 40% of synaptosomes (synaptophysin-positive) were labeled with the anti-α4 nAChR subunit and with anti-DAT antibodies (Fig. 2b,c). The average data displayed in the histogram of Fig. 2c shows that 30% of the dopaminergic terminals (DAT-positive) were α4-immunopositive and near 30% of the α4-positive terminals were dopaminergic (Fig. 2c).

The pharmacological characterization of the NMDARs stimulating DA release from NAc synaptosomes is presented in Fig. 3. The NMDA (100 μM)-evoked DA release was almost completely antagonized in presence of MK801 (1 μM) and partially inhibited by CGS-19755 (10 μM). Notably, two GluN2B-selective antagonists, RO 25-6981 (1 μM) and

ifenprodil (1 µM), inhibited the NMDA (100 µM)-evoked DA release, whereas two GluN2A-preferring antagonists, CPP-19755 (1 µM) and $ZnCl_2$ (1 nM), were devoid of effects (Fig. 3).

3.4. Co-localization and pharmacological characterization of the nAChR controlling NMDAR in dopaminergic terminals

Figure 4a shows that only the pre-treatment with 5IA85380 (10 nM), but neither with varenicline (30 µM), cotinine (30 µM) or choline (1 mM), caused a significant reduction of the NMDA (100 µM)-evoked overflow of DA. This ability of the selective α4β2- containing nAChR agonist to decrease NMDAR responses was next confirmed at the single synaptosomal level by following Ca^{2+} levels in individual NAc synaptosomes. As shown in Fig. 4b,c, the NMDA (100 µM)-induced elevation of intracellular calcium levels were reduced by the previous exposure to 5IA85380 (10 nM), an effect which was blunted by the pre-exposure to 5IA85380 (10 nM) together with the selective α4 containing nAChR antagonist DHβE (1 µM) (Fig. 4d,e). All together, these data indicate that the nAChR-induce modification of the NMDA-induced DA release involves α4-containing nAChRs.

To additionally confirm this contention, we analyzed the calcium levels in NAc terminals and then subsequently tested by immunocytochemistry if they were GluN1- and α4-immunopositive. Indeed, as shown in Fig. 5, we could identify individual nerve terminals (terminal 1) that are dopaminergic (DAT-positive) and contain both GluN1 and α4 subunits (Fig. 5b, c), where the pre-treatment with 5IA85380 (10 nM) attenuated the NMDA (100 µM)-induced elevation of intracellular calcium levels (NMDA enhanced [Ca2+]i by 33±2%, n=3 and NMDA+5IA85380 by 17±1%, n=3; $p<0.01$) (Fig. 5a). In parallel, we also recorded other terminals (terminal 2) containing both GluN1 and α4 subunits but that were not dopaminergic (Fig. 5b), where 5IA85380 (10 nM) failed to modify the NMDA (100 µM)-induced elevation of intracellular calcium levels (NMDA enhanced [Ca2+]i by 33±2%, n=3

and NMDA+5IA85380 by 29±3%, n=3) (Fig. 5a). Finally, there were also nerve terminals responding only to NMDA (terminals 3) or to the □4 nAChR agonist (terminal 4) (Fig 5 a,b).

3.5. nAChR control GluN2B responses in dopaminergic terminals through a control of GluN2B endocytosis

To further confirm that nAChR pre-treatment selectively affected NR2B-mediated responses in NAc dopaminergic terminals, we tested if nAChR pretreatment still caused any effect in the NMDA-induced DA release from NAc synaptosomes in the presence of the selective NR2B antagonists RO 25-6981. As shown in Fig. 6a, the ability of nicotine (30 µM) pre-treatment to decrease the NMDA (100 µM)-induced DA release from NAc synaptosomes pre-exposed to nicotine (30 µM) was eliminated in the presence of RO 25-6981 (1 µM) (black bars of Fig. 6a), indicating that nAChRs only control the NR2B-mediated responses in NAc dopaminergic terminals.

In view of the our previous observations that the control of the response of ionotropic glutamate receptors in nerve terminals might involve a control of their trafficking in and out of the plasma membrane (Grilli et al., 2012), we posted that nicotine-induced inhibition of NMDA responses in NAc dopaminergic terminals could rely on the internalization of GluN2b receptor subunit. To test this hypothesis, we quantified the surface levels of GluN2B subunit proteins in the plasma membrane of NAc synaptosomes without and after nicotine pre-treatment. As shown in Fig. 6b and c, the pretreatment with nicotine (30 µM for 10 min) decreased the density of biotin-tagged GluN2B proteins (Fig. 6b, lane Nic) when compared to control (Fig. 6b, lane Ctr).

4. Discussion

The present results show that the pre-treatment with nicotine decreases the NMDA-induced dopamine release from NAc nerve terminals. This involves a functional interaction

likely within the same individual dopaminergic terminal between α4-containing nAChR and NR2B-containing NMDAR, through an ability of these nAChRs to induce the endocytosis of NR2B receptors.

Interactions between receptors localized on the same neuronal membrane represent an important mechanism of neurotransmitter control and several receptor systems seem to be involved in such a modulatory effect (Marchi and Grilli, 2010; Cong et al., 2011, Brown et al., 2012; Delille et al., 2013). The nicotinic receptors are among those receptors involved in these mechanisms. As previously shown, at the presynaptic level, nicotine may not only stimulate neurotransmitter release, but, through the interaction with other co-existing receptors, may exert also a functional modulatory role (reviewed in Vizi and Lendvai, 1999; Sher et al., 2004; Marchi and Grilli, 2010). Of particular interest are the interactions that have been shown to occur between nAChRs and glutamate receptors, in particular when considering the role of glutamate in several aspects of nicotine dependence (Wang et al., 2010; Gao et al., 2010) and also because both systems have been implicated in neuronal plasticity as well as in the processes of learning and memory (Timofeeva and Levin, 2011). As far as the nicotinic modulation of glutamate receptors is concerned, it has been previously demonstrated that the acute administration of nicotine *in vitro* positively modulates the function of both NMDA and glutamate metabotropic receptors co-existing on the same nerve endings (Risso et al., 2004b; Parodi et al., 2006). Here we show that a brief pretreatment of NAc synaptosomes with nicotine caused, on the contrary, a significant reduction of the NMDA-evoked release of [^{3}H]DA. This interaction was blunted in presence of mecamylamine indicating that activation of nAChRs represents the triggering event accounting for the loss of function of NMDARs. Moreover, since the nicotine pre-incubation failed to modify the 4AP-induced [^{3}H]DA overflow, changes in the exocytotic machinery of release do not account for nicotine-induced modifications of the NMDAR function.

In accordance with the control of DA release, we found that the pre-activation of nAChR also inhibited the ability of NMDARs to elevate the intracellular calcium content of individual nerve terminals identified as dopaminergic. In spite of the different time course of the changes in intracellular calcium content (1 min) and the changes of DA release (10 min), the parallel qualitative effects observed in both types of measurements is suggestive of an ability of nAChR to control NMDAR-mediated calcium entry to control the release of DA, under the assumption that the nAChR-mediated inhibition of NMDAR-mediated control of the intracellular calcium content may occur in a shorter time frame and persist to influence DA release because of the continuous presence of nAChR and NMDAR ligands in the medium. Importantly, as further re-enforced by the morphological evidence of co-localization of nAChR and NMDAR in the same dopaminergic terminal, the modulation of DA release under superfusion conditions unequivocally shows that the interaction between nAChR and NMDAR occurs in the same dopaminergic terminal. However, our results do not allow defining if there is a direct interaction between nAChR and NMDAR. Thus, the explanation for the decreased NMDAR-induced elevation of intracellular calcium levels after nicotine pre-treatment could be manifold. The NMDAR is proposed to exist as a heterotetramer composed of a GluN1 subunit responsible for ion flow together with modulatory GluN2 (GluN2a–GluN2d) and/or GluN3 (GluN3a–GluN3b) subunits (Köhr, 2006; Lau and Zukin, 2007; Traynelis et al., 2010), which dictate NMDAR-mediated functions. By using selective GluN2 antagonists, we here provide evidence that GluN2B subunits are critical members of presynaptic NMDARs located on NAc dopaminergic nerve endings. These GluN2B subunits can undergo phosphorylation and their stoichiometry plays an essential role in the regulation of NMDAR channel properties (Wang and Salter, 1994; Wang et al., 2006; Chen and Roche, 2007; Nakajima, 2012), leaving open the possibility that the altered NMDA responses might involve either a rearrangement of subunits stoichiometry or a modification of the

phosphorylation of NMDARs or alternatively a change in NMDAR trafficking, typified by a reduction of the number of NMDARs present at the plasma membrane level of DA nerve endings. The latter hypothesis is clearly favored since biotinylation studies unveiled a reduced density of GluN2B subunits in NAc synaptosomal plasma membranes following nicotine pre-treatment. Furthermore, our previous observation that the acute co-administration of nicotine and NMDA actually enhances NMDAR function (Risso et al., 2004b) is converted into a negative interaction when the exposure of nicotine is prolonged, is also suggestive of a nAChR-induced control of the trafficking (endocytosis) of NR2B-containing NMDAR after pre-treatment with nicotine.

An additional goal of this study was the pharmacological characterization of the nAChRs responsible for the control of NMDAR-mediated responses in DA terminals of the NAc. It is well known that different nAChR subtypes including αconotoxin MII-sensitive (α6β2β3, α4α6β2β3, α6β2, and α4α6β2) and αconotoxin MII-insensitive (α4β2 and α4α5β2) subtypes mediate nicotine-evoked DA release from striatal synaptosomes (Salminen et al., 2004; Gotti et al., 2007). Since the inhibitory effect of nicotine pre-treatment was partially counteracted by α-conotoxin MII, it is likely that nAChRs containing the α6 subunit are involved since this subunit is abundant in the NAc (Dwoskin et al., 2008; Papke et al., 2008; Exley et al., 2008a,b). Importantly, our data using the selective nAChR agonist 5IA85380 indicate that the functional decrease of the NMDAR function is a consequence of the prolonged activation of an α4β2-subtype of nAChRs. This suggests that different nAChRs might fulfill different roles in NAc DA terminals, with α6-containing nAChRs mostly dedicated to directly promoting DA release, whereas α4-containing nAChRs mostly fine-tune other incoming signals such as glutamatergic NMDAR-mediated responses. This selective association of α4β2-containing nAChRs with NMDAR also suggests that these nAChRs might be located extra-synaptically (Lendvai and Vizi, 2008), since NR2B-containing NMDARs are mainly located extra-

synaptically (Vizi et al., 2013). Additionally, it is worth noting that α7*-nAChRs do not seem to play a role in NAc DA terminals, as heralded by the absence of effects of choline, and in agreement with the lack of effect of α7-containing nAChR ligands found by others (Salminen et al., 2004, Gotti et al., 2007). This contrasts with glutamatergic synapses, where it was found that α7*-nAChRs may enhance the presynaptic surface expression of NMDAR leading to an increased glutamate release during early synaptic development (Lin et al., 2010). However, the NMDARs modulating glutamate release are different from those modulating DA release (Pittaluga et al., 2001). This suggests that the presynaptic interaction between nAChR and NMDAR can be differently organized in different types of nerve terminals, possibly depending on the different nAChR and NMDAR subtypes involved.

The present findings of a nicotine-induced control of NMDAR responses in NAc DA terminals may be of relevance for the adaptive changes taking place in the brain of smokers. We previously reported that nicotine, at a concentration achieved by smokers or after nicotine replacement therapy, is able to stimulate DA release *in vivo* (Millar and Gotti, 2009). We now found a decrease of NMDAR function upon pre-treatment with a low concentration of nicotine compatible with that achieved by smokers (Fant et al., 1999; Henningfield et al., 2003; Table 1). It is therefore reasonable to hypothesize that nicotine could cause *in vivo* the kind of modulatory changes of NMDAR function reported in this paper. It can be speculated that the nicotine-induced neuro-adaptation of NMDARs present on NAc DA terminals could represent a drug-induced impairment in the glutamatergic control of DA release, which may play a role in the mechanism of nicotine addiction. In this respect, the finding that varenicline pre-treatment was unable to produce any effect on NMDAR function is of interest since this drug has been recently introduced for smoking cessation.

The present results may also be relevant to understand the interplay between nAChR and NMDAR in neurodegenerative diseases. Thus, the activation of NR2B-containing NMDARs,

at either synaptic or extrasynaptic sites, leads to excitotoxicity, whereas activation of NR2A-containing NMDARs promotes neuronal survival and is neuroprotective (Liu et al., 2007). In contrast, nicotine is suggested to afford neuroprotection, a concept that emerged from several studies documenting an inverse association between cigarette smoking and Parkinson's disease (Ritz et al., 2007). Nicotine-associated neuroprotection may be linked to the control of NMDARs since the stimulation of α7-containing nAChRs decreased glutamate neurotoxicity through a down regulation of NMDARs (Shen et al., 2010) and donepezil, an cholinesterase inhibitor, exerts neuroprotective effects via the nicotinic receptor-mediated cascade (Takada et al., 2003) possibly by reducing surface NMDARs and consequently glutamate mediated calcium entry (Shen et al., 2010). This is notable agreement with our finding that nicotine pre-treatment decreased the function of the NMDARs by reducing the NR2B-containing NMDARs.

In conclusion, the present results demonstrate, with combined functional and immunocytochemical evidence, the co-localization and functional interaction of nAChRs and NMDARs in NAc dopaminergic terminals. Our results also show that the NMDAR function can be dynamically negatively regulated in neurons in response to a brief activation of nAChRs present on the same nerve endings. This dynamic control of NMDA and also of AMPA receptors (Grilli et al., 2012) operated by the cholinergic nicotinic system at the level of nerve ending may therefore represent an important neuronal adaptation associated with nicotine reward and reinforcement.

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Legend to the figures

Fig. 1. (a) Immunocytochemical co-localization of synaptophysin (green), dopamine transporters (DAT, red) and GluN1 receptors (blue) in nerve terminals from the rat nucleus accumbens, as gauged by the merged image (white). (b) Histograms represent this averaged co-localization, with data expressed as mean ± S.E.M. of at least 3 animals**.** (c) Effect of nicotine pre-treatment on the release of [^{3}H]DA evoked by nicotine, NMDA and 4-aminopyridine (4AP) from rat NAc synaptosomes. White bar: control synaptosomes; black bar: NIC pre-treated synaptosomes; dashed bar: synaptosomes pretreated with 20 μM mecamylamine together with nicotine 10 min before adding NMDA. Data are means ± S.E.M. of six experiments run in triplicate. ***$p < 0.001$ vs. non-treated synaptosomes challenged with nicotine, $^{\$\$\$}p < 0.001$ vs. non-treated synaptosomes challenged with NMDA, $^{\#\#}p < 0.01$ vs. nicotine pre-treated synaptosomes challenged with NMDA, all using a one-way ANOVA followed by Tukey-Kramer *post-hoc* test (d) Time course of FURA-2 fluorescence emission in an individual nerve terminal from the rat NAc that was challenged twice with NMDA (100 μM), before and 60 seconds after pre-treatment with nicotine (100 μM). (e) Average attenuation of the elevation of intracellular calcium levels evoked by NMDA (100 μM) after a pre-treatment with 100 μM nicotine (white bar: control; black bar: pre-treatment with nicotine). Data are expressed as mean ± S.E.M. of at least 3 animals**.** *$p<0.05$ vs control synaptosomes using a paired Student's *t* test.

Fig. 2. (a) Effect of different nAChR agonists on the release of [^{3}H]DA from nerve terminals of the rat nucleus accumbens. Data are means ± S.E.M. of five experiments run in triplicate. **$p < 0.01$, ***$p < 0.001$, vs. nicotine (30 μM) using a one-way ANOVA followed by Tukey-Kramer *post-hoc* test. (b) Immunocytochemical co-localization of synaptophysin (blue), dopamine transporters (DAT, red) and α4 subunit of nAChRs (green) in nerve terminals from the rat nucleus accumbens, as gauged by the merged image

(white). (c) Histograms represent this averaged co-localization, with data expressed as mean ± S.E.M. of at least 3 animals**.**

Fig. 3. Effect of different NMDA receptor antagonists on the NMDA (100 μM)-evoked [^{3}H]DA release from rat NAc synaptosomes. Data are means ± S.E.M. of five experiments run in triplicate. $^{**}p < 0.01$, $^{***}p < 0.001$ vs. 100 μM NMDA without any other drug using a one-way ANOVA followed by Tukey-Kramer *post-hoc* test.

Fig. 4. (a) Impact of the pre-treatment with different nAChR agonists on the ability of NMDA (100 μM) to trigger [^{3}H]DA from nerve terminals of the rat nucleus accumbens. Each nAChR was added 10 minutes before challenging with NMDA. Data are means ± S.E.M. of five experiments run in triplicate. $^{***}p < 0.001$ vs. control. (b) Time course of FURA-2 fluorescence emission in an individual nerve terminal from the rat NAc that was challenged twice with NMDA (100 μM), before and 60 seconds after pre-treatment with 5IA85380 (10 nM). (c) Average attenuation of the elevation of intracellular calcium levels evoked by NMDA (100 μM) after a pre-treatment with 10 nM 5IA85380 (white bar: control; black bar: pre-treatment with 5IA85380). Data are expressed as mean ± S.E.M. of at least 3 animals**.** $^{**}p<0.01$ vs control synaptosomes using a paired Student's *t* test. (d) Time course of FURA-2 fluorescence emission in an individual nerve terminal from the rat NAc that was challenged twice with NMDA (100 μM), before and 60 seconds after pre-treatment with 5IA85380 (10 nM) together with DHβE (1 μM). (e) Average attenuation of the elevation of intracellular calcium levels evoked by NMDA (100 μM) after a pre-treatment with 10 nM 5IA85380 together with 1 μM DHβE (white bar: control; dotted bar: pre-treatment with 5IA85380 and DHβE). Data are expressed as mean ± S.E.M. of at least 3 animals**.**

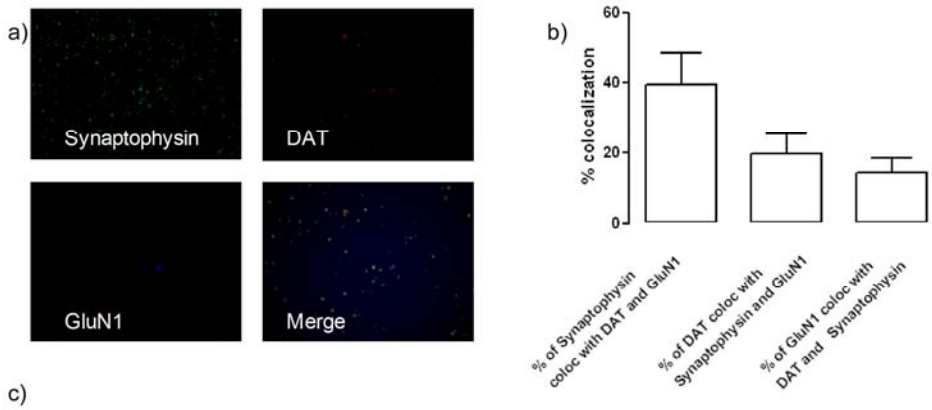
Fig. 5. Different modification upon pretreatment with 10 nM 5IA85380 of ability of NMDA

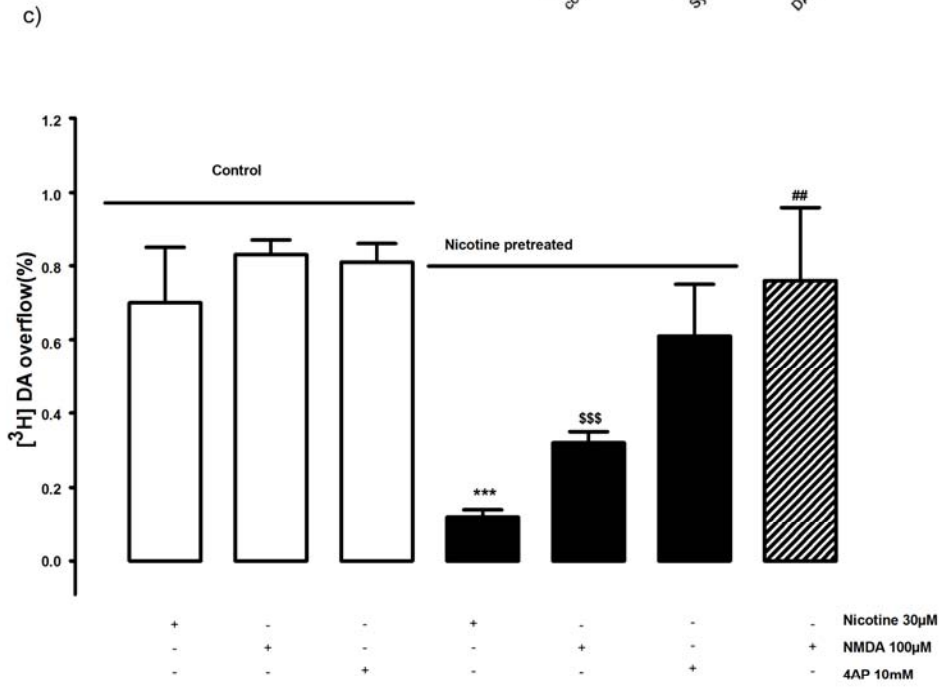
(100 μM) to trigger an elevation of intracellular calcium levels in individual terminals from the rat NAc. (a) Time course of FURA-2 fluorescence emission in each individual nerve terminal (terminal 1 – terminal 4) from the rat NAc that was challenged twice with NMDA (100 μM), before and 60 seconds after pre-treatment with 5IA85380 (10 nM). (b) Fluorescence image of FURA-2-labelled synaptosomes. (c) Co-localization of α4*nAChRs, DAT and NR1 in terminal 1.

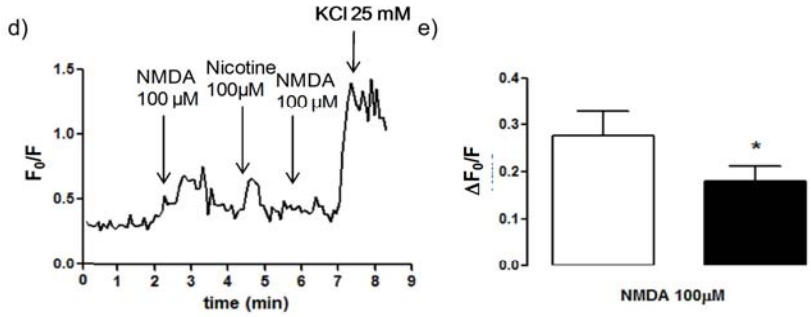
Fig. 6. (a) Effects of the selective NR2B receptor subunit antagonist Ro 25-6981 on the NMDA(100 μM)-evoked [^{3}H]DA release from control (white bars) and nicotine (30 μM)-pre-treated synaptosomes from the rat NAc (black bars). Data are means ± S.E.M. of six experiments run in triplicate. ***$p < 0.001$ vs. control effect of NMDA using a one-way ANOVA followed by Tukey-Kramer *post-hoc* test. (b) Representative Western blot of NR2B subunit surface density in NAc terminals. The Western blot compares total synaptosomal membranes before adding biotin (Syn Tot), synaptosomal membranes that were not treated with biotin and were subject to a streptavidin pull-down (B), synaptosomal membranes incubated with biotin and subject to a streptavidin pull-down (Control) and nicotine pre-treated synaptosomal membranes incubated with biotin and subject to a streptavidin pull-down (Nicotine). The blots are representative of four different experiments carried out with synaptosomal preparations from different rats. (c) The histogram shows the mean ± SEM of surface NR2B intensity. n = 4 for each condition (white bar, control; black bar, nicotine (30 μM)- pre-treated synaptosomes. **$p < 0.01$ using a paired Student's *t* test vs surface NR2B intensity in control.

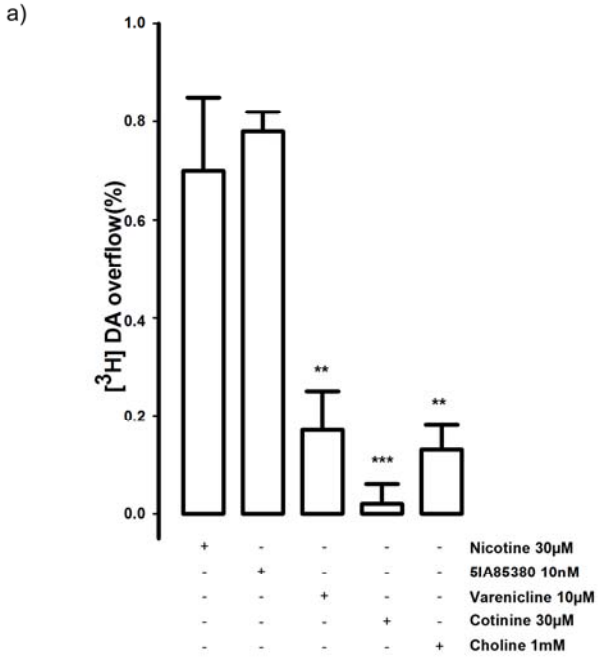
	Pretreatment	Overflow (%)	% of Inhibition
NMDA 100μM+ Gly 1μM		0.83 ± 0.04	
NMDA 100μM+ Gly 1μM	Nicotine 100μM	0.39 ± 0.04**	53 ± 0.04
NMDA 100μM+ Gly 1μM	Nicotine 30μM	0.33 ± 0.04***	60 ± 0.04
NMDA 100μM+ Gly 1μM	Nicotine 0.1μM	0.41 ± 0.04*	51 ± 0.04
NMDA 100μM+ Gly 1μM	Nicotine 0.001μM	0.57 ± 0.14	31 ± 0.14

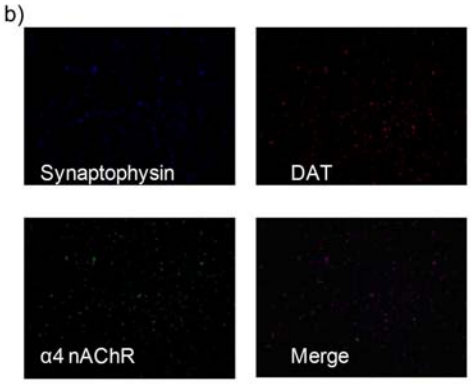
Effect of pretreatment with different concentrations of nicotine on NMDA-evoked [^{3}H]DA release. Results are expressed as induced overflow. Data are means ± S.E.M. of six experiments run in triplicate. .*p < 0.05 ,**p < 0.01, ***p < 0.001, vs. 100 μM NMDA-evoked [^{3}H]DA release. One-Way ANOVA followed by Tukey-Kramer *post-hoc* test.

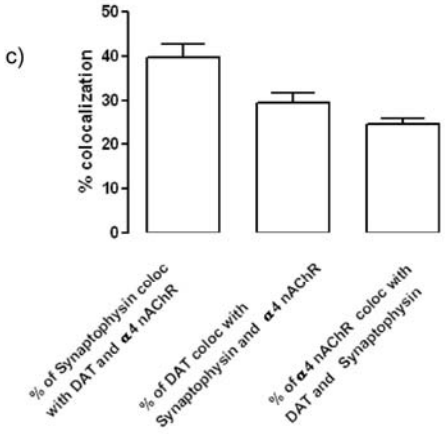

a)
Synaptophysin
DAT
GluN1
Merge
b)
% colocallization
60
40
20
0
% of Synaptophysin coloc with DAT and GluN1
% of DAT coloc with Synaptophysin and GluN1
% of GluN1 coloc with DAT and Synaptophysin

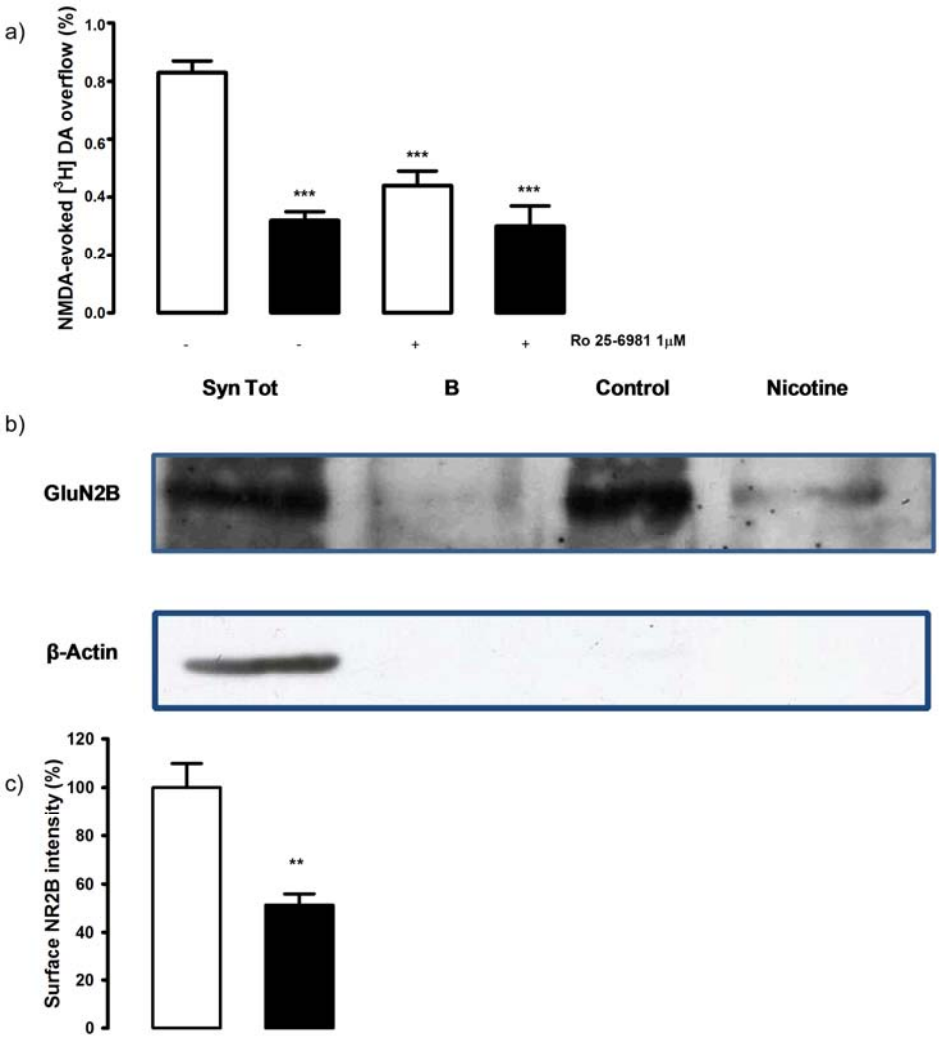

c)
[3H] DA overflow(%)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Control
Nicotine pretreated
##
$$$
***
Nicotine 30μM
NMDA 100μM
4AP 10mM

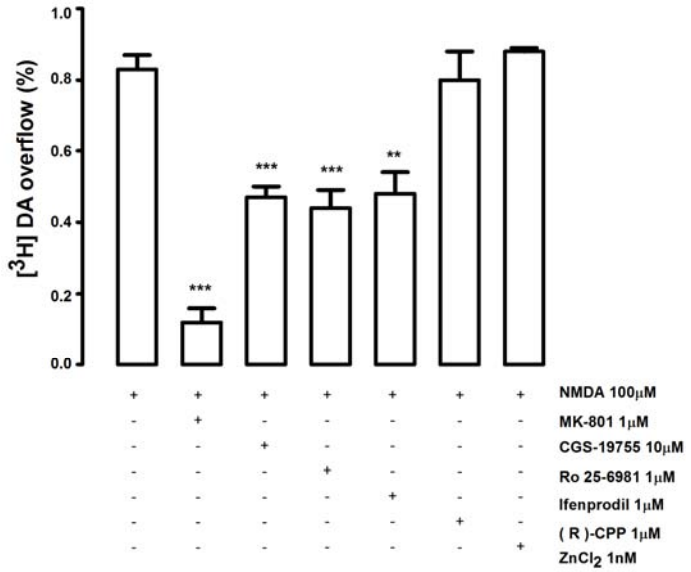

d)
KCl 25 mM
NMDA 100 μM
Nicotine 100μM
NMDA 100 μM
F0/F
time (min)
e)
ΔF0/F
*
NMDA 100μM

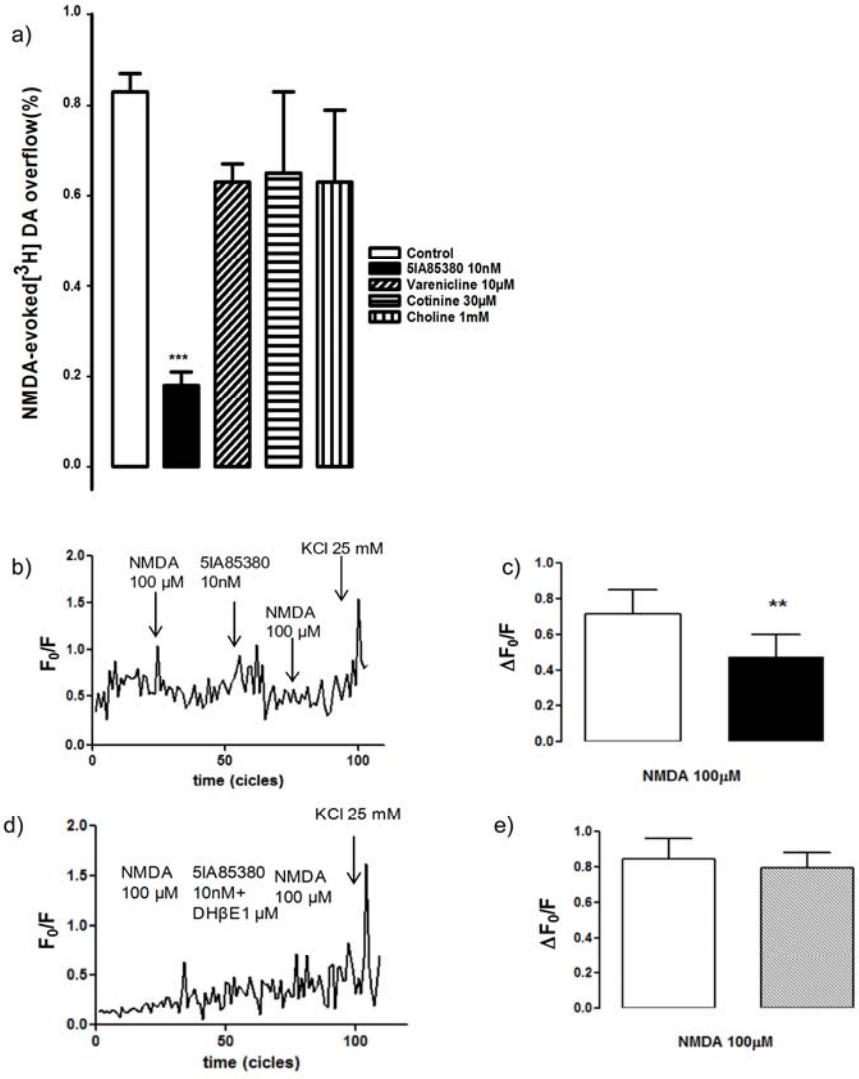

a)
1.0
0.8
0.6
0.4
0.2
0.0
[3H] DA overflow(%)
**
***
**
+ - - - - Nicotine 30μM
- + - - - 5IA85380 10nM
- - + - - Varenicline 10μM
- - - + - Cotinine 30μM
- - - - + Choline 1mM

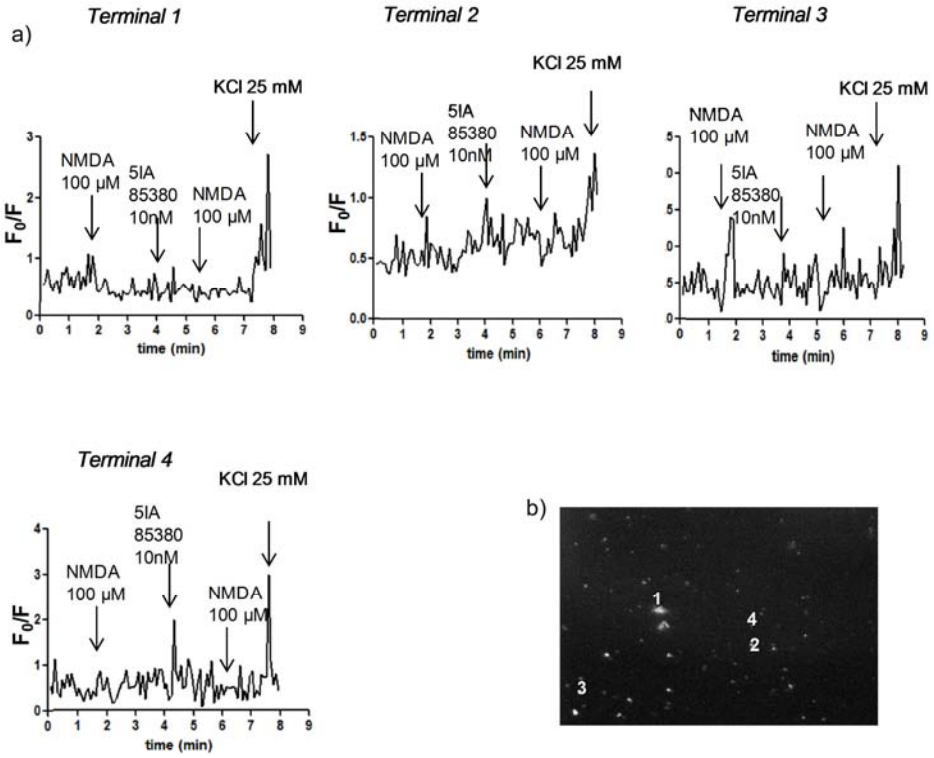

b)
Synaptophysin
DAT
α4 nAChR
Merge


c)
50
40
30
20
10
0
% colocalization
% of Synaptophysin coloc with DAT and α4 nAChR
% of DAT coloc with Synaptophysin and α4 nAChR
% of α4 nAChR coloc with DAT and Synaptophysin






a)
NMDA-evoked[3H] DA overflow(%)
1.0
0.8
0.6
0.4
0.2
0.0
***
Control
5IA85380 10nM
Varenicline 10μM
Cotinine 30μM
Choline 1mM
b)
F0/F
NMDA 100 μM
5IA85380 10nM
NMDA 100 μM
KCl 25 mM
time (cicles)
c)
ΔF0/F
**
NMDA 100μM
d)
F0/F
NMDA 100 μM
5IA85380 10nM+ DHβE1 μM
NMDA 100 μM
KCl 25 mM
time (cicles)
e)
ΔF0/F
NMDA 100μM


a)
Terminal 1
Terminal 2
Terminal 3
Terminal 4
KCl 25 mM
NMDA 100 µM
5IA 85380 10nM
F0/F
time (min)
b)
1
2
3
4
c)
α4 nAChR
DAT
GluN1
Merge
1

- NMDAR coexist and functionally interact with α4*nAChR on DA terminals
- Nicotine pre-treatment reduced the NMDA-evoked DA overflow from NAc nerve terminals
- Nicotine pre-treatment reduced the NMDA -evoked calcium transients in single nerve terminals
- Nicotine pretreatment decreased the density of biotin-tagged GluN2B proteins in NAc.