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DOPAMINE D₂ AND ACETYLCHOLINE α 7 NICOTINIC RECEPTORS HAVE SUBCELLULAR DISTRIBUTIONS FAVORING MEDIATION OF CONVERGENT SIGNALING IN THE MOUSE VENTRAL TEGMENTAL AREA

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Abstract

Alpha7 nicotinic acetylcholine receptors (α 7nAChRs) mediate nicotine-induced burst-firing of dopamine neurons in the ventral tegmental area (VTA), a limbic brain region critically involved in reward and in dopamine D₂ receptor (D₂R)-related cortical dysfunctions associated with psychosis. The known presence of α 7nAChRs and Gi-coupled D₂Rs in dopamine neurons of the VTA suggests that these receptors are targeted to at least some of the same neurons in this brain region. To test this hypothesis, we used electron microscopic immunolabeling of antisera against peptide sequences of α 7nACh and D₂ receptors in the mouse VTA. Dual D₂R and α 7nAChR labeling was seen in many of the same somata (co-localization over 97%) and dendrites (co-localization over 49%), where immunoreactivity for each of the receptors was localized to endomembranes as well as to non-synaptic or synaptic plasma membranes often near excitatory-type synapses. In comparison with somata and dendrites, many more small axons and axon terminals were separately labeled for each of the receptors. Thus, single-labeled axon terminals were predominant for both α 7nAChR (57.9%) and D₂R (89.0%). The majority of the immunolabeled axonal profiles contained D₂R-immunoreactivity (81.6%) and formed either symmetric or asymmetric synapses consistent with involvement in the release of both inhibitory and excitatory transmitters. Of 160 D₂R-labeled terminals, 81.2% were presynaptic to dendrites

that expressed $\alpha 7$ nAChR alone or together with the D₂R. Numerous glial processes inclusive of those enveloping either excitatory- or inhibitory-type synapses also contained single labeling for D₂R ($n = 152$) and $\alpha 7$ nAChR ($n = 561$). These results suggest that classic antipsychotic drugs, all of which block the D₂R, may facilitate $\alpha 7$ nAChR-mediated burst-firing by elimination of D₂R-dependent inhibition in neurons expressing both receptors as well as by indirect pre-synaptic and glial mechanisms.

Keywords

electron microscopic immunolabeling; mesocorticolimbic; reward; schizophrenia; addiction

INTRODUCTION

Mesocorticolimbic dopaminergic neurons within the midbrain ventral tegmental area (VTA) play a key role in the reinforcing actions of natural rewards and many drugs of abuse, including nicotine, the main addictive component of tobacco (Corrigall et al., 1992; Di Chiara, 2000; Balfour, 2009; Mark et al., 2011). Nicotine acts through nicotinic acetylcholine receptors (nAChRs), which are ligand-gated cationic channels consisting of various combinations of alpha (α) and beta (β) subunits, whose mRNAs are widely distributed throughout the VTA and other brain regions (for review see Azam et al., 2002; Collins et al., 2009). nAChR homomers comprising exclusively of the $\alpha 7$ subunit exist as functional $\alpha 7$ nicotinic acetylcholine receptors ($\alpha 7$ nAChRs) that selectively bind the nicotinic antagonist α -bungarotoxin (Orr-Urtreger et al., 1997; Klink et al., 2001; Collins et al., 2009). Binding of α -bungarotoxin is seen in both dopaminergic and non-dopaminergic neurons as well as in many glutamatergic and non-glutamatergic axon terminals in the VTA (Jones and Wonnacott, 2004). In this region of the rodent brain, the mRNA transcript and protein for $\alpha 7$ nAChRs are also expressed in many dopaminergic neurons (Seguela et al., 1993; Dominguez del Toro et al., 1994; Charpantier et al., 1998; Azam et al., 2002; Wooltorton et al., 2003). Electrophysiological and pharmacological studies provide further evidence that $\alpha 7$ nAChRs are present and functional in dopamine neurons of rat midbrain (Pidoplichko et al., 1997; Wu et al., 2004; Yang et al., 2009).

Activation of $\alpha 7$ nAChRs augments burst firing of dopaminergic neurons in the VTA (Schilström et al., 2003), an effect that is opposed by dopamine D₂ receptor (D₂R)-mediated auto-inhibition (Mercuri et al., 1997; Li et al., 2012). The D₂Rs belong to a family of Gi-coupled receptors that similarly to $\alpha 7$ nAChR express their mRNAs in many dopamine as well as in non-dopamine neurons in the VTA of rat and other species including human (Meador-Woodruff et al., 1991; Hurd et al., 1994; Haber et al., 1995). Furthermore, electron microscopy has established the presence of D₂R immunolabeling in dopamine neurons and in their presynaptic terminals in the rat VTA (Sesack et al., 1994; Pickel et al., 2002). Mesolimbic dopamine neurons in the VTA that project to the nucleus accumbens (NAc) express high levels of D₂Rs (Nimitvilai and Brodie, 2010), and are thought to be largely responsible for the reduction in nicotine reward produced by local microinjection of D₂R antagonists in the VTA (Sziraki et al., 2002). Intra-VTA administration of the $\alpha 7$ nAChR-selective nicotinic antagonist, methyllycaconitine (MLA), also attenuates the conditioned

rewarding effects of nicotine and, even more strikingly, reverses the motivational valence of nicotine from rewarding to aversive (Laviolette and van der Kooy, 2003). In addition, the blockade of $\alpha 7$ nAChRs markedly reduces the intracranial self-stimulation threshold-reducing action of nicotine (Panagis et al., 2000), as well as intravenous nicotine self-administration (Markou and Paterson, 2001). Taken together, these observations indicate that $\alpha 7$ nAChRs in the VTA are critically involved in the underlying mechanisms of nicotine addiction, which may be ascribed in part to direct or indirect activation of the mesolimbic dopaminergic neurons under the influence of D_2 R autoreceptors in this brain region.

In contrast with the well-characterized efficacy of classic antipsychotic drugs, all of which block the D_2 R (Levin and Rezvani 2007), there is controversy as to whether activation of $\alpha 7$ nAChR through cigarette smoking is effective in alleviating either positive or negative symptoms of schizophrenia (Olincy and Freedman, 2012; Wylie et al., 2012; Zhang et al., 2012). A benefit of nicotine as self-medication is supported by the comparatively high incidence of smoking in schizophrenic patients and the greater nicotine dependence in schizotypic smokers, who endorse greater positive and fewer negative consequences of smoking (Stewart et al., 2013). Moreover, the *CHRNA7* gene has been associated with schizophrenia (Freedman et al. 1997; Olincy et al. 2006; Severance and Yolken 2008). These observations, together with the known presence of the D_2 and $\alpha 7$ nACh receptors in mesocorticolimbic dopamine neurons suggest that these receptors are strategically positioned for involvement in dual control of the output of single neurons in the VTA. We tested this hypothesis using dual-labeling immunoelectron microscopy. The results provide new evidence that in the mouse VTA, $\alpha 7$ nAChR and D_2 R are frequently coexpressed in somata and dendrites, but they are also targeted to separate axon terminals with the D_2 R terminals being most abundant and most often presynaptic to dendrites containing $\alpha 7$ nAChR. These associations, together with the detection of both receptors in perisynaptic glia suggest multiple mechanisms for functional associations between the $\alpha 7$ nACh and D_2 R systems in the VTA.

EXPERIMENTAL PROCEDURES

Animals

The experimental procedures were carried out in accordance with the National Institutes of Health *Guidelines for the Care and Use of Laboratory Animals*, and approved by the Institutional Animal Care and Use Committees (IACUC) at the Weill Cornell Medical College. The studies were conducted in adult male C57BL/6J mice (20–25 g; Charles River, Kingston, NY, USA). All efforts were made to minimize the number of animals used and their suffering. The mice were anesthetized by an intraperitoneal (i.p.) injection of sodium pentobarbital (150 mg/kg) prior to fixation of their brain tissue by vascular perfusion with an aldehyde solution.

Antisera

A mouse monoclonal antibody against the $\alpha 7$ nAChR (clone mAb306; Sigma, St. Louis, MO, USA) was generated using affinity-purified native chicken and rat α -bungarotoxin-binding proteins (Schoepfer et al., 1990). Epitope mapping has shown that mAb306

recognizes the sequence corresponding to the cytoplasmic loop residues 380–400 of the $\alpha 7$ nAChR from rat and chicken (Schoepfer et al., 1990; McLane et al., 1992). This antibody was used previously to study the $\alpha 7$ nAChR distribution, in (1) rat and mouse brain using bright field (Dominguez del Toro et al., 1994; Duffy et al., 2009) or electron microscopy (Fabian-Fine et al., 2001; Levy and Aoki, 2002; Duffy et al., 2009) and (2) macaque brain using fluorescent microscopy (Centeno et al., 2006). The $\alpha 7$ nAChR antibody has been examined using Western blotting of cultured hippocampal neurons, mouse, rat and macaque brain extracts (Dominguez del Toro et al., 1994; Centeno et al., 2006; Arnaiz-Cot et al., 2008; Duffy et al., 2009).

The D₂R antiserum was generated in rabbit using a GST fusion protein containing amino acids 216–311 of the human D₂R long isoform (Brana et al., 1997), which was cloned into the pET30c plasmid (EMD Millipore, Billerica, MA, USA) and confirmed by sequencing. The antiserum was affinity-purified first by exclusion on a GST column, and then by retention on a GST-fusion protein (i.e., a GST fusion protein containing the sequence used for immunization) column. This antiserum was shown to be specific by positive immunolabeling in human embryonic kidney cells transiently transfected with the pcDNA-FLAG-D2L plasmid (Kearn et al., 2005). In Western blot analysis, a single band at 50 kDa was recognized by the D₂R antiserum, and preadsorption with antigen eliminated the D₂R immunoreactive band as well as the immunolabeling seen in sections of the NAc, a region highly enriched in D₂Rs (Pickel et al., 2006). In the present study, we also demonstrate an absence of D₂R immunoreactivity in dorsal striatum (Fig. 1) of brain tissue from conditional knock-out MSN-D₂ KO mice (*Drd2^{loxP/loxP}; Adora2a-Cre^{+/-}*), which was generously provided by Dr. Veronica Alvarez at the National Institute on Alcohol Abuse and Alcoholism. These mutant mice which lack D₂R in medium spiny neurons of the striatum were generated by crossing *Drd2^{loxP/loxP}* mice with *Adora2a-Cre^{+/-}* mice (B6.FVB(Cg)-Tg(*Adora2a-cre*) KG139Gsat/Mmucd) that express Cre recombinase in medium spiny neurons of the indirect pathway. *Drd2^{loxP/loxP}* mice (*Drd2^{tm1Mrub}*, also known as *Drd2^{loxP/loxP}*) carry two targeted loxP sites flanking *Drd2* exon 2 and were generated in the laboratory of Dr. Marcelo Rubinstein at INGEBI Bello et al., 2011). MSN-D₂ KO mice show a pronounced reduction of *Drd2* mRNA and D₂R binding in the striatum (Kaplan et al., 2012) and thus, the epitope used for generation of the D₂R antibody in the present study, amino acids 216–311 of the human D₂ long isoform, is expected to be missing in the striatum, but not the VTA as confirmed in Fig. 1.

Monoclonal Anti-S-100 (β -subunit) from (Sigma–Aldrich, St. Louis, MO) was derived from the SH-B1 hybridoma produced by the fusion of mouse myeloma cells and splenocytes from mice immunized with purified bovine brain S-100b preparation. This antibody recognizes an epitope located on the β -chain (i.e., in S-100a and S-100b), but not on the α -chain of S-100 (i.e., in S-100a and S-100ao). In enzyme-linked immunosorbent assay (ELISA), recognition of S-100 β -subunit is independent of Ca⁺⁺ ion. It is also reactive in dot blot using denatured-reduced preparations, and in immunohistochemical staining. Cross-reactivity has been observed with S-100 from human, bovine, porcine, rabbit, cat and rat. The product does not react with other members of the EF-hand family such as calmodulin, and

parvalbumin (Baudier et al., 1986), and has previously been shown to be specific by prior adsorption with S-100 protein (Castagna et al., 2003).

Fixation and tissue preparation

The brain tissue was preserved for immunolabeling by vascular perfusion of aldehyde fixatives through the left ventricle of the heart. This was achieved using a Masterflex pump (Cole Parmer, Vernon Hills, IL, USA) to sequentially deliver solutions 5–10 ml of normal saline (0.9%) containing 1000 units/ml of heparin followed by approximately 150 ml of 4% paraformaldehyde for light microscopy or 30 ml of 0.1 M phosphate buffer (PB; pH 7.4) containing 3.75% acrolein and 2% paraformaldehyde, and 150 ml of 2% paraformaldehyde in PB. The brains were removed from the cranium, dissected and postfixed for 30 min in 2% paraformaldehyde. Coronal sections were collected through the midbrain region including the VTA at -3.4 mm from bregma (Paxinos and Franklin, 2001). These were cut at $40\text{-}\mu\text{m}$ thickness into 0.1 M PB at 4°C on a Leica Vibratome (Leica Microsystems, Bannockburn, IL, USA), incubated for 30 min in a solution of 1% sodium borohydride in 0.1 M PB to remove excess of active aldehydes, and rinsed in 0.1 M PB until bubbles disappeared. After extensive rinsing in 0.1 M Tris-buffered saline (TS), the sections were incubated for 30 min in 0.5% bovine serum albumin (BSA) in 0.1 M TS to minimize nonspecific staining, and then processed for dual-immunocytochemical labeling.

Immunolabeling

For immunocytochemical localization of $\alpha 7\text{nACh}$ and D_2R , sections prepared as described above were processed for single immunoperoxidase or dual immunoperoxidase and immunogold–silver labeling (Chan et al., 1990). The primary antiserum against D_2R was raised in rabbit and both the $\alpha 7\text{nAChR}$ and S100 antibodies were raised in mouse and hence could be distinguished by appropriate species-specific secondary antibodies. Peroxidase and gold markers were switched in alternate sections to maximize the detection of either antigen with methods differing in resolution and sensitivity (Leranth and Pickel, 1989). The sections were incubated for 4 days at 4°C in a solution of 0.1% BSA in TS-containing mouse monoclonal antibodies against $\alpha 7\text{nAChR}$ (diluted 1:1000 for immunoperoxidase and 1:250 for immunogold) or S100 (1:500 for immunoperoxidase and 1:100 for immunogold). The rabbit polyclonal antiserum for D_2R (diluted 1:800 for immunoperoxidase and 1:200 for immunogold) was added and the incubation continued for 24 more hours at 4°C . After incubation in these primary antisera, sections were first processed for immunoperoxidase and afterward for immunogold labeling. All the incubations were carried out at room temperature with continuous agitation on a rotator and were followed by several rinses in 0.1 M TS and 0.1 M PB.

For the immunoperoxidase visualization of antigens, the avidin–biotin complex (ABC) method (Hsu et al., 1981) was used. For this, the incubation in primary antisera was followed by incubation in secondary biotinylated antibody (horse anti-mouse IgG for $\alpha 7\text{nAChR}$, Vector Laboratories, Burlingame, CA, US or goat anti-rabbit IgG for D_2R , Jackson, West Grove, PA, USA 1:200) for 30 min and then in ABC (1:100, Vectastain Elite Kit, Vector Lab.) for another 30 min. The immunoreactivity bound to the tissue was

visualized by a 6-min incubation in 0.022% 3,3'-diaminobenzidine and 0.003% hydrogen peroxide in 0.1 M TS.

These sections were then prepared for silver-enhanced immunogold labeling by the method of Chan et al. (1990). Thus, they were rinsed in 0.1 M TS, transferred to 0.1 M PB, blocked for 10 min in 0.8% BSA and 0.1% gelatin in 0.05 M phosphate-buffered saline (PBS), and incubated overnight in colloidal gold (0.8 nm)-labeled antibody (donkey anti-mouse IgG for $\alpha 7$ nAChR, or goat anti-rabbit IgG for D₂R, Electron Microscopy Sciences, Fort Washington, PA, USA, 1:50). After this, the sections were reacted with a silver solution IntenSEM kit (GE Lifesciences, Piscataway, NJ, USA) for 6–7 min for electron microscopy.

Light and electron microscopy

Immunolabeled VTA sections used for light microscopy were mounted on glass slides, dehydrated and coverslipped prior to viewing with a Nikon microscope. Immunolabeled tissue used for electron microscopy was postfixed in 2% osmium tetroxide in 0.1 M PB for 1 h, dehydrated through a series of graded ethanols and propylene oxide, and incubated overnight in a 1:1 mixture of propylene oxide and Epon (EMbed-812; Electron Microscopy Sciences). The sections were transferred to 100% Epon for 2 h and flat-embedded in Epon between two sheets of Aclar plastic (Honeywell, Pottsville, PA, USA). The flat-embedded plastic sections were glued on Epon blocks and trimmed to approximately 1–2 mm² surface-truncated pyramids in the VTA. Ultrathin sections (60 nm) were cut from the outer surface of the tissue with a diamond knife (Diatome, Fort Washington, PA, USA) by using an ultramicrotome (Leica Microsystems, Buffalo Grove, IL, USA). The regions examined were located in the VTA at the levels of anteroposterior planes –3.4 to –3.6 mm from Bregma of the mouse brain atlas of Paxinos and Franklin (2001). The sections were collected on 400-mesh copper grids, counterstained with uranyl acetate and lead citrate (Reynolds, 1963), and examined with a Tecnai Biotwin 12 (Serial # D271) electron microscope (FEI Company, Hillsboro, OR, USA).

Data analysis

Only ultrathin sections near the surface of the tissue at the Epon-tissue interface were examined in order to reduce false negatives due to inadequate penetration of antisera. The classification of identified cellular elements was based on the descriptions of Peters et al. (1991). Axon terminals were identified by the presence of numerous synaptic vesicles and were at least 0.2 μ m in diameter. Small unmyelinated axons were <0.2 μ m and rarely contained small vesicles. Neuronal somata were identified by the presence of a nucleus, Golgi apparatus, and rough endoplasmic reticulum. Dendrites usually contained abundant endoplasmic reticulum, and were distinguished from unmyelinated axons by their larger diameter and/or abundance of uniformly distributed microtubules. In addition, dendrites were in many cases postsynaptic to axon terminals. Glial profiles were distinguished by their irregular shape, which followed the contours of neuronal profiles. The glial cytoplasm also often contained bundles of filaments. Asymmetric synapses were recognized by thick postsynaptic densities (asymmetric synapses, type Gray I), while symmetric synapses had thin pre- and postsynaptic specializations (symmetric synapses, type Gray II; Gray, 1959). Zones of closely spaced parallel plasma membranes, which lacked discernible synaptic

densities, but were otherwise not separated by glial processes, were defined as appositions or non-synaptic contacts, and were not included in the quantification unless specifically stated. A profile was considered to be selectively immunoperoxidase labeled when it contained cytoplasmic precipitates making it appear more electron dense than morphologically similar profiles located within the surrounding neuropil. A profile was considered to contain immunogold labeling when two or more gold particles were observed within large profiles. However, in dendrites smaller than 0.5- μm diameter and in small unmyelinated axons a single particle was considered positive immunogold-labeling, since almost no gold–silver deposits were seen over myelin and other tissue elements not known to express the receptors under examination. The validity of this approach was established previously for other receptors (Garzón et al., 1999; Garzón and Pickel, 2006) and has also been used for $\alpha 7\text{nAChR}$ and D_2R in the cerebral cortex (Duffy et al., 2009, 2011). In addition, however, we examined the number of gold particles over dendritic and axonal profiles in control experiments in which the tissue was processed for dual immunolabeling with the omission of one of the respective primary antisera to assess the level of background spurious gold–silver particles.

The ultrastructural quantitative analysis of receptor distributions was carried out in 17 vibratome sections with $\alpha 7\text{nAChR}$ - and D_2R -immunolabeling that were obtained from four mice. All immunoreactive processes ($n=4983$) were counted in randomly sampled electron micrographs at magnifications of 18,500 $\times$ from an area of 24,912.2 μm^2 . The tissue was quantitatively examined to determine the relative frequencies with which the immunoreactive products were localized within neuronal somata, dendrites, axons or glial cells. Morphologically recognizable synaptic relationships of each labeled profile, as well as non-synaptic appositional contacts among immunoreactive profiles for $\alpha 7\text{nAChR}$ and/or D_2R were also quantified in a smaller sample taken from six vibratome sections through the VTA in two mice. One-way analysis of variance (ANOVA) was performed using JMP (SAS, Cary, NC, USA) to ensure that there was no significant inter-animal variability in the area density (number of immunolabeled processes per area of examined surface) of immunoreactive profiles for $\alpha 7\text{nAChR}$ and/or D_2R . Variations in the density (mean number per cross-sectional surface unit) of asymmetric and symmetric synapses established by $\alpha 7\text{nAChR}$ - and/or D_2R -immunolabeled profiles were assessed using Student *t*-tests.

The light and electron microscopic images used for the figures were acquired respectively from a (1) Nikon Microphot-FX microscope equipped with a digital SNAP 2 camera (Photometrics Inc.) and (2) Tecnai 12 electron microscope (FEI, Mahwah, NJ) equipped with an AMT digital camera (Advanced Microscopy Techniques Corporation, Danvers, MA, USA) on a computer using a Windows XP operating system. Adobe Photoshop (Adobe Systems Inc., Mountain View, CA, USA) was used for adjustment of brightness, contrast and sharpness. The final images were then imported to PowerPoint software for assembly and labeling of composite figures.

RESULTS

Electron microscopy established that $\alpha 7\text{nAChR}$ and D_2R are localized within many of the same somatodendritic and in a few of the same as well as in many separate pre-synaptic

neuronal and glial profiles in the mouse VTA. The well-known high sensitivity of the immunoperoxidase method allowed detection of immunoreactivity in more profiles compared with immunogold. The global distribution of immunolabeling for $\alpha 7$ nAChR- and/or D₂R in different cellular compartments with either method was, however, rather similar, and there were no apparent differences in frequencies or types of associations between differentially labeled profiles. Hence, for statistical analysis we grouped the numbers obtained using both markers. The data from different animals were also pooled, since there were no significant variations in the number of $\alpha 7$ nAChR- and/or D₂R-labeled profiles per unit area (immunolabeling area density) among different animals ($\alpha 7$ nAChR, $F_{3,5}=2.7161$, $p=0.1548$; D₂R, $F_{3,5}=0.9736$, $p=0.4745$; dual $\alpha 7$ nAChR + D₂R, $F_{3,5}=1.7750$, $p=0.2681$; Table 1). The requirements for parametric statistical analysis, such as random sampling, normal distribution of data and finite measuring level were fulfilled in our data.

Somatodendritic distribution of $\alpha 7$ nAChR and D₂R immunoreactivity

In somata, $\alpha 7$ nAChR immunoreactivity was located most frequently in tubulovesicular endomembranes within the cytoplasm. In tissue processed for immunoperoxidase, $\alpha 7$ nAChR was observed as cytoplasmic aggregates that tended to be associated with cisterns of the endoplasmic reticulum and Golgi complex, but were also sometimes seen on restricted portions of the plasma membrane (Fig. 2A). The $\alpha 7$ nAChR-labeled somata received mainly symmetric synaptic contacts from axon terminals that were unlabeled or contained D₂R immunoreactivity (Fig. 2A). Most of the $\alpha 7$ nAChR-immunolabeled somata also expressed D₂R ($n=34$, 97.1%; Table 2), which had a similar cellular distribution to that reported previously by Sesack et al. (1994) and Pickel et al. (2002).

In small dendrites, $\alpha 7$ nAChR immunoperoxidase labeling was typically seen in aggregates having a restricted distribution within the cytoplasm (Fig. 2B, E) or on the inner face of the plasma membrane (Fig. 2C, E, F), although in some dendrites the labeling had a more diffuse cytoplasmic distribution. This diffuse localization of $\alpha 7$ nAChR was more common in larger dendrites (Fig. 2D). The $\alpha 7$ nAChR immunogold labeling was also localized to select segments of the dendritic plasma membrane contacted by axon terminals (Fig. 3) and sometimes seen on the postsynaptic densities of synaptic specializations established with those terminals (Fig. 3B, C). The dendrites containing exclusively $\alpha 7$ nAChR-immunoreactivity received twice as many asymmetric compared with symmetric synapses from either D₂R-labeled (Figs. 3B, C and 4C, D) or unlabeled (Figs. 2B and 3B) axon terminals.

Dual $\alpha 7$ nAChR- and D₂R-labeled dendrites were as prevalent as single $\alpha 7$ nAChR-immunoreactive dendrites and almost as numerous as D₂R-labeled dendrites (Table 2). Accordingly, dually-labeled dendrites comprised about half of the total dendrites containing $\alpha 7$ nAChR or D₂R labeling (Fig. 5). The $\alpha 7$ nAChR and D₂R were seen both in plasmalemmal and cytoplasmic localizations within the same dendrite. The immunolabeling for $\alpha 7$ nAChR and D₂R were observed in close proximity within the cytoplasm in some dendrites (Fig. 3A), and also at a distance in others (Fig. 2C, E). Dual $\alpha 7$ nAChR- and D₂R-labeled dendrites received mainly asymmetric inputs ($n=173$, 81.3%) from either unlabeled

(Fig. 2C, E, F) or D₂R-labeled (Figs. 3A, 5B and 7A) axon terminals; synaptic contacts from terminals expressing $\alpha 7$ nAChR were much less commonly seen (Table 3).

D₂R immunoreactivity was seen alone in many dendrites ($n=831$, 50.9%), where the labeling was localized often to cytoplasmic endomembranes (Figs. 2D and 3A) or to segments of the plasma membrane (Figs. 2D and 5C). Immunogold particles for D₂R also were identified on postsynaptic sides of synaptic junctions in dendrites that were either labeled (Fig. 2F) or not (Fig. 3B) for $\alpha 7$ nAChR. D₂R-labeled dendrites received many asymmetric-type synaptic contacts from unlabeled ($n=94$, 70.7%; Fig. 2D) or D₂R-immunoreactive ($n=30$, 22.6%; Fig. 3A, B) axon terminals, and much more infrequently from $\alpha 7$ nAChR-labeled terminals ($n=5$, 3.7%; Table 3).

Axonal distribution of $\alpha 7$ nAChR and D₂R immunoreactivities

As compared with dendrites, axonal profiles more rarely contained $\alpha 7$ nAChR immunoreactivity (Table 2). Only 16.4% of the total $\alpha 7$ nAChR labeling was localized to axonal profiles, either to small unmyelinated axons ($n=142$; 5.2%) or to axon terminals ($n=309$; 11.2%). Small unmyelinated axons labeled for $\alpha 7$ nAChR usually were dispersed in the neuropil within bundles containing other small axons that were unlabeled (Fig. 6). However, some of the $\alpha 7$ nAChR-labeled axons apposed terminals that either were unlabeled or contained D₂R (Fig. 3D). Within these axons, the $\alpha 7$ nAChR-immunoperoxidase reaction product was diffusely distributed in the cytoplasm (Fig. 6A, B), whereas the $\alpha 7$ nAChR-immunogold was more discretely localized to the plasma membrane (Figs. 3B, 5C). The $\alpha 7$ nAChR-immunolabeled axon terminals, of which 42.1% also contained D₂R ($n=130$), formed either asymmetric ($n=43$) or symmetric ($n=20$) synaptic specializations with dendrites (Fig. 6C).

Axonal profiles containing D₂R immunoreactivity were abundant in the VTA ($n=1377$). Most of the D₂R-immunolabeled axonal profiles were axon terminals ($n=1178$, 85.5%), the majority of which did not contain $\alpha 7$ nAChRs ($n=1048$, 89%). The D₂R immunoperoxidase reaction product was seen as a dense precipitate that rimmed synaptic vesicles (Figs. 3, 7C). Aggregates of D₂R immunoperoxidase labeling were also detected on and near the presynaptic membrane specialization (Figs. 3C, D and 6C) or within the cytoplasm (Fig. 5B). In addition, D₂R immunoreactivity was evident on select segments of the plasma membrane away from synaptic contacts, and opposed by glial profiles (Fig. 7C, D). D₂R-immunogold had a similar distribution, to that of the immunoperoxidase labeling, but was more frequently localized to small synaptic vesicles within axon terminals (Figs. 4 and 7A, B).

About half of the D₂R-labeled terminals (48.2%) had synaptic junctions in the plane of section, showing a prevalence of asymmetric (Fig. 4; $n=201$, 74.4%) over symmetric (Fig. 6C; $n=69$, 25.6%) synapses. The postsynaptic targets were mainly unlabeled dendrites, but also included dendrites immunoreactive for D₂R and/or $\alpha 7$ nAChR (Fig. 8; Table 3). Each of these types of dendrites was rather similar in their receipt of asymmetric and symmetric synapses.

Glial distribution of $\alpha 7$ nAChR and D₂R immunoreactivities

Many glial processes resembling those of astrocytes contained $\alpha 7$ nAChR and/or D₂R immunolabeling (Figs. 6 and 7A–C). These glial processes comprised one-third of all single $\alpha 7$ nAChR-labeled profiles ($n=561$, 33.3%; Table 2). The $\alpha 7$ nAChR-immunoperoxidase in glial processes was distributed in expansions wrapping presynaptic terminals and dendrites immunolabeled for $\alpha 7$ nAChR with or without D₂R (Fig. 7A–C). These axon terminals were unlabeled (Fig. 7B) or D₂R-labeled (Fig. 7A). The $\alpha 7$ nAChR-immunogold labeling was often localized to glial processes enveloping synapses (Fig. 7C). Glial-like profiles also contained D₂R labeling, and over one-third of these profiles also contained $\alpha 7$ nAChR immunoreactivity ($n=85$, 35.9%; Table 2 and Fig. 7C–E). In a separate series of dual labeling experiments 26/ 177 of the D₂R-labeled profiles contained S100-immunoreactivity. These glial profiles were frequently opposed to dendritic profile with (Fig. 7D) or without (Fig. 7E) D₂R labeling.

DISCUSSION

Our results provide immunocytochemical evidence that $\alpha 7$ nACh and D₂R are targeted to many of the same, as well as to synaptically linked neurons and their opposing glia in the mouse VTA. The co-localization of $\alpha 7$ nAChR and D₂R within some of the same somatodendritic profiles in this region suggests that there may be convergent signaling between the $\alpha 7$ -subunit and D₂R G-protein pathways in postsynaptic neurons of the VTA. In contrast with somata and dendrites, presynaptic $\alpha 7$ nAChR and D₂R labeling was largely found in separate axons and axon terminals with the D₂R being more prevalent than the $\alpha 7$ nAChR in these presynaptic sites. In addition, we have shown that axon terminals containing the D₂R often form symmetric or asymmetric synapses or are opposed to dendrites expressing $\alpha 7$ nAChR and/or D₂R, indicating a key role of the D₂R in the presynaptic release of different neurotransmitters or modulators affecting the activity of VTA neurons responsive to $\alpha 7$ nAChR and/or D₂R ligands. The further localization of $\alpha 7$ nAChR alone or together with the D₂R in perisynaptic glia in this brain region also suggests a non-neuronal mechanism by which these ligands can affect synaptic transmission in the VTA. The findings have important implications for the understanding the shared effectiveness of nicotine and D₂R antagonists in ameliorating some of the symptoms of schizophrenia.

Methodological considerations

Dual ultrastructural localization of $\alpha 7$ nAChR and D₂R was achieved by combining immunogold and immunoperoxidase pre-embedding techniques for identification of their respective antisera in single sections of tissue (Chan et al., 1990). The specificity of these antisera was described previously (Pickel et al., 2006; Duffy et al., 2009, 2011) and that of the D₂R further confirmed in the dorsal striatum of MSN-D2R KO. Each receptor had a similar subcellular location when detected with either the immunogold or immunoperoxidase method, and reversal of markers assured that each antigen was optimally identified by methods having different sensitivities and resolution. Profiles were considered immunolabeled when they contained one or more gold particles or a peroxidase reaction product with an electron density greater than that seen in nearby profiles. The validity of

considering small profiles with only one immunogold particle as specifically labeled has been established for other neurotransmitter receptors (Garzón and Pickel, 2006; Hara et al., 2006) and has proved to be valid for $\alpha 7$ nAChR in the cerebral cortex (Duffy et al., 2009, 2011). However, the method is dependent on first establishing that there are a minimal number of particles overlying myelin and other tissue elements expected not to contain the antigen of interest (Wang et al., 2003). In the present study, this criterion was met for the immunogold labeling of both $\alpha 7$ nAChR and D₂R. Moreover, we observed no immunolabeling in VTA tissue processed with the omission of the primary antibody thus assuring a minimal false-positive labeling. However, the often sparse labeling for both the $\alpha 7$ nAChR and D₂R may have contributed to an underestimation of the number of both neuronal and glial profiles that express $\alpha 7$ nAChR and D₂R in the VTA. To circumvent the false-negative data resulting from inadequate penetration of immunoreagents, ultrathin sections were collected exclusively from the tissue-plastic interface from vibratome sections.

Somatodendritic distribution of $\alpha 7$ nAChR and D₂R

The $\alpha 7$ nAChR-immunoreactivity was seen in large cytoplasmic aggregates in somata and proximal dendrites that also frequently contained D₂R, a location comparable to that reported previously in the prefrontal cortex (Duffy et al., 2011). The cytoplasmic aggregates of $\alpha 7$ nAChR labeling may reflect large intracellular receptor reserve pools providing a mechanism for the rapid ACh-induced up-regulation of the nAChR by mobilization from their cytoplasmic stores (Drenan et al., 2008; Millar and Harkness, 2008).

Both $\alpha 7$ nAChR and D₂R were also distributed along tubulovesicular membranes of the endoplasmic reticulum or endosomal multivesicular bodies, suggesting sites of synthesis/transfer and degradation, respectively, of these receptor proteins (Keller et al., 2001; Prou et al., 2001; Piper and Katzmann, 2007; Geetha and Wooten, 2008; Kim et al., 2008; Rezvani et al., 2010). In some cases, both the $\alpha 7$ nAChR and D₂R were localized to single segments of endomembranes suggesting their involvement in trafficking and assembly of the nAChRs (Gelman et al., 1995; Green and Millar, 1995; Wanamaker and Green, 2007) and D₂R (Free et al., 2007). Collectively, these observations support a postsynaptic neuronal affiliation between nAChR $\alpha 7$ subunits and the G-protein coupled D₂R in the VTA, which is similar to that proposed in the prefrontal cortex (Duffy et al., 2011). Likewise, analogous interactions including many cellular G-protein pathways had been demonstrated between nAChR beta-2 subunits and nAChR-interacting proteins (Kabbani et al., 2007). Thus, $\alpha 7$ nAChR labeling in somata and large dendrites most likely reflect dynamic sites involved in intracellular trafficking of the surface receptors.

Our observation of $\alpha 7$ nAChR in dendrites is consistent with nicotine activation of VTA dopaminergic neurons *in vivo* (Grenhoff et al., 1986; Mereu et al., 1987) or *in vitro* in deafferented slice preparations (Calabresi et al., 1989; Pidoplichko et al., 1997; Picciotto et al., 1998). There are some data suggesting that the stimulatory effect of nicotine on VTA dopaminergic neurons could largely be due to the activation of beta2-subunit containing nAChR (Picciotto et al., 1998; Schilström et al., 2003). However, there is also substantial evidence that the underlying mechanism involves an increase in Ca²⁺ entry through $\alpha 7$ nAChR present on dopaminergic neuronal somata or dendrites and the subsequent

activation of Ca^{2+} -dependent K^+ channels (Kitai et al., 1999; Mameli-Engvall et al., 2006). Furthermore, our finding of $\alpha 7\text{nAChR}$ in the somatodendritic compartment of VTA neurons agrees with the identification of α -bungarotoxin- and MLA-sensitive whole-cell currents evoked by nicotine in dissociated VTA neurons using patch-clamp recordings (Yang et al., 2009).

The observed, dendritic distribution of $\alpha 7\text{nAChR}$ on extra- or peri-synaptic plasma membranes close to mainly asymmetric but also symmetric contacts, suggests a prominent role of $\alpha 7\text{nAChR}$ as postsynaptic modulator of excitatory as well as inhibitory signaling in VTA neurons. Excitatory input to this region arises prominently from the cerebral cortex, but also derives from the mesopontine tegmentum and subthalamic nucleus (Geisler et al., 2007). These glutamate-containing inputs to VTA are known to partially drive the synaptic plasticity underlying neural adaptation to addictive drugs (Saal et al., 2003; Jin et al., 2011). The present localization of $\alpha 7\text{nAChR}$ to postsynaptic membrane specializations in dendrites of the VTA is comparable to that of glutamate N-methyl-D-aspartate receptor (NMDA) receptors and supports the idea that activation of the postsynaptic $\alpha 7\text{nAChR}$ can initiate a Ca^{2+} signal that produces a calmodulin-dependent reduction in the responsiveness of glutamate NMDA receptors (Fisher and Dani, 2000). In addition, the localization of $\alpha 7\text{nAChR}$ to postsynaptic densities of asymmetric synapses is consistent with the blocking of nicotinic activation of mesolimbic dopaminergic neurons by antagonists of ionotropic glutamate receptors (Schilström et al., 1998; Grillner and Svensson, 2000). Our observation of $\alpha 7\text{nAChR}$ labeling in dendrites that receive symmetric inputs is consistent with reported involvement of the $\alpha 7\text{nAChR}$ in postsynaptic modulation of responses mediated by GABA-A (Zhang and Berg, 2007) and GABA-B receptors in the VTA (Amantea and Bowery, 2004).

The present localization of D_2R on or near the dendritic plasma membrane may indicate sites relevant to coupling of Gi/o -like proteins involved in modulating ion channels and/or decreasing adenylate cyclase activity, which can elicit inhibitory postsynaptic currents and inhibition in these dendrites (Johnson and North, 1992; Missale et al., 1998; Beckstead et al., 2004). The prevalence of D_2R labeling on plasma membranes near asymmetric synapses that are typical of glutamatergic terminals is consistent with involvement of D_2Rs in the modulation of synaptic plasticity of excitatory synapses in the VTA (Thomas et al., 2000). Psychostimulants binding to the D_2R can produce long-term potentiation of excitatory postsynaptic current mediated through AMPA (Saal et al., 2003; Bellone and Lüscher, 2006) and NMDA (Ahn et al., 2010) receptors in the VTA. Our data, thus, support D_2R -mediated effects of dopamine on VTA neurons (Lacey et al., 1987), which may be ascribed in part to modulation of postsynaptic glutamate currents affecting both basal firing and bursting activity of mesolimbic dopamine neurons (Beckstead et al., 2004).

Somatodendritic processes accounted for the majority of the profiles containing both the $\alpha 7\text{nAChR}$ and the D_2R in the VTA. When the two receptors were co-expressed in common soma, both proteins were occasionally seen in overlapping endomembrane structures, suggesting shared synthesis and trafficking machinery. In dendritic processes, $\alpha 7\text{nAChR}$ and D_2R were usually located more in plasma membranes near asymmetric inputs, advocating for co-modulation of postsynaptic excitatory transmission in the VTA.

Localization of $\alpha 7$ nACh and D₂ receptors to excitatory postsynaptic densities

The present localization of $\alpha 7$ nACh to postsynaptic membrane specializations at mainly excitatory synapses is similar to previous observations in the cerebral cortex (Duffy et al., 2009, 2011). This distribution is shared by NMDA receptors and is consistent with evidence showing that activation of the postsynaptic $\alpha 7$ nAChR can initiate a Ca²⁺ signal that produces a calmodulin-dependent reduction in the responsiveness of glutamate NMDA receptors (Fisher and Dani, 2000).

The D₂R was also seen on and near excitatory-type postsynaptic membrane specializations, consistent with G-protein pathways that can modulate glutamate NMDA currents (Martina and Bergeron, 2008). In addition, D₂R stimulation can decrease the surface and synaptic expression of GluR1, an obligatory subunit of glutamate AMPA receptors (Sun et al., 2005). Thus, the activation of the D₂R may modulate both the NMDA and AMPA receptor-mediated postsynaptic responses in VTA neurons. Moreover, the less commonly observed expression of plasmalemmal D₂R near symmetric synapses suggests that these receptors are potentially involved in modulating slow GABA-dependent inhibitory currents, through activation of GABA receptors and G-protein-coupled inwardly rectifying potassium (GIRK) channels (Johnson and North, 1992; Cruz et al., 2004; Labouèbe et al., 2007).

Presynaptic distribution of $\alpha 7$ nACh and D₂ receptors

Nicotine increases extracellular concentrations of glutamate and aspartate in the VTA (Schilström et al., 2000). Supporting this, numerous binding sites for the $\alpha 7$ nAChR agonist α -bungarotoxin have been observed in immunocytochemically-identified glutamatergic axons within the VTA (Jones and Wonnacott, 2004). The present localization of $\alpha 7$ nAChR to axon terminals making asymmetric synapses is consistent with presynaptic facilitation of glutamate release that has been proposed to be mediated mainly by $\alpha 7$ nAChR in the VTA (Mansvelder and McGehee, 2000; Schilström et al., 2000; Jones and Wonnacott 2004; Good and Lupica 2009). The $\alpha 7$ nAChR is known to be involved in the modulation of presynaptic NMDA receptor expression and in the structural plasticity of glutamatergic axons in the VTA (Gao et al., 2010; Mao et al., 2011). The paucity of $\alpha 7$ nAChR-labeled terminals forming excitatory-type synapses in this region suggests, however, that there may be alternative mechanisms contributing to nicotine-induced potentiation of glutamatergic transmission as is discussed in the section on perisynaptic astrocytes containing $\alpha 7$ nAChR.

Unlike the $\alpha 7$ nAChR, D₂R in the VTA was observed in many axon terminals. Within these terminals, D₂R was localized mainly over membranes of synaptic vesicles, a distribution similar to that of the inhibitory Gi-protein (Aronin and DiFiglia, 1992). The D₂R-labeled terminals were often without recognizable synaptic junctions, although some formed symmetric or more commonly asymmetric synapses. This morphological heterogeneity suggests involvement of the D₂R in presynaptic regulation of the release of functionally diverse transmitters.

Our results suggest a predominant involvement of the D₂R in presynaptic release of glutamate or other excitatory transmitters in axon terminals forming asymmetric excitatory-type synapses on dendrites including those that express the $\alpha 7$ nAChR. D₂R activation

inhibits presynaptic release of glutamate in the VTA (Koga and Momiyama, 2000). Classic antipsychotics blocking D₂R may enable, thus, disinhibition of glutamate release onto dendrites also responsive to α 7nAChR agonists, which is critically involved in behaviors such as instrumental response selection and psychostimulant-induced euphoria (Koch et al., 2000). The D₂R-labeled terminals that form excitatory-type synapses in the VTA may derive either from glutamatergic local neurons (Dobi et al., 2010) or glutamatergic inputs from other brain regions, specifically from the cerebral cortex (Geisler et al., 2007; Omelchenko and Sesack, 2007) or the mesopontine tegmentum (Charara et al., 1996; Parent et al., 1999) among others. Cholinergic terminals are also a recognized source of excitatory input to the VTA, which that might be modulated by D₂R (Garzón et al., 1999; Omelchenko and Sesack, 2005).

D₂R-labeled axon terminals also formed, albeit in a much lesser degree, symmetric synapses typical of those containing GABA (Torrealba and Müller, 1999). Inhibitory-type terminals may derive from local GABAergic interneurons (Steffensen et al., 1998; Omelchenko and Sesack, 2009) or from GABAergic neurons located in multiple structures, such as striatum, pallidum, periaqueductal gray, rostromedial tegmental nucleus or mesopontine tegmentum among others (Kalivas et al., 1993; Geisler and Zahm, 2005; Tripathi et al., 2010; Xia et al., 2011). The conclusion that GABA is present in some of the D₂R-labeled terminals that contact dendrites is consistent with studies showing D₂R-dependent regulation of GABAergic currents in VTA neurons (Michaeli and Yaka, 2010, 2011). This observation supports prior studies demonstrating presynaptic D₂R-mediated inhibition of GABA release in this area through GIRK channel activation (Michaeli and Yaka, 2010). The expression of D₂R in axon terminals that contact dendrites containing the α 7nAChR is consistent with studies showing D₂R-dependent regulation of GABAergic currents in cortical pyramidal cells (Trantham-Davidson et al., 2008).

We cannot exclude that some of the non-synaptic axon terminals that contain D₂R, or both D₂R and α 7nAChR may have a synaptic specialization not seen in the plane of section, but those terminals may also belong substantially to axons that typically release non-synaptically their transmitters, such as those containing dopamine (Nirenberg et al., 1997; Turner, 2004), serotonin (Hervé et al., 1987; Levin and Rezvani, 2007), or acetylcholine (ACh) (Descarries et al., 1997; Garzón et al., 1999) neurons. These neuromodulators are often stored in axon terminals without recognizable synapses (Drenan et al., 2008; Millar and Harkness, 2008), and elicit many of their physiological effects by volume transmission, activating their respective receptors at a distance after diffusing through the extracellular space (Descarries and Mechawar, 2000; Fuxe et al., 2010).

Glial distribution of α 7nAChR and D₂R

Our results show that α 7nAChR-labeled glial processes in the VTA are in contact with perisynaptic portions of dendrites containing α 7nAChR, suggesting that α 7nAChR activation in glia might modulate postsynaptic responses of adjacent α 7nAChR-expressing dendrites to bound ligands by glial-neuronal coupling (Perea and Araque, 2005). Thus, signaling between neurons and astrocytes is a reciprocal communication within the conceptual framework of the tripartite synapse, where astrocytes not only respond to

neuronal activity but also actively regulate neuronal and synaptic activity (Perea et al., 2009; Perea and Araque, 2010).

The present detection of $\alpha 7$ nAChR alone or occasionally together with the D₂R in perisynaptic glial processes in the VTA suggests that these receptors may play an important role in astrocytic uptake and clearance of glutamate and in the conversion of glutamate to glutamine, the substrate for neuronal synthesis of glutamate (Sharma and Vijayaraghavan, 2001; Oikawa et al., 2005; Patti et al., 2007). An increase in glial intracellular Ca²⁺ evokes glutamate release (Simard and Nedergaard, 2004), and also elicits glutamate-dependent currents in surrounding neurons (Pasti et al., 1997; Araque et al., 1998; Bezzi et al., 1998; Stigliani et al., 2006). Functional $\alpha 7$ nAChR-mediated astrocytic current responses have been observed in acute slices of hippocampal CA1 region (Shen and Yakel, 2012), and $\alpha 7$ nAChR activation produces glutamate release in cortical mice gliosomes (Patti et al., 2007). These observations, together with the known $\alpha 7$ nAChR-mediated enhancement of intracellular Ca²⁺ in astrocytes, support the hypothesis that perisynaptic astrocytes expressing $\alpha 7$ nAChR are critical modulators of neural transmission in the VTA. In contrast with $\alpha 7$ nAChR, D₂R immunolabeling was rarely seen alone, but more often co-expressed with $\alpha 7$ nAChR and with S100B, an astrocytic marker whose release can be regulated through D₂R activation (Nardin et al., 2011). The co-expression of $\alpha 7$ nAChR and D₂R in perisynaptic glia suggests that *in vivo* activation of both receptors may synergistically enhance intracellular Ca²⁺ levels, as has been shown in cultured astrocytes (Finkbeiner, 1993; Khan et al., 2001; Reuss and Unsicker, 2001; Oikawa et al., 2005).

Our demonstration of glial labeling for these receptors in mouse VTA is inconsistent with prior studies that failed to detect α -bungarotoxin binding for the $\alpha 7$ nAChR (Jones and Wonnacott, 2004) or D₂R immunolabeling in glial profiles in this region of rat VTA (Sesack et al., 1994). The apparent discrepancies may reflect either species or methodological differences as well as false positive or false negative results that are inherent to all immunocytochemical studies. Our belief in the authenticity of the glial labeling in our study is based on its absence in control tissue and identification using two visually distinct, immunogold and immunoperoxidase markers.

Implications

The present findings contribute to further understanding of the neurobiological cellular and subcellular bases for mutual integration of ACh- and DA-signaling in the VTA, which underlies reinforcement and cognition (Levin and Simon, 1998; Mark et al., 2011). They identify dendrites as major neuronal sites for convergent $\alpha 7$ nAChR- and D₂R-mediated effects on the postsynaptic excitability of VTA neurons. Our study also provides evidence that the presynaptic release of both excitatory and inhibitory transmitters is controlled in part through D₂R in axon terminals that provide input to $\alpha 7$ nAChR-labeled dendrites in this region. The scarcity of $\alpha 7$ nAChR-labeled excitatory-type axon terminals and the observed localization of $\alpha 7$ nAChR and/or D₂R in many perisynaptic astrocytic profiles suggest the involvement of astrocytes in ACh- and/or DA-evoked changes in the synaptic availability of neurotransmitters in the VTA (Navarrete et al., 2012).

Our results have implications for understanding the therapeutic differential effectiveness as antipsychotic drugs of $\alpha 7$ nAChR agonists and D_2 R antagonists. Beneficial effects of some atypical antipsychotics, such as clozapine, involve $\alpha 7$ nAChR activation in the VTA (Schwielers and Erhardt, 2003). Clozapine normalizes sensory gating, a characteristic deficit in schizophrenia, in both human patients (Becker et al., 2004) and mice models (Simosky et al., 2003); and in mice this effect is selectively blocked by the $\alpha 7$ nAChR antagonist α -bungarotoxin (Simosky et al., 2003). Furthermore, $\alpha 7$ nAChR has been extensively implicated in cognitive processes, such as attention, learning and memory (for review see Leiser et al., 2009). In contrast with $\alpha 7$ nAChR agonists, classical antipsychotic drugs block the D_2 R and are clinically effective in reducing positive psychotic symptoms of schizophrenia (Guillin et al., 2007). The mechanisms involved in this action definitely comprise a subcortical DA decline in which D_2 R targeting in the VTA causing DA inhibitory autoregulation could play a role, as well as actions in other regions (Goto and Grace, 2007). Our data suggest that the excitability of VTA neurons inclusive of those that express DA and D_2 R is highly influenced by $\alpha 7$ nAChR activation. The prominent expression of the $\alpha 7$ nAChR in VTA astrocytes suggests that these astrocytes may play a pivotal role in nicotine-induced enhancement of the activity of VTA neurons.

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Abbreviations

ABC	Avidin–biotin complex
ACh	acetylcholine
$\alpha 7$nAChRs	alpha7 nicotinic acetylcholine receptors
BSA	bovine serum albumin
D_2R	dopamine D_2 receptor
GIRK	G-protein-coupled inwardly rectifying potassium
MLA	methyllycaconitine
NAc	nucleus accumbens
nAChR	nicotinic acetylcholine receptor
NMDA	N-methyl-D-aspartate receptor
PB	phosphate buffer
PBS	phosphate-buffered saline
TS	Tris-buffered saline
VTA	ventral tegmental area

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Fig. 1. Photomicrographs showing immunoperoxidase labeling for the D₂R in the caudate-putamen nucleus (CPu) of wild-type mice (A) is largely gone in the CPu of MSN-D₂ KO mice (B). In contrast, there is no noticeable difference in the density of the labeling in the VTA of wild-type (C) and KO mice (D). Corner arrows point dorsal (d) and medial (m). Scale bars=0.02 μm.

Fig. 2.

$\alpha 7$ nAChR distribution in somata and dendrites with or without D_2 R. (A) A dark precipitous $\alpha 7$ nAChR-immunoperoxidase reaction product (white block arrows) is observed rimming a multivesicular body (mvb) and the cisterns of endoplasmic reticulum and other endomembranes, as well as the plasma membrane of a neuronal soma ($\alpha 7$ -soma). The soma receives synaptic contact from an axon terminal showing D_2 R gold particles (D_2 -t). (B) Aggregates of $\alpha 7$ nAChR-immunoperoxidase (white block arrows) are observed within a dendrite receiving synaptic input from an unlabeled axon terminal (ut). (C) A clump of $\alpha 7$ nAChR-immunoperoxidase reaction product (white block arrow) is localized in a dendrite ($\alpha 7+D_2$ -d) that also contains one cytoplasmic immunogold particle (small black arrow) for D_2 R. The dendrite receives an asymmetric synapse (black curved arrow) from an unlabeled axon terminal (ut). (D) Diffuse $\alpha 7$ nAChR-immunoperoxidase labeling is seen in a dendrite ($\alpha 7+D_2$ -d) that also contains D_2 R-immunogold particles (small black arrows). The $\alpha 7+D_2$ -d shows an extensive plasmalemmal apposition with a dendrite exclusively labeled for D_2 R (D_2 -d), which receives what appears to be an asymmetric synapse from an unlabeled axon terminal (ut). (E) $\alpha 7$ nAChR-immunoperoxidase aggregates (white block arrows) are clustered in an otherwise diffusely labeled dendrite ($\alpha 7+D_2$ -d) that also contains both cytoplasmic and plasmalemmal gold particles (small black arrow) for D_2 R. The dually-labeled $\alpha 7+D_2$ -d receives an asymmetric synapse (curved black arrow) from an unlabeled terminal (ut). (F) An unlabeled axon terminal (ut) makes a thick synaptic contact with a dendrite ($\alpha 7+D_2$ -d) showing D_2 R gold particles (small black arrows). The D_2 R immunogold particles are located beneath the postsynaptic specialization and distant from the synapse near an aggregate of $\alpha 7$ nAChR-immunoperoxidase reaction product. Scale bars=500 nm.

Fig. 3.

D_2R immunoperoxidase in axon terminals presynaptic to $\alpha 7nAChR$ -labeled dendrites. (A) An axon terminal containing D_2R immunoperoxidase labeling (D_2-t) forms an asymmetric synapse with a dendrite ($\alpha 7+D_2-d$) showing multiple cytoplasmic gold particles for $\alpha 7nAChR$ (small black arrows) and also immunoperoxidase precipitate for D_2R in tubulovesicular membranes (white block arrows). (B) A D_2R -labeled terminal (D_2-t) is presynaptic to dendrite showing plasmalemmal gold particles (small black arrows) for $\alpha 7nAChR$ ($\alpha 7-d$) and receiving convergent synaptic input from an unlabeled axon terminal (ut). (C) D_2R -immunoperoxidase labeling is seen in an axon terminal that forms an asymmetric synapse with a dendrite containing $\alpha 7nAChR$ -immunogold particles (small black arrows) in the cytoplasm and in extrasynaptic and synaptic portions of the plasma membrane ($\alpha 7-d$). (D) D_2R -immunoperoxidase precipitate is seen within several axon terminals (D_2-t) making contacts with dendrites showing immunogold particles (small black arrows) for $\alpha 7nAChR$ ($\alpha 7-d$). In two of those terminals ($D_2-t_{1,2}$) showing discernible synapses with the dendrites, the D_2R -immunoperoxidase is seen concentrated in the presynaptic sides facing $\alpha 7-d_{1,2}$. The surrounding neuropil contains two small unmyelinated axons ($\alpha 7-a_{1,2}$) with plasmalemmal $\alpha 7nAChR$ immunogold particles (small black arrows). Scale bars=500 nm.

Fig. 4.

Subcellular distribution of D₂R in axon terminals providing input to either D₂R- or α7nAChR-immunolabeled dendrites. (A) D₂R immunogold particles are seen within the cytoplasm and on small synaptic vesicles of an axon terminal (D₂-t) that makes a symmetric synapse onto a dendrite (D₂-d) containing also D₂R-immunogold (small black arrows). (B) An axon terminal (D₂-t) containing D₂R-immunogold makes an asymmetric synapse with a dendrite (D₂-d) showing synaptic D₂R-immunogold particles (small black arrows). (C) D₂R gold particles are observed rimming the membranes of small synaptic vesicles (ssv) within an axon terminal (D₂-t) establishing an apparently asymmetric synapse with a dendrite (α7-d) containing a dense intracytoplasmic aggregate of α7nAChR-immunoperoxidase. (D) α7nAChR-immunoperoxidase clumps are seen beneath extrasynaptic portions of the plasma membrane of a dendrite (α7-d) receiving asymmetric synaptic input from a D₂R immunogold-labeled terminal (D₂-t₁). The D₂-t₁ shows an appositional contact with another axon terminal (D₂-t₂) containing D₂R gold particles (small black arrows). D₂R immunogold=small black arrows; Scale bars=500 nm.

Fig. 5.

Co-localization of D₂R and α7nAChR labeling in dendrites. (A) α7nAChR immunogold particles (small black arrows) are seen within the cytoplasm and on the postsynaptic density of an incoming synapse from an unlabeled terminal (ut) within a dendrite (α7+D2-d) that also contains diffuse D₂R-immunoperoxidase labeling. (B) An axon terminal (D₂-t) showing dense D₂R immunoperoxidase makes what appears to be an obliquely sectioned asymmetric synapse with a dendrite (α7+D2-d) containing both light D₂R immunoperoxidase and α7nAChR immunogold particles (small black arrows). (C) α7nAChR immunogold particles (small black arrows) and D₂R immunoperoxidase are seen within a dendrite (α7+D2-d) that receives convergent input from two unlabeled axon terminals (ut_{1,2}). A dendrite containing exclusively D₂R immunoperoxidase labeling (D2-d) and a small unmyelinated axon (α7-a) showing plasmalemmal α7nAChR immunogold particles (small black arrow) are observed in the nearby neuropil. Scale bars=500 nm.

Fig. 6.

$\alpha 7$ nAChR distribution in unmyelinated axons and glia. (A) $\alpha 7$ nAChR immunoperoxidase is seen in transversally sectioned unmyelinated axons that show organized arrangement of microtubules ($\alpha 7$ -a₁₋₃). (B) Diffuse $\alpha 7$ nAChR immunoperoxidase is observed in a small unmyelinated axon ($\alpha 7$ -a) contacting a dendrite ($\alpha 7$ +D2-d) showing $\alpha 7$ nAChR immunoperoxidase and D₂R immunogold (small black arrows). The $\alpha 7$ nAChR immunoperoxidase is also observed near the plasma membrane of an astrocytic expansion ($\alpha 7$ -g₁). Another astrocytic process ($\alpha 7$ -g₂), lightly gray-shaded and depicting contour in discontinuous trace for easier recognition, contains aggregates of $\alpha 7$ nAChR immunoperoxidase). The $\alpha 7$ -g₂ opposes and partially wraps the dually-labeled $\alpha 7$ +D2-d. (C) Immunogold-labeled $\alpha 7$ nAChR (small black arrows) is seen in a terminal ($\alpha 7$ -t) making an asymmetric synapse (curved arrow) onto a $\alpha 7$ -labeled dendrite ($\alpha 7$ -d). A peroxidase-labeled D₂R-labeled terminal is located adjacent to the same dendrite (curved white arrow). A small tubulovesicle (tv) is associated with two $\alpha 7$ gold particles. Unlabeled Dendrite=ud, $\alpha 7$ axon= $\alpha 7$ -a. Scale bars=500 nm.

Fig. 7.

Glial distribution of $\alpha 7$ nAChR, D_2 receptor and S100. (A) An intensely $\alpha 7$ nAChR-immunoperoxidase-labeled glial profile ($\alpha 7$ -g) opposes a dendrite ($\alpha 7$ + D_2 -d) containing D_2 R immunogold (small black arrows) and light $\alpha 7$ nAChR-immunoperoxidase reaction product. The labeled glial process also partially contacts a D_2 R immunogold (small black arrows)-labeled axon terminal (D_2 -t) presynaptic to the $\alpha 7$ + D_2 -d. (B) The $\alpha 7$ nAChR-immunoperoxidase is seen in a glial profile ($\alpha 7$ -g) opposing a dendrite ($\alpha 7$ -d) that also contains clumps of $\alpha 7$ nAChR peroxidase reaction product. The $\alpha 7$ -g has thin expansions that partially form a common coating for $\alpha 7$ -d and a D_2 R immunogold (small black arrows)-labeled axon terminal (D_2 -t) presynaptic to an unlabeled dendrite. (C) Glial profiles containing both $\alpha 7$ nAChR gold particles (small black arrows) and immunoperoxidase D_2 R labeling ($\alpha 7$ nAChR+ D_2 R-g) are opposed to a dual-labeled $\alpha 7$ nAChR+ D_2 R-d. The dually labeled dendrite is receptive to an axon terminal containing peroxidase labeling for the D_2 R (D_2 -t). Insert shows a comparable dual-labeled glial process in another plane of section. (D and E) Immunogold D_2 R labeling (small arrows) is seen in glial profiles containing immunoperoxidase labeling for S100 (S100+ D_2 -g). In D, the dually labeled glial process opposes many unlabeled axon terminals (ut) and a dendrite that also contains D_2 R immunogold (D_2 -d). In E, the dually labeled glial profile opposes many unlabeled dendritic and axonal profiles, while D_2 R (small arrows) is seen in a more distant dendrite (D_2 -d) and presynaptic terminal (D_2 -t). Scale bar=500 nm.

Fig. 8.

Bar graphs showing the synaptic area density (number of synapses per surface unit area) distribution of asymmetric or symmetric synapses made by axon terminals immunolabeled for D_2R (D_2R) and/or $\alpha 7\text{nAChR}$ ($\alpha 7\text{R}$) with dendrites in the VTA. These dendrites are unlabeled (Un) or contain immunolabeling for $\alpha 7\text{R}$, D_2R or both receptors (dual). Synaptic area density, shown in the Y axis, is expressed as the number of synapses per 1000 μm^2 of analyzed tissue, calculated from the tallied micrograph area in which the synapses were counted for each group. The raw numbers of synapses are shown on top of each bar as the ratio value with respect to the total number of synapses within the group. Data were collected from six vibratome sections through the VTA in two mice. In three sections markers were reversed (immunogold or immunoperoxidase) to the other three, and both animals included sections of either type.

Table 1

Immunolabeling density for $\alpha 7$ nAChR and/or D₂R within the mouse VTA in each animal included in the study

Animal	$\alpha 7$ nAChR	D ₂ R	$\alpha 7$ nAChR+D ₂ R
Animal 1	0.61±0.49	2.74 ±0.38	0.67±0.28
Animal 2	1.18±0.69	3.45 ±0.54	1.16±0.39
Animal 3	2.26±0.35	3.28 ±0.27	1.45±0.20
Animal 4	1.83±0.49	2.66 ±0.38	1.22±0.28

Area density (number of immunoreactive profiles per square micron) for different VTA immunolabeling ($\alpha 7$ nAChR and/or D₂R) in each of four animals. Data are expressed as mean±standard error. Data were collected from 14 vibratome sections in four mice processed for dual labeling.

Table 2

Subcellular distribution of $\alpha 7$ nAChR and D₂R in the VTA

Profile Type	$\alpha 7$ nAChR labeling			D ₂ R labeling		
	Total	Single ($\alpha 7$)	Dual	Total	Single (D ₂)	Dual
Somata	35	1 (2.9%)	34 (97.1%)	34	0 (0%)	34 (100%)
Dendrites	1602	800 (49.9%)	802 (50.1%)	1633	831 (50.9%)	802 (49.1%)
Dendritic Spines	13	10 (76.9%)	3 (23.1%)	19	16 (84.2%)	3 (15.8%)
Axon Terminals	309	179 (57.9%)	130 (42.1%)	1178	1048 (89.0%)	130 (11.0%)
Unmyelinated Axons	142	132 (93.0%)	10 (7.0%)	199	189 (95.0%)	10 (5.0%)
Glia	646	561 (86.8%)	85 (13.2%)	237	152 (64.1%)	85 (35.9%)
TOTAL	2747	1683 (61.3%)	1064 (38.7%)	3300	2236 (67.8%)	1064 (32.2%)

Immunolabeling for $\alpha 7$ nAChR and/or D₂R in different neuronal compartments within the mouse VTA. Profiles containing single $\alpha 7$ nAChR- and dual $\alpha 7$ nAChR + D₂R-labelings are given as raw numbers and as percentage of the total $\alpha 7$ nAChR and/or total D₂R immunolabeled profiles in each category. Data were collected from 14 vibratome sections in four mice processed for dual labeling.

Table 3

Distribution of synaptic inputs to $\alpha 7$ nAChR- and/or D_2 R-immunolabeled dendrites in the VTA

Type of dendrite	Synapses	Presynaptic terminal (%)		
		Unlabeled	D_2 R	$\alpha 7$ nAChR+ D_2 R
D_2 R	244	159 (65.2%)	67 (27.4%)	9 (3.7%)
$\alpha 7$ nAChR+ D_2 R	267	161 (60.3%)	84 (31.5%)	11 (4.1%)
$\alpha 7$ nAChR	309	141 (45.6%)	118 (38.2%)	33 (10.7%)
Total	820	461 (56.2%)	269 (32.8%)	37 (4.5%)

Distribution of synapses made by dendrites labeled for $\alpha 7$ nAChR and/or D_2 R in the mouse VTA with respect to the immunoreactivity contained in the presynaptic axon terminal. Synapses are given as raw numbers and as percentage of the total synapses in each category. Data were collected from six vibratome sections in two mice processed for dual labeling.