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Acetylcholine $\alpha 7$ Nicotinic and Dopamine D_2 Receptors Are Targeted to Many of the Same Postsynaptic Dendrites and Astrocytes in the Rodent Prefrontal Cortex

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Abstract

The alpha-7 nicotinic acetylcholine receptor ($\alpha 7$ nAChR) and the dopamine D_2 receptor (D_2 R) are both implicated in attentional processes and cognition, mediated in part through the prefrontal cortex (PFC). We examined the dual electron microscopic immunolabeling of $\alpha 7$ nAChR and either D_2 R or the vesicular acetylcholine transporter (VACHT) in rodent PFC to assess convergent functional activation sites. Immunoreactivity (ir) for $\alpha 7$ nAChR and/or D_2 R was seen in the same as well as separate neuronal and glial profiles. At least half of the dually labeled profiles were somata and dendrites, while most labeled axon terminals expressed only D_2 R-ir. The D_2 R-labeled terminals were without synaptic specializations or formed inhibitory or excitatory-type synapses with somatodendritic profiles, some of which expressed the $\alpha 7$ nAChR and/or D_2 R. Astrocytic glial processes comprised the majority of nonsomatodendritic $\alpha 7$ nAChR or $\alpha 7$ nAChR and D_2 R-labeled profiles. Glial processes containing $\alpha 7$ nAChR-ir were frequently located near VACHT-labeled terminals and also showed perisynaptic and perivascular associations. We conclude that in rodent PFC $\alpha 7$ nACh and D_2 R activation can dually modulate (1) postsynaptic dendritic responses within the same or separate but synaptically linked neurons in which the D_2 R has the predominately presynaptic distribution, and (2) astrocytic signaling that may be crucial for synaptic transmission and functional hyperemia.

Keywords

electron microscopic immunolabeling; tripartite synapse; schizophrenia; functional hyperemia

INTRODUCTION

Basal forebrain derived cholinergic inputs to the prefrontal cortex (PFC; Henny and Jones, 2008; Sato et al., 2004; Zaborszky et al., 1997) play a key role in attentional and cognitive processes, which are often dysfunctional in schizophrenia (Levin and Simon, 1998; Robbins, 2000). Moreover, the alpha-7 nicotinic acetylcholine receptor ($\alpha 7$ nAChR) gene CHRNA7 is

genetically deficient in schizophrenia (Freedman et al., 1997; Olincy et al., 2006; Severance and Yolken, 2008). *In vivo* and *in vitro* microdialysis suggests that the $\alpha 7$ nAChR-mediated effects of acetylcholine (ACh) are ascribed in part to modulation of the cross-talk between glutamate and dopamine in the PFC (Livingstone et al., 2010). This modulation may occur in dopamine receptive neurons that are subject to pre- or postsynaptic regulation through activation of $\alpha 7$ nACh receptors (Duffy et al., 2009; Gault et al., 2003; Lubin et al., 1999).

Dopamine D₁ and D₂ receptor (D₂R) family members have been identified in both axon terminals and dendrites of PFC neurons (Muly et al., 1998; Negyessy and Goldman-Rakic, 2005). The D₂R is of special interest with respect to the $\alpha 7$ nAChR because of the effectiveness of both D₂R antagonists and nicotine in ameliorating many of the symptoms of schizophrenia (Levin and Rezvani, 2007). Imaging studies suggest that the antipsychotic effectiveness of D₂R blockade reflects suppression of the D₂R-mediated inhibition of glutamatergic transmission in the PFC (Laruelle et al., 2005; Wang and Goldman-Rakic, 2004). However, whether the D₂R is contained in the same or different neuronal compartments as $\alpha 7$ nAChRs in the PFC has not been explored.

The D₂R also is present in non-neuronal cells of the PFC (Negyessy and Goldman-Rakic, 2005). The glial expression of the D₂R has been extensively described (Bal et al., 1994; Khan et al., 2001; Negyessy and Goldman-Rakic, 2005) and shown to have a laminar-specific distribution comparable to that of neurons in the PFC (Khan et al., 2001; Pinto and Sesack, 2008). Moreover, the stimulation of D₂R in astrocytes increases their intracellular levels of Ca²⁺ (Khan et al., 2001; Li et al., 2006; Parpura and Haydon, 2000). Similarly, the $\alpha 7$ nAChR is both expressed and functionally active in enhancing intracellular Ca²⁺ in cortical astrocytes (Oikawa et al., 2005; Parri and Crunelli, 2003). However, it is not known whether the D₂R and $\alpha 7$ nAChR are present in the same astrocyte, an essential requirement for their synergistic involvement in intracellular calcium signaling in these cells.

The functional relevance of astrocytic D₂R and/or $\alpha 7$ nAChR in the PFC is dependent not only on the expression of these receptors, but also on the location of the astrocytes with respect to neighboring neurons and to the vasculature. Perisynaptic astrocytes participate in neurotransmission through their exchanges with pre- and postsynaptic neurons, which together form “the tripartite synapse” (Araque et al., 1999). The activation of these and other cortical astrocytes including those associated with microvessels is controlled in large part by intracellular Ca²⁺ signaling (Halassa et al., 2007; Perea and Araque, 2005). A single astrocyte may contact innumerable synapses and at the same time also form end feet on neighboring blood vessels, thereby being optimally positioned for regulation of synaptic transmission and neurovascular coupling (Haydon and Carmignoto, 2006). The reduction of cerebral blood flow (CBF) seen in the PFC of schizophrenic patients (Khan et al., 2001) whose symptoms are lessened by blockade of the D₂R or by activation of the $\alpha 7$ nAChR suggests that these receptors are co-expressed not only in some of the same neurons, but also in perisynaptic and/or perivascular astrocytes. The perivascular astrocytes are instrumental in functional hyperemia, the matching of CBF with ongoing synaptic activity (Gonzalez-Islas and Hablitz, 2003; Iadecola and Nedergaard, 2007).

We tested the hypothesis that $\alpha 7$ nACh and D₂ receptors are expressed within the same or synaptically associated neurons and within the perisynaptic and/or perivascular astrocytes having access to neuron-derived ACh in the PFC. The neuronal source of ACh was assessed by determining whether those astrocytes expressing the $\alpha 7$ nAChR are in contact with cholinergic terminals, or located within the diffusion sphere (<0.2 μ m) of ACh (Descarries et al., 1997; Descarries and Mechawar, 2000; Duffy et al., 2009). These objectives were achieved by using electron microscopic immunocytochemistry in two sets of dual labeling experiments: (1) $\alpha 7$ nACh and D₂ receptors and (2) $\alpha 7$ nAChR with the vesicular ACh

transporter (VACHT), a protein that is expressed in cholinergic terminals of the PFC (Descarries and Mechawar, 2000; Duffy et al., 2009). The analysis was conducted in the prelimbic PFC of rats and mice, the latter of which enabled the use of transgenic animals expressing green fluorescent protein (EGFP) for the glial fibrillary acidic protein (GFAP).

MATERIALS AND METHODS

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 Weill Cornell Medical College. The following animals were used: 16 male Sprague-Dawley rats (180–200 g; Charles River, Kingston, NY), 20 male C57BL/6J mice (20–25 g; Charles River), and 3 male transgenic mice EGFP under the control of the GFAP promoter (Zhou et al., 15–20 g; Jackson Laboratory, Bar Harbor, ME). All animals were deeply anesthetized by *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) 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 (McLane et al., 1992; Schoepfer et al., 1990). The $\alpha 7$ nAChR antibody has been shown to recognize a band at ~50 kDa molecular weight in Western blots of cultured hippocampal neurons and mouse, rat and macaque brain extracts (Arnaiz-Cot et al., 2008; Centeno et al., 2006; Dominguez Del Toro et al., 1994; Duffy et al., 2009). This antibody also yields discrete immunolabeling in (1) rat and mouse brain using bright field microscopy (Dominguez Del Toro et al., 1994; Duffy et al., 2009) or electron microscopy (Duffy et al., 2009; Fabian-Fine et al., 2001; Levy and Aoki, 2002) and (2) macaque brain using fluorescent microscopy (Centeno et al., 2006). The $\alpha 7$ nAChR-ir in the PFC is absent when the tissue is processed with omission of the primary antibody (Duffy et al. 2009), or with antigen pre-adsorbed $\alpha 7$ nAChR antibody (unpublished observation).

A commercial polyclonal VACHT antiserum (Chemicon, Temecula, CA) was used to identify cholinergic processes in the PFC. This antiserum was raised in goat against a synthetic peptide corresponding to amino acids 511–530 from cloned rat VACHT (Erickson et al., 1994; Roghani et al., 1994). Studies have been performed previously to characterize the antiserum, which selectively labels CV-1 cells transfected with VACHT cDNA (Arvidsson et al., 1997). This antiserum shows a specific band at the predicted 70 kDa molecular weight in Western blots of tissue (Brunelli et al., 2005). The VACHT antiserum has been used extensively in electron microscopy studies in the rat to identify cholinergic processes (Duffy et al., 2009; Garzon and Pickel, 2000; Kaplan et al., 2004; Pickel et al., 2000; Svingos et al., 2001; Towart et al., 2003).

The dopamine D₂R antipeptide antiserum was produced and affinity purified in the laboratory of coauthor Dr. Kenneth Mackie, Indiana University. This antiserum was generated in rabbit against amino acids 216–311 of the human D₂R long (D₂RL) isoform (Brana et al., 1997), which was cloned into the pET30c plasmid (Novagen, Madison, WI) and confirmed by sequencing. This antiserum recognizes a single band at 50 kDa, the predicted molecular mass of the D₂R, in Western blots of homogenates from rat brain and produces immunolabeling in cortical and striatal tissue that is eliminated by prior adsorption

with the antigenic peptide (Pickel et al., 2006). Western blot analysis and immunolabeling have also shown that the D₂R antiserum specifically identifies the D₂RL isoforms in human embryonic kidney (HEK) cells transiently transfected with the pcDNA-FLAG-D2L plasmid (Kearn et al., 2005), an observation that is extended and confirmed in the present study.

Fluorescence immunolabeling of HEK cells transiently expressing FLAG-D₂R long

HEK cells growing on glass coverslips were transiently transfected with FLAG-tagged mouse D₂RL isoform D₂LR (Kearn et al., 2005). The following day they were washed three times with ice-cold 0.1 M PB and then fixed with 4% paraformaldehyde in PB for 20 min at room temperature. Cells were then washed twice with PB and three times with 150 mM NaCl + PB (0.1 M PBS). Next, cells were incubated with a blocking solution (5% donkey serum + 0.1% saponin in 0.1 M PBS) for 30 min at room temperature. Cells were incubated with affinity-purified D₂R antibody (Kearn et al., 1999), diluted 1:500 in the blocking solution and anti-FLAG antibody (raised in mouse, 1:500, Sigma-Aldrich, St. Louis, MO) for 3 h at room temperature and then washed six times with 0.1 M PBS. Cells were then incubated with fluorescein isothiocyanate (FITC)-conjugated donkey anti-rabbit secondary antibody (1:150, Jackson ImmunoResearch Laboratories, West Grove, PA) and Texas Red-conjugated donkey anti-mouse secondary antibody (1:150) for 1 h at room temperature. Finally, cells were washed three times with 0.1 M PBS, twice with 0.1 M PB, and three times with water, and air-dried. Dried coverslips were inverted and mounted on a drop of Vectashield containing DAPI (Vector Laboratories, Inc., Burlingame, CA) and sealed with nail polish. Stained cells were examined with a Nikon Eclipse (TE2000-E) confocal microscope (Fryer Company, Inc., Huntley, IL) with the appropriate excitation filters for FITC (D₂RL) and Texas Red (FLAG). The 4',6-diamidino-2-phenylindole (DAPI) staining was used for the identification of HEK cell nuclei (Schmued et al., 2005).

Fixation and tissue preparation

Brain tissue was aldehyde fixed for immunolabeling by perfusion through the ascending aorta (rat) or the left ventricle of the heart (mouse). This was achieved using a Masterflex pump (Barnat Company, Barrington, IL) to rapidly fix tissue (<5 min) using sequentially delivered solutions of (1) normal saline (0.9%) containing 1000 units/ml of heparin (10–15 ml), (2) 40–50 ml of 0.1 M phosphate buffer (PB; pH 7.4) containing 3.75% acrolein and 2% paraformaldehyde, and (3) 150 ml of 2% paraformaldehyde in PB. The transgenic mice expressing GFAP-EGFP were fixed by trans-cardiac perfusion with the heparin-saline solution followed by 100 ml of 4% paraformaldehyde in PB. The brains were then removed from the cranium, cut into 4–5 mm coronal blocks and postfixated for 30 min in the latter aldehyde solution.

Coronal sections (40 µm thick) were cut through the PFC using a Leica Vibratome (Leica Microsystems, Bannockburn, IL). Sections through the PFC corresponded to +2.8 mm from Bregma in rat (Paxinos and Watson, 1998) and +2.2 mm from Bregma in the mouse (Franklin and Paxinos, 1997). Sections through the PFC from both rat and mouse were initially processed for light microscopic immunolabeling of α 7nAChR, VACHT or the D₂R in the presence of 0.25% Triton X-100 to determine the optimal dilution for each primary antiserum and to assess the laminar distribution of the receptors.

Astrocytic Immunofluorescence labeling

The astrocytic localization α 7nAChR and D₂R was ascertained by examining the immunofluorescent labeling of their respective antisera in coronal sections through the PFC of the GFAP-EGFP transgenic mice. The labeling method was modified from that described by Drake and Milner (2002) for other receptor proteins. The PFC sections were incubated with the α 7nAChR (1:500) antibody or D₂R (1:200) antiserum for 24 hours at 4°C. They

were then incubated in either Cy5 goat anti-mouse IgG (1:200; Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA) for the detection of $\alpha 7$ nAChR or Cy5 goat anti-rabbit IgG (1:200; Jackson ImmunoResearch Laboratories Inc.) for detection of the D₂R. Immunofluorescence serial Z-stack images were acquired sequentially using a confocal laser-scanning microscope (Leica, Nussloch, Germany). Cy5 was pseudocolored red while the native GFAP-EGFP positive cells were pseudocolored green. Labeling for each receptor and native GFAP-EGFP in astrocytic processes was confirmed by confocal Z-stack analysis and rotation of the stacks in multiple planes. Images were analyzed and collapsed into single images in Image J (Rasband, 1997–2004 Image J, NIH). Photomicrographs were collected and assembled in Power Point software (Power-Point 2007, Microsoft Corporation, Redmond, WA) after adjusting images in Adobe Photoshop 7.0 (Adobe Systems, San Jose, CA).

Immunoperoxidase and Immunogold labeling

Tissue sections through rat and mouse PFC were coprocessed for light and electron microscopy after optimization of the immunolabeling. Dual-immunogold and immunoperoxidase labeling of $\alpha 7$ nAChR with either VAcHT or D₂R was carried out according to the methods described in Duffy et al. (2009). The markers were reversed in a few sections to ensure maximal detection of each antiserum as there are known differences in sensitivity for each method (Chan et al., 1990). To obtain optimal dilutions of each primary antiserum, PFC sections were initially processed for single immunogold and immunoperoxidase labeling of $\alpha 7$ nAChR, VAcHT and D₂R. Other controls consisted in processing the tissue for immunoperoxidase and immunogold labeling, omitting each of the primary antisera.

Electron microscopic tissue processing and data analysis

Immunolabeled PFC sections used for electron microscopy were processed according to the methods described by Chan et al. (1990). Ultrathin sections from PFC Layers III–V, were collected on 400-mesh copper grids, counterstained with uranyl acetate and Reynold's lead citrate and examined with a CM10 electron microscope (FEI, Mahwah, NJ).

Images were collected only from ultrathin sections near the plastic-tissue interface (i.e., surface of the tissue), a precaution that reduces false negatives resulting from inadequate penetration of antisera (Leranth et al., 1989). The classification of identified cellular elements was based on the descriptions of Peters et al. (1991). Axon terminals were defined by the presence of numerous synaptic vesicles and were greater than 0.1 μ m in diameter. Small, unmyelinated axons were <0.1 μ m and rarely contained small vesicles. Neuronal somata were identified by the presence of a nucleus, Golgi apparatus, and rough endoplasmic reticulum. The dendrites also contained abundant endoplasmic reticulum, but few, if any small vesicles. Dendrites and dendritic spines were postsynaptic to axon terminals. Glial processes were identified by their irregular shape, which often followed the contours of neuronal or vascular structure. Larger astrocytic processes often contained intermediate filaments and were connected with each other by gap junctions. Asymmetric synapses were recognized by thick postsynaptic densities, whereas symmetric synapses had thin pre- and postsynaptic membrane specializations. The use of electron microscopy in this study was essential for the identification of the type of synapse at which the receptors of interest were present. In addition, electron microscopy is the only technique that allows for the characterization and quantification of receptors in different cellular compartments, on different cells types and the appositions of the labeled cells. These techniques have been used extensively for such characterization (Drake and Milner, 2002; Duffy et al., 2009; Levy and Aoki, 2002; Pickel et al., 2000, 2006; Wang et al., 2003), which cannot be identified using light or confocal microscopy. Zones of closely spaced parallel neuronal and plasma

membranes, without discernible synaptic densities or glial interventions, were defined as appositions or non-synaptic contacts.

A profile was considered to be selectively labeled for immunoperoxidase when it contained electron dense cytoplasmic precipitate not seen in morphologically similar profiles located within the surrounding neuropil. Large profiles $>0.5 \mu\text{m}$ were considered immunogold labeled when they contained two or more gold particles. In small ($<0.5 \mu\text{m}$ diameter) dendrites or in small axons ($<0.1 \mu\text{m}$ diameter), a single particle was considered positive for immunogold labeling when no gold particles were detected over myelin or other tissue components not expected to express the receptor. The validity of this approach has been established previously for several other tissue antigens (Garzon and Pickel, 2006; Hara et al., 2006; Wang et al., 2003). Previous studies from our laboratory have shown that immunogold processing without addition of primary antibody to the tissue results in very few randomly scattered gold particles, a density of $<0.001 \mu\text{m}^{-2}$ gold particles was observed in an area comparable to the area examined in the present study (Wang et al., 2003). This approach combined with the extensive characterization of the antisera used in this study helps to limit the possibility of false positive results, but may have contributed to an underestimation of the number of neuronal and glial profiles that express $\alpha 7\text{nACh}$ and/or D_2 receptors in levels that are below the sensitivity of the detection methods.

For quantification, electron microscopic images were captured from at least three immunolabeled vibratome sections through the PFC of each animal ($n = 4$ rats and 4 mice). These sections included those processed for $\alpha 7\text{nAChR}$ -immunoperoxidase and D_2R -immunogold, or VAcHT -immunoperoxidase and $\alpha 7\text{nAChR}$ -immunogold labeling. All immunoreactive processes ($n = 2430$) were counted in randomly sampled electron microscopic images taken at a magnification of 13,500–34,000 $\times$. The total area of tissue examined was 9321 μm^2 . The tissue was examined quantitatively to determine the relative frequencies with which the immunoreactive products were localized within neuronal somata, dendrites, axons, and glial profiles. In addition, each $\alpha 7\text{nAChR}$ -labeled neuronal profile was defined with respect to appositional contacts and distance from nearest VAcHT -labeled axon. One-way analysis of variance (ANOVA) was performed using SPSS (SPSS, Chicago, IL) to ensure there was no significant inter-animal variability in the number of immunoreactive $\alpha 7\text{nAChR}$ -labeled profiles, D_2R -labeled profiles or VAcHT -labeled axons.

The electron microscopic images used for the analysis and figures were acquired with an AMT digital camera (Advanced Microscopy Techniques Corporation, Danvers, MA) on a Microsmart computer with a Windows 2000 operating system (Microsoft Corporation). Adobe Photoshop 7.0 (Adobe Systems) was used for adjustment of sharpness, contrast and brightness. The final images were then imported to PowerPoint 2007 software (Microsoft Corporation) for assembly and labeling composite figures.

RESULTS

Light microscopy revealed $\alpha 7\text{nAChR}$ -ir primarily in pyramidal cells and processes having a distinct laminar distribution in layers III and V of the prelimbic cortex, as defined by Gabbott et al. (1997) for the rat and Van De Werd et al. (2010) for the mouse (Fig. 1A). The D_2R -ir was also seen by light microscopy in many perikarya and processes in layers III and V of the prelimbic cortex. However, this labeling was less notably laminar and appeared to include both pyramidal cells and interneurons (Fig. 1B).

Ultrastructural analysis established that $\alpha 7\text{nAChR}$ -ir and D_2R -ir are located within some of the same, and also in many separate neuronal and glial profiles in the middle layers of the PFC. The neuronal and glial labeling for D_2 and $\alpha 7\text{nACh}$ receptors are jointly described in

the PFC of rat and mouse, since their immunolabeling patterns were qualitatively similar in both species.

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

Many large somata showed organized stacks of rough endoplasmic reticulum, typical features of cortical pyramidal cells, and contained one or both receptors (Peters et al., 1991). In somata (Fig. 2A) and proximal dendrites (Fig. 2B), the $\alpha 7$ nAChR-ir was often seen in cytoplasmic aggregates that were near, but did not overlap with the D₂R immunogold. This D₂R immunogold was dispersed in the cytoplasm or associated with cytoplasmic membranes such as those of the rough and smooth endoplasmic reticulum, or endosome/multivesicular bodies (Figs. 2A and 2B). Occasionally, the labeling for each of the receptors could be seen on separate portions of some of the same segments of endoplasmic reticulum or different surfaces of a single multivesicular body (Fig. 2B). In contrast with the pyramidal neurons, other dually labeled somata were characterized by a more diffuse cytoplasmic distribution of $\alpha 7$ nAChR-ir and low abundance of stacked rough endoplasmic reticulum (Fig. 2C). In these cells, the D₂R-ir also was dispersed in the cytoplasm, but was additionally localized to the endomembranes and portions of the plasmalemma contacted by axon terminals. These morphological features are similar to those described for D₂R-containing inhibitory interneurons (LeMoine and Gaspar, 1998; Van De Werd et al., 2010).

The differential subcellular distributions of $\alpha 7$ nAChR-ir seen in somata also were evident in dendritic shafts (Figs. 3A–3F). These dendritic shafts were among the most abundant of all labeled profiles and included those containing exclusively $\alpha 7$ nAChR-ir (Figs. 3A, 3C, and 3D) or D₂R-ir (Figs. 3A, 3E, and 3F) as well as those containing immunolabeling for both receptors (Fig. 3B). The $\alpha 7$ nAChR-ir has an aggregated distribution within the cytoplasm in many of the large (Fig. 3A) as well as in some of the small-medium diameter (Fig. 3D) dendrites. In other small-medium diameter dendritic shafts, the $\alpha 7$ nAChR-ir was more diffusely distributed throughout the cytoplasm or accumulated on the cytoplasmic surface of the postsynaptic membrane specialization at asymmetric excitatory-type synapses (Figs. 3B and 3C). These more diffusely labeled dendritic shafts were among those that contained both $\alpha 7$ nAChR-ir and D₂R-immunogold (Fig. 3B). Within dendritic shafts, the D₂R immunogold was located within and near postsynaptic membrane specializations of symmetric (Figs. 3B and 3E) or asymmetric (Fig. 3F) synapses. More commonly, however, these immunogold particles appeared to be randomly distributed within the cytoplasm or aligned near endomembranes running parallel to nonsynaptic portions of the plasma membrane (Figs. 3A and 3B).

Dendritic spines also contained $\alpha 7$ nAChR-ir and/or D₂R-ir (Figs. 3G–3J), and these spines received synaptic input from axon terminals that exhibited D₂R-ir (Fig. 3G), $\alpha 7$ nACh-ir (Fig. 3H), or no detectable immunoreactivity (Figs. 3I and 3J). In dendritic spines, $\alpha 7$ nACh-ir was intensely localized to postsynaptic membrane specializations at asymmetric excitatory-type synapses on spine heads (Figs. 3H and 3I), whereas D₂R immunogold was more often seen within the cytoplasm just beneath this type of synapse (Figs. 3G–3J). The $\alpha 7$ nAChR-ir was also lightly distributed throughout the cytoplasm or accumulated on largely nonsynaptic portions of the plasma membrane in dendritic spines (Fig. 3J). The subcellular location of $\alpha 7$ nAChR-ir in dendritic spines appeared independent D₂R-ir (Fig. 3H and 3J).

Species comparison of the number of $\alpha 7$ nAChR- and D₂R-labeled somatodendritic profiles

Quantitative analysis of the somatodendritic and spinous distribution of $\alpha 7$ nAChR and/or D₂R immunoreactivity revealed a similar labeling pattern in rats and mice (Table I). Of all dually labeled profiles, over 50% ($n = 228$ in mouse and 148 in rat; Table I) were somata

and dendrites and about 6% ($n = 142$; Table I) were dendritic spines. However, of all single $\alpha 7$ nAChR-labeled profiles, less than 25% ($n = 177$ in mouse and 264 in rat; Table I) were somata and dendrites and ~20% were dendritic spines. Similarly, somata and dendrites comprised approximately 35% ($n = 619$ in mouse and $n = 853$ in rat; Table I) of all single D₂R-labeled profiles, and less than 10% of these profiles were dendritic spines. These distributions suggest that in the rodent PFC, the D₂R is coexpressed with the $\alpha 7$ nAChR in spiny dendrites, and is also present in other largely aspiny dendrites that do not contain the $\alpha 7$ nAChR.

Presynaptic location of D₂R and/or 7nAChR labeling

D₂R-immunogold was seen within the cytoplasm overlying synaptic vesicles (Fig. 3B) or in contact with mainly non-synaptic portions of the plasma-lemma in many axon terminals (Fig. 3G). The axon terminals containing exclusively D₂R-ir were the most abundant of all single or dually labeled profiles in the PFC of either mouse or rat. These terminals comprise 54% ($n = 619$) in mouse and 61% ($n = 853$) in rat of all single D₂R-labeled profiles, but only <7% of the single $\alpha 7$ nAChR-labeled and almost none of the dually labeled profiles in the PFC of either species (Table I).

The single D₂R-labeled axonal varicosities in either mouse or rat were largely without recognizable synaptic membrane specialization within a single plane of section. Of those forming synapses ($n = 206$) in the mouse, 35% showed symmetric junctions mainly on dendritic shafts, while 55% were characterized by asymmetric, excitatory-type junctions located primarily on dendritic spines, and the remaining contacts were not readily distinguished. Similarly, of 293 single D₂R-labeled terminals forming synapses in rat PFC, 35% showed symmetric (Fig. 4A) and 65% asymmetric (Fig. 4B) membrane specializations. The dendritic profiles targeted by the D₂R-containing axon terminals were often without detectable immunoreactivity for either the D₂R or $\alpha 7$ nACh receptor. However, approximately 10% ($n = 206$ in mouse and 293 in rat) of the synaptic D₂R-labeled axon terminals contacted dendritic profiles containing D₂R-ir (Fig. 3G). In addition, ~25% (mouse) and 16% (rat) of these D₂R-labeled terminals were presynaptic to dendritic shafts (Fig. 4A) or dendritic spines (Fig. 4B) containing $\alpha 7$ nAChR-ir.

Despite their small number, about 2% ($n = 40$ in mouse and 72 in rat) of single $\alpha 7$ nAChR-labeled axon terminals formed asymmetric excitatory-type synapses on D₂R-containing dendritic spines (Fig. 3H), and about 5% of them showed either symmetric synapses or appositional contacts with D₂R-containing dendritic shafts (Fig. 4C). Because of their scarcity, the dually labeled axon terminals were not characterized with respect to the symmetry or asymmetry of their synaptic junctions. Indeed most of the dually labeled axonal profiles were small varicosities without recognizable membrane specializations (Fig. 4D).

Astrocytic localization of $\alpha 7$ nAChR and/or D₂R immunoreactivity

Electron microscopy revealed the presence of $\alpha 7$ nAChR-ir alone or together with the D₂R-ir in many glial processes (Fig. 6; Table I). These processes constituted around 50% of all single $\alpha 7$ nAChR or dual labeled profiles in the mouse PFC. In rat, however, 75% of all single $\alpha 7$ nAChR and 20% of the dual labeled profiles were glial processes (Table I). In contrast with the prevalence of D₂R-labeled axon terminals, glial processes comprised <5% of the single D₂R-labeled profiles. No labeling was observed in morphologically identifiable oligodendrocytes.

The expression of $\alpha 7$ nAChR and D₂R in astrocytic glial processes was confirmed using confocal microscopy in the PFC of transgenic mice expressing native GFAP-EGFP. Some

GFAP-containing astrocytes showed immunofluorescent labeling for the $\alpha 7$ nAChR (Figs. 5A–5C) or the D₂R (Figs. 5D–5F). Both $\alpha 7$ nAChR-ir and D₂R-ir (Figs. 5D and 5E) were localized to the perinuclear regions of the cytoplasm and to thick-branched processes of many astrocytes in the PFC of the transgenic mice (Figs. 5B–5E). HEK cells transfected with FLAG-tagged mouse D₂RL long isoform show identical patterns of immunoreactivity with D₂R and FLAG antisera, further validating the specificity of our D₂R antibody (Figs. 5G–5I).

Localization of $\alpha 7$ nAChR and/or D₂R to perisynaptic astrocytic processes

The $\alpha 7$ nAChR was present alone or together with the D₂R in many astrocytic glial processes (Figs. 6C and 6D; Table I). Electron microscopic immunolabeling established that the $\alpha 7$ nAChR had a restricted subcellular location in astrocytic processes, many of which were inserted near asymmetric excitatory-type synapses on dendritic spines (Figs. 6A–6D) and small dendrites (Fig. 6E). In sections through the PFC that were processed for dual labeling of the $\alpha 7$ nAChR and the VAcHT (Fig. 6B), the $\alpha 7$ nAChR-labeled astrocytic processes were observed in direct contact with vesicle-filled axonal profiles containing VAcHT immunoreactivity. More frequently, however, the $\alpha 7$ nAChR-containing astrocytic processes were located at short distances ($<0.2 \mu\text{m}$) from the VAcHT-labeled axon terminals in both rat and mouse (data not shown).

Perivascular astrocytic distribution of $\alpha 7$ nACh and D₂ receptors

The $\alpha 7$ nAChR-ir was detected in astrocytic end feet contacting the basement membrane of microvessels (Fig. 7A; Table II). The perivascular astrocytic processes comprised 15% of the total $\alpha 7$ nAChR-labeled glial processes (Table II). The $\alpha 7$ nAChR-labeled perivascular astrocytic processes were either in contact with or short distances from vesicle-filled axonal profiles immunoreactive for the VAcHT (Fig. 7B; Table II). In addition, the $\alpha 7$ nACh and D₂ receptors were coexpressed in the perivascular astrocytes (Figs. 7C and 7D; Table II). Since the D₂R labeling was seen alone in relatively few glial processes exclusive of those that also contained the $\alpha 7$ nAChR, we can extrapolate that the D₂R-containing perisynaptic and perivascular astrocytes are proportionally similar in number to those expressing the $\alpha 7$ nAChR.

DISCUSSION

Our results provide immunocytochemical evidence that $\alpha 7$ nACh and dopamine D₂ receptors are targeted to many of the same as well as separate, but synaptically linked, cortical neurons and to both perisynaptic and perivascular astrocytes in rodent PFC (Fig. 8). The observed colocalization of $\alpha 7$ nAChR-ir and D₂R-ir within many of the same somatodendritic and glial profiles in this region suggests that there may be convergent signaling between the $\alpha 7$ -subunit and the G-protein pathway within postsynaptic neurons and in astrocytic glia in the PFC. In addition, we have shown that in contrast with dendritic and glial profiles, D₂R-ir is more commonly found alone in axon terminals having diverse morphologies. These morphologies are consistent with a major involvement of dopamine D₂ receptors in regulation of the presynaptic release of (1) modulators from non-synaptic axon terminals and (2) both inhibitory and excitatory transmitters from axon terminals that respectively form symmetric and asymmetric synapses. These results are discussed with respect to their implications for understanding and treating schizophrenia as well as control of cerebral blood flow affecting imaging studies.

Somatodendritic distribution of $\alpha 7$ nACh and D₂R

The $\alpha 7$ nAChR-ir was seen in large cytoplasmic aggregates in somata and proximal dendrites that also sometimes contained D₂R-ir and had the morphological features of

cortical pyramidal neurons. The identification of these cells as pyramidal neurons is consistent with the many $\alpha 7$ nAChR- and/or D₂R-labeled dendritic spines that are characteristic of pyramidal neurons and rarely seen on cortical interneurons (Peters et al., 1991). The cytoplasmic aggregates of $\alpha 7$ nAChR-ir may reflect large intracellular receptor pools, which have been suggested to provide a mechanism for the rapid ACh-induced upregulation of the nAChR (Drenan et al., 2008; Millar and Harkness, 2008).

The pyramidal-like neurons also showed $\alpha 7$ nAChR-ir and D₂R-ir distributed along portions of the endoplasmic reticulum (ER) and endosome/multivesicular bodies that are respectively involved in the synthesis and degradation of receptor proteins (Geetha and Wooten, 2008; Keller et al., 2001; Kim et al., 2008; Prou et al., 2001; Piper and Katzmann, 2007; Rezvani et al., 2010). In some cases, both the $\alpha 7$ nAChR-ir and D₂R-ir were localized to single segments of ER. This commonality in distribution may reflect the involvement of the ER chaperone, calnexin, in the clathrin-dependent subunit trafficking and assembly of nAChRs (Gelman et al., 1995; Wanamaker and Green, 2007) and both the ER retention and surface trafficking of the D₂R (Free et al., 2007). Together, these observations suggest a postsynaptic neuronal affiliation between nAChR $\alpha 7$ subunits and the G-protein coupled D₂R in the PFC. This may be similar to the previously demonstrated cellular link between nAChR beta-2 subunits and other G-protein pathways (Kabbani et al., 2007).

The $\alpha 7$ nACh and D₂R labeling also was detected in neurons having the morphological features of interneurons, which appear to be among those cells showing the more diffuse distribution of $\alpha 7$ nAChR-ir and receiving many asymmetric excitatory-type axodendritic synapses (Rotaru et al., 2005). This finding confirms and extends evidence that these receptors are expressed in parvalbumin containing interneurons of the PFC (LeMoine and Gaspar, 1998; Lubin et al., 1999; Porter et al., 1999). In contrast with pyramidal neurons, however, the $\alpha 7$ nAChR-ir in the putative interneurons was less aggregated and more diffusely distributed in the cytoplasm, suggesting that the interneurons may have a more limited capacity for rapidly up-regulating their responses to ACh by mobilization of these receptors from cytoplasmic pools of receptors (Drenan et al., 2008; Millar and Harkness, 2008).

The D₂R-ir was prevalent on membranes of the endoplasmic reticulum and on both synaptic and non-synaptic plasmalemmal surfaces of the dendritic shafts whose diffusely distributed $\alpha 7$ nAChR-ir resembled that of the putative interneurons. As in pyramidal cells, the D₂R-mediated effects of dopamine on parvalbumin-containing interneurons within the PFC may be ascribed in part to modulation of postsynaptic glutamate currents (Porter et al., 1999). The diverse D₂R distribution may facilitate spatially and temporally diffuse dopamine signaling in the PFC (Lapish et al., 2007).

Localization of $\alpha 7$ nACh and D₂R to dendritic spines

The present localization $\alpha 7$ nACh-ir to postsynaptic membrane specializations in dendritic spines of the PFC confirms and extends our prior evidence for preferential localization of these receptors in dendritic spines of pyramidal neurons within the diffusion sphere of ACh (Duffy et al., 2009). This distribution is comparable to that of NMDA receptors and supports the idea 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-ir was also located in dendritic spines, some of which showed labeling for both receptors. Unlike the $\alpha 7$ nAChR, however, the D₂R-ir was usually distributed in the cytoplasm below or lateral to, but not within, the excitatory-type postsynaptic densities. The differential postsynaptic distribution of the $\alpha 7$ nACh and D₂ receptors is consistent with the

respective coupling of these receptors to Ca^{2+} channels (Collins et al., 2009) and G-protein pathways that can modulate glutamate NMDA currents (Martina and Bergeron, 2008). In addition, D_2R stimulation can also decrease the surface and synaptic expression of GluR1, an obligatory subunit of glutamate AMPA receptors (Sun et al., 2005). Thus, the activation of the D_2R may modulate both the NMDA and AMPA receptor-mediated postsynaptic responses in PFC neurons.

Presynaptic distribution of $\alpha 7\text{nAChR}$ and D_2 receptors

The $\alpha 7\text{nAChR}$ -ir was rarely detected alone or together with D_2R -ir in axon terminals of the PFC. However, the single $\alpha 7\text{nAChR}$ -labeled terminals in the PFC included those that were nonsynaptic as well as those forming inhibitory- or excitatory-type synapses on D_2R -containing dendritic shafts and spines, respectively. The location of $\alpha 7\text{nAChR}$ in excitatory-type terminals supports prior evidence that nicotine potentiates excitatory transmission through activation of presynaptic $\alpha 7\text{nAChRs}$ in the PFC (Wang et al., 2006) and other brain regions (Good and Lupica, 2009; Jones and Wonnacott, 2004). However, the scarcity of $\alpha 7\text{nAChR}$ -labeled terminals forming excitatory-type synapses in this region suggests that there may be alternate mechanisms contributing to nicotine-induced potentiation of glutamatergic transmission.

Unlike the $\alpha 7\text{nAChR}$, the D_2R -ir in the PFC was located in many axon terminals. In these terminals, the D_2R -ir was seen mainly over membranes of synaptic vesicles, a distribution similar to that of the inhibitory Gi-protein (Aronin and DiFiglia, 1992). The majority of the D_2R -labeled terminals were without recognizable synaptic junctions, although many others formed either symmetric or asymmetric synapses, with the latter being more prevalent. This morphological heterogeneity suggests involvement of D_2Rs in the presynaptic regulation of the release of functionally diverse transmitters. Conceivably, the nonsynaptic axon terminals that contain D_2R or D_2R and $\alpha 7\text{nAChR}$ may be those belonging to either dopaminergic (Turner, 2004), serotonergic (Artigas, 2010; Levin and Rezvani, 2007), or cholinergic (Descarries et al., 1997; Mechawar et al., 2000) neurons. These neuromodulators are often stored in axon terminals without recognizable synapses (Drenan et al., 2008; Millar and Harkness, 2008), which elicit many of their physiological effects by volume transmission (Descarries et al., 1997; Fuxe et al., 2010).

Our results suggest a predominant involvement of the D_2R in the presynaptic release of glutamate or other excitatory transmitter in axon terminals forming asymmetric excitatory-type synapses on dendritic spines, some of which express the $\alpha 7\text{nAChR}$ in the PFC. However, the larger number of D_2R -labeled terminals forming asymmetric compared with symmetric synapses may also reflect in part the greater number of the excitatory-type axospinous synapses in the cerebral cortex (Peters et al., 1991). The D_2R -labeled terminals forming excitatory-type synapses in the PFC may derive either from the glutamatergic pyramidal neurons or glutamatergic inputs from other brain regions, and specifically those from the thalamus (Lambe et al., 2003). The presence of D_2R -ir in axon terminals forming excitatory-type synapses in the PFC supports evidence that activation of these receptors can enhance glutamate release, and thereby increase the release of dopamine in this brain region (Livingstone et al., 2010).

D_2R -labeled axon terminals also formed symmetric synapses typical of those containing GABA (Torrealba and Müller, 1999), even though the number of these terminals was somewhat smaller than those forming asymmetric synapses. The inhibitory-type terminals may derive from GABAergic interneurons in the PFC or from cortically projecting GABAergic neurons located in the ventral tegmental area (Carr and Sesack, 2000). The conclusion that GABA is present in many of the D_2R -labeled terminals that contact dendritic shafts, and particularly those shafts containing the $\alpha 7\text{nAChR}$ is consistent with

prior studies showing D₂R-dependent regulation of GABAergic currents in pyramidal cells within the PFC (Trantham-Davidson et al., 2008). However, mesocortical dopamine terminals also sometimes form symmetric synapses with dendritic shafts of interneurons in the PFC (Sesack et al., 1998). The shafts of these interneurons are likely to be among those showing both pre- and postsynaptic distributions of D₂R-ir.

Proximity between $\alpha 7$ nACh-ir astrocytes and cholinergic terminals

Our results showing that $\alpha 7$ nAChR-labeled astrocytic processes in the PFC are in contact with varicose axon terminals that express VAcHT-ir suggest that cholinergic terminals are a major source of the endogenous ligand for the $\alpha 7$ nAChR in cortical astrocytes. The proximity between the VAcHT-labeled terminals and $\alpha 7$ nAChR-containing astrocytic processes is comparable to that shown previously for neuronal $\alpha 7$ nAChRs in the PFC (Duffy et al., 2009). Volume transmission may facilitate the activation of all glial and/or neuronal $\alpha 7$ nAChRs that are located at distances within the diffusion sphere of ACh (Coggan et al., 2005; Descarries and Mechawar, 2000). In addition, $\alpha 7$ nAChRs can be activated by other factors, such as choline derived from phosphatidylcholine, which is found on the plasma membrane and a binding protein secreted by glia is also involved in mediating cholinergic transmission (Bramanti et al., 2008; Smit et al., 2001; Uteshev et al., 2003).

The VAcHT-labeled terminals contacted both perisynaptic and perivascular astrocytic processes containing $\alpha 7$ nAChR-ir. Thus, these cholinergic terminals are likely the primary source of ACh needed for $\alpha 7$ nAChR activation. However, non-neuronal cells including the vascular endothelium also have the capacity to produce ACh (Arneric et al., 1988; Fujii et al., 2009), which is another potential source of the endogenous ligand for $\alpha 7$ nAChRs in perivascular astrocytes.

Perisynaptic and perivascular distribution of $\alpha 7$ nACh and D₂ receptors in astrocytes

The present detection of $\alpha 7$ nAChR-ir in perisynaptic astrocytic processes enclosing excitatory-type synapses in the PFC suggest that in this brain region astrocytic $\alpha 7$ nAChR may play an important role in the $\alpha 7$ nAChR-mediated effects of ACh on glutamatergic transmission. Astrocytes are responsible for the uptake and clearance of glutamate as well as the conversion of glutamate to glutamine, the substrate for neuronal synthesis of glutamate (Oikawa et al., 2005; Patti et al., 2007; Sharma and Vijayaraghavan, 2001). An increase in intracellular Ca²⁺ can evoke glutamate release (Simard and Nedergaard, 2004), and glutamate-dependent currents in surrounding neurons (Araque et al., 1998; Bezzi et al., 1998; Pasti et al., 1997; Stigliani et al., 2006). These observations together with the known $\alpha 7$ nAChR-mediated enhancement of intracellular Ca²⁺ in astrocytes (Oikawa et al., 2005; Sharma and Vijayaraghavan, 2001) support our working hypothesis that the perisynaptic astrocytes expressing the $\alpha 7$ nAChR are critical modulators of excitatory transmission in the PFC. In other brain regions, the $\alpha 7$ nAChR is rarely detected in glia and prevalent in excitatory-type terminals (Jones and Wonnacott, 2004; Serova and Sabban, 2002) suggesting that the glial $\alpha 7$ nAChR in the PFC is at least partially region specific.

The perisynaptic and perivascular astrocytic processes expressing the $\alpha 7$ nAChR were among those that also showed both plasmalemmal and cytoplasmic D₂R-ir, which was rarely seen in glial profiles without detectable $\alpha 7$ nAChR-ir. This suggests that the expression of the $\alpha 7$ nAChR identifies a unique subgroup of PFC astrocytes, whose activities are dually controlled by ACh and by D₂R-activation. The D₂R is coupled to inhibitory Gi proteins (LeMoine and Gaspar, 1998; Missale et al., 1998). Thus, the presence of the D₂R in astrocytic processes around excitatory-type axospinous synapses is consistent with known expression of Gi proteins in perisynaptic astrocytes (Aronin and DiFiglia, 1992). The colocalization of D₂ and $\alpha 7$ nACh receptors in perisynaptic astrocytes of the PFC suggest

that the in vivo activation of both receptors may synergistically enhance intracellular Ca^{2+} levels as has been shown in cultured astrocytes (Khan et al., 2001; Finkbeiner, 1993; Oikawa et al., 2005; Reuss and Unsicker, 2001).

The presence of $\alpha 7\text{nAChR}$ -ir in perivascular astrocytes in contact with the endothelial cells of microvessels in the PFC is consistent with evidence that astrocytes cocultured with endothelial cells have higher expression levels of the $\alpha 7\text{nAChR}$ and a more highly developed signal transduction system (Delbro et al., 2009). In addition, the cocultured astrocytes are more sensitive to nicotine, which induces a pronounced increase in intracellular Ca^{2+} signaling that is dysfunctional in inflammatory reactive astrocytes (Delbro et al., 2009). Many of the $\alpha 7\text{nAChR}$ containing perivascular astrocytes also expressed the D_2R suggesting that like the perisynaptic astrocytes, an increase in intracellular Ca^{2+} can be induced by activation of one or both receptors. However, in contrast with the perisynaptic astrocytes where enhanced intracellular Ca^{2+} signaling may be more directly related to glutamatergic transmission, the enhanced intracellular Ca^{2+} in perivascular astrocytes may promote the release of prostanoids to produce vasodilation and enhance cerebral blood flow (Choi et al., 2006; Marota et al., 2000; Mandeville et al., 2001; Sato et al., 2004; Uchida et al., 1997).

Implications

Our results provide new insight into the mechanisms by which ACh and dopamine integrate neuronal signals that are crucial for cognitive processes (Gonzalez-Islas and Hablitz, 2003). They specifically identify dendrites and spines as the major neuronal sites for convergent $\alpha 7\text{nAChR}$ - and D_2R -mediated effects on the postsynaptic excitability of cortical neurons, while suggesting that the presynaptic release of both excitatory and inhibitory transmitters is mainly ascribed to the activation of the D_2R in axon terminals. Furthermore, the observed scarcity of $\alpha 7\text{nAChR}$ -labeled excitatory-type axon terminals and colocalization of $\alpha 7\text{nAChR}$ and D_2R immunoreactivities in many perisynaptic astrocytic processes suggests involvement of astrocytes in ACh- and/or dopamine-evoked changes in the synaptic availability of glutamate in the PFC.

The findings of our study have important implications for understanding the potential therapeutic effectiveness of nicotine and other nAChR agonists in treating the cognitive and attentional deficits in schizophrenic patients (Olincy et al., 2006). The specific involvement of $\alpha 7\text{nAChRs}$ in mediating these effects is suggested by the fact that nicotine and 3-[(2,4-dimethoxy)benzylidene]-anabaseine (DMXB-A), two partial $\alpha 7\text{nAChR}$ agonists, significantly improve neurocognitive disturbances in schizophrenia (Olincy et al., 2006). In contrast with $\alpha 7\text{nAChR}$ agonists, all classical antipsychotic drugs block D_2Rs and produce a reduction in the positive psychotic features of schizophrenia (Pratt et al., 2008). Haloperidol is a well known classic antipsychotic compound that can reverse the burst firing produced by dopamine and D_2R agonist activation of D_2Rs in a subset of cortical pyramidal cells (Wang and Goldman-Rakic, 2004). Together, these observations suggest that the antipsychotic effectiveness of $\alpha 7\text{nAChR}$ agonists and D_2R antagonists may be ascribed in part to their actions in distinct subpopulations of neurons in the PFC.

The prominent expression of the $\alpha 7\text{nAChR}$ in PFC astrocytes suggests that these astrocytes may play a pivotal role in nicotine-induced enhancement of PFC activity and improvement of cognitive functions (Mansvelder et al., 2006). The specific presence of the $\alpha 7\text{nAChR}$ alone or together with the D_2R in perisynaptic and perivascular astrocytes also implicates these receptors in functional hyperemia and in cerebral blood flow. The involvement of $\alpha 7\text{nACh}$ and D_2Rs in control of cerebral blood flow may have a significant impact on the interpretation of data from imaging studies of psychotic patients receiving medications that target one or both receptors. This impact would be evident not only when using positron

emission tomography (PET) scans (Uchida et al., 2009), but also functional and pharmacological magnetic resonance imaging (MRI) (Alves et al., 2008; Toga et al., 2006).

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REFERENCES

- Alves FS, Figue M, Vamelsvoort T, Veltman D, de Haan L. The revised dopamine hypothesis of schizophrenia: evidence from pharmacological MRI studies with atypical antipsychotic medication. *Psychopharmacol Bull.* 2008; 41:121–132. [PubMed: 18362875]
- Araque A, Parpura V, Sanzgiri R, Haydon P. Glutamate-dependent astrocyte modulation of synaptic transmission between cultured hippocampal neurons. *Eur J Neurosci.* 1998; 10:2129–2142. [PubMed: 9753099]
- Araque A, Parpura V, Sanzgiri RP, Haydon PG. Tripartite synapses: glia, the unacknowledged partner. *Trends Neurosci.* 1999; 22:208–215. [PubMed: 10322493]
- Arnaiz-Cot J, Gonzalez J, Sobrado M, Baldelli P, Carbone E, Gandia L, Garcia A, Hernandez-Guijo J. Allosteric modulation of alpha 7 nicotinic receptors selectively depolarizes hippocampal interneurons, enhancing spontaneous GABAergic transmission. *Eur J Neurosci.* 2008; 27:1097–1110. [PubMed: 18312591]
- Arneric S, Honig M, Milner T, Greco S, Iadecola C, Reis D. Neuronal and endothelial sites of acetylcholine synthesis and release associated with microvessels in rat cerebral cortex: ultrastructural and neurochemical studies. *Brain Res.* 1988; 454:11–30. [PubMed: 3408997]
- Aronin N, DiFiglia M. The subcellular localization of the G-protein Gi alpha in the basal ganglia reveals its potential role in both signal transduction and vesicle trafficking. *J Neurosci.* 1992; 12:3435–3444. [PubMed: 1527588]
- Artigas F. The prefrontal cortex: a target for antipsychotic drugs. *Acta Psychiatr Scand.* 2010; 121:11–21. [PubMed: 20059453]
- Arvidsson U, Riedl M, Elde R, Meister B. Vesicular acetylcholine transporter (VACHT) protein: a novel and unique marker for cholinergic neurons in the central and peripheral nervous systems. *J Comp Neurol.* 1997; 378:454–467. [PubMed: 9034903]
- Bal A, Bachelot T, Savasta M, Manier M, Verna J, Benabid A, Feuerstein C. Evidence for dopamine D2 receptor mRNA expression by striatal astrocytes in culture: in situ hybridization and polymerase chain reaction studies. *Mol Brain Res.* 1994; 23:204–212. [PubMed: 8057777]
- Bezzi P, Carmignoto G, Pasti L, Vesce S, Rossi D, Rizzini B, Pozzan T, Volterra A. Prostaglandins stimulate calcium-dependent glutamate release in astrocytes. *Nature.* 1998; 391:281–285. [PubMed: 9440691]
- Bramanti V, Bronzi D, Tomassoni D, Li Volti G, Cannavo G, Raciti G, Napoli M, Vanella A, Campisi A, Ientile R, Avola R. Effect of choline-containing phospholipids on transglutaminase activity in primary astroglial cell cultures. *Clin Exp Hypertens.* 2008; 30:798–807. [PubMed: 19021029]
- Brana C, Aubert I, Charron G, Pellevoisin C, Bloch B. Ontogeny of the striatal neurons expressing the D2 dopamine receptor in humans: an in situ hybridization and receptor-binding study. *Mol Brain Res.* 1997; 48:389–400. [PubMed: 9332736]
- Brunelli G, Spano P, Barlati S, Guarneri B, Barbon A, Bresciani R, Pizzi M. Glutamatergic reinnervation through peripheral nerve graft dictates assembly of glutamatergic synapses at rat skeletal muscle. *Proc Natl Acad Sci USA.* 2005; 102:8752–8757. [PubMed: 15937120]
- Carr D, Sesack S. GABA-containing neurons in the rat ventral tegmental area project to the prefrontal cortex. *Synapse.* 2000; 38:114–123. [PubMed: 11018785]
- Centeno M, Henderson J, Pau K, Bethea C. Estradiol increases alpha7 nicotinic receptor in serotonergic dorsal raphe and noradrenergic locus coeruleus neurons of macaques. *J Comp Neurol.* 2006; 497:489–501. [PubMed: 16736471]

- Chan J, Aoki C, Pickel V. Optimization of differential immunogold-silver and peroxidase labeling with maintenance of ultrastructure in brain sections before plastic embedding. *J Neurosci Methods*. 1990; 33:113–127. [PubMed: 1977960]
- Choi J, Chen Y, Hamel E, Jenkins B. Brain hemodynamic changes mediated by dopamine receptors: Role of the cerebral microvasculature in dopamine-mediated neurovascular coupling. *Neuroimage*. 2006; 30:700–712. [PubMed: 16459104]
- Coggan J, Bartol T, Esquenazi E, Stiles J, Lamont S, Martone M, Berg D, Ellisman M, Sejnowski T. Evidence for ectopic neurotransmission at a neuronal synapse. *Science*. 2005; 309:446–451. [PubMed: 16020730]
- Collins A, Salminen O, Marks M, Whiteaker P, Grady S. The road to discovery of neuronal nicotinic cholinergic receptor subtypes. *Handb Exp Pharmacol*. 2009; 192:85–112. [PubMed: 19184647]
- Delbro D, Westerlund A, Bjorklund U, Hansson E. In inflammatory reactive astrocytes co-cultured with brain endothelial cells nicotine-evoked Ca(2+) transients are attenuated due to interleukin-1 β release and rearrangement of actin filaments. *Neuroscience*. 2009; 159:770–779. [PubMed: 19171179]
- Descarries L, Gisiger V, Steriade M. Diffuse transmission by acetylcholine in the CNS. *Prog Neurobiol*. 1997; 53:603–625. [PubMed: 9421837]
- Descarries L, Mechawar N. Ultrastructural evidence for diffuse transmission by monoamine and acetylcholine neurons of the central nervous system. *Prog Brain Res*. 2000; 125:27–47. [PubMed: 11098652]
- Dominguez Del Toro E, Juiz J, Peng X, Lindstrom J, Criado M. Immunocytochemical localization of the $\alpha 7$ subunit of the nicotinic acetylcholine receptor in the rat central nervous system. *J Comp Neurol*. 1994; 349:325–342. [PubMed: 7852628]
- Drake C, Milner T. Mu opioid receptors are in discrete hippocampal interneuron subpopulations. *Hippocampus*. 2002; 12:119–136. [PubMed: 12000113]
- Drenan RM, Nashmi R, Imoukhuede P, Just H, McKinney S, Lester HA. Subcellular trafficking, pentameric assembly, and subunit stoichiometry of neuronal nicotinic acetylcholine receptors containing fluorescently labeled $\alpha 6$ and $\beta 3$ subunits. *Mol Pharmacol*. 2008; 73:27–41. [PubMed: 17932221]
- Duffy A, Zhou P, Milner T, Pickel V. Spatial and intracellular relationships between the $\alpha 7$ nicotinic acetylcholine receptor and the vesicular acetylcholine transporter in the prefrontal cortex of rat and mouse. *Neuroscience*. 2009; 161:1091–1103. [PubMed: 19374941]
- Erickson J, Varoqui H, Schafer M, Modi W, Diebler M, Weihe E, Rand J, Eiden L, Bonner T, Usdin T. Functional identification of a vesicular acetylcholine transporter and its expression from a "cholinergic" gene locus. *J Biol Chem*. 1994; 269:21929–21932. [PubMed: 8071310]
- Fabian-Fine R, Skehel P, Errington M, Davies H, Sher E, Stewart M, Fine A. Ultrastructural distribution of the $\alpha 7$ nicotinic acetylcholine receptor subunit in rat hippocampus. *J Neurosci*. 2001; 21:7993–8003. [PubMed: 11588172]
- Finkbeiner S. Glial calcium. *Glia*. 1993; 9:83–104. [PubMed: 8244537]
- Fisher J, Dani J. Nicotinic receptors on hippocampal cultures can increase synaptic glutamate currents while decreasing the NMDA-receptor component. *Neuropharmacology*. 2000; 39:2756–2769. [PubMed: 11044745]
- Franklin, K.; Paxinos, G. The mouse brain in stereotaxic coordinates. San Diego: Academic Press; 1997.
- Free R, Hazelwood L, Cabrera D, Spalding H, Namkung Y, Rankin M, Sibley D. D1 and D2 dopamine receptor expression is regulated by direct interaction with the chaperone protein calnexin. *J Biol Chem*. 2007; 282:21285–21300. [PubMed: 17395585]
- Freedman R, Coon H, Myles-Worsley M, Orr-Urtreger A, Olincy A, Davis A, Polymeropoulos M, Holik J, Hopkins J, Hoff M, Rosenthal J, Waldo M, Reimherr F, Wender P, Yaw J, Young D, Breese C, Adams C, Patterson D, Adler L, Kruglyak L, Leonard S, Byerley W. Linkage of a neurophysiological deficit in schizophrenia to a chromosome 15 locus. *Proc Natl Acad Sci USA*. 1997; 94:587–592. [PubMed: 9012828]
- Fujii T, Masai M, Misawa H, Okuda T, Takada-Takatori Y, Moriwaki Y, Haga T, Kawashima K. Acetylcholine synthesis and release in NIH3T3 cells coexpressing the high-affinity choline

- transporter and choline acetyltransferase. *J Neurosci Res.* 2009; 87:3024–3032. [PubMed: 19405101]
- Fuxe K, Dahlstrom A, Jonsson G, Marcellino D, Guescini M, Dam M, Manger P, Agnati L. The discovery of central monoamine neurons gave volume transmission to the wired brain. *Prog Neurobiol.* 2010; 90:82–100. [PubMed: 19853007]
- Gabbott PL, Dickie BG, Vaid RR, Headlam AJ, Bacon SJ. Local-circuit neurones in the medial prefrontal cortex (areas 25, 32 and 24b) in the rat: morphology and quantitative distribution. *J Comp Neurol.* 1997; 377:465–499. [PubMed: 9007187]
- Garzon M, Pickel V. Dendritic and axonal targeting of the vesicular acetylcholine transporter to membranous cytoplasmic organelles in laterodorsal and pedunculopontine tegmental nuclei. *J Comp Neurol.* 2000; 419:32–48. [PubMed: 10717638]
- Garzon M, Pickel V. Subcellular distribution of M2 muscarinic receptors in relation to dopaminergic neurons of the rat ventral tegmental area. *J Comp Neurol.* 2006; 498:821–839. [PubMed: 16927256]
- Gault J, Hopkins J, Berger R, Drebing C, Logel J, Walton C, Short M, Vianzon R, Olincy A, Ross R, Adler L, Freedman R, Leonard S. Comparison of polymorphisms in the alpha7 nicotinic receptor gene and its partial duplication in schizophrenic and control subjects. *Am J Med Genet B Neuropsychiatr Genet.* 2003; 123:39–49. [PubMed: 14582144]
- Geetha T, Wooten MW. TrkA receptor endolysosomal degradation is both ubiquitin and proteasome dependent. *Traffic.* 2008; 9:1146–1156. [PubMed: 18419753]
- Gelman MS, Chang W, Thomas DY, Bergeron JJ, Prives JM. Role of the endoplasmic reticulum chaperone calnexin in subunit folding and assembly of nicotinic acetylcholine receptors. *J Biol Chem.* 1995; 270:15085–15092. [PubMed: 7797492]
- Gonzalez-Islas C, Hablitz J. Dopamine enhances EPSCs in layer II–III pyramidal neurons in rat prefrontal cortex. *J Neurosci.* 2003; 23:867–875. [PubMed: 12574415]
- Good C, Lupica C. Properties of distinct ventral tegmental area synapses activated via pedunculopontine or ventral tegmental area stimulation in vitro. *J Physiol.* 2009; 587:1233–1247. [PubMed: 19188251]
- Halassa MM, Fellin T, Haydon PG. The tripartite synapse: roles for gliotransmission in health and disease. *Trends Mol Med.* 2007; 13:54–63. [PubMed: 17207662]
- Hara Y, Yakovleva T, Bakalkin G, Pickel V. Dopamine D1 receptors have subcellular distributions conducive to interactions with prodynorphin in the rat nucleus accumbens shell. *Synapse.* 2006; 60:1–19. [PubMed: 16575853]
- Haydon PG, Carmignoto G. Astrocyte control of synaptic transmission and neurovascular coupling. *Physiol Rev.* 2006; 86:1009–1031. [PubMed: 16816144]
- Henny P, Jones B. Projections from basal forebrain to prefrontal cortex comprise cholinergic, GABAergic and glutamatergic inputs to pyramidal cells or interneurons. *Eur J Neurosci.* 2008; 27:654–670. [PubMed: 18279318]
- Iadecola C, Nedergaard M. Glial regulation of the cerebral microvasculature. *Nat Neurosci.* 2007; 10:1369–1376. [PubMed: 17965657]
- Jones I, Wonnacott S. Precise localization of alpha7 nicotinic acetylcholine receptors on glutamatergic axon terminals in the rat ventral tegmental area. *J Neurosci.* 2004; 24:11244–11252. [PubMed: 15601930]
- Kabbani N, Woll MP, Levenson R, Lindstrom JM, Changeux JP. Intracellular complexes of the beta2 subunit of the nicotinic acetylcholine receptor in brain identified by proteomics. *Proc Natl Acad Sci USA.* 2007; 104:20570–20575. [PubMed: 18077321]
- Kaplan T, Skyers P, Tabori N, Drake C, Milner T. Ultrastructural evidence for mu-opioid modulation of cholinergic pathways in rat dentate gyrus. *Brain Res.* 2004; 1019:28–38. [PubMed: 15306235]
- Kearn C, Blake-Palmer K, Daniel E, Mackie K, Glass M. Concurrent stimulation of cannabinoid CB1 and dopamine D2 receptors enhances heterodimer formation: a mechanism for receptor cross-talk? *Mol Pharmacol.* 2005; 67:1697–1704. [PubMed: 15710746]
- Keller SH, Lindstrom J, Ellisman M, Taylor P. Adjacent basic amino acid residues recognized by the COP I complex and ubiquitination govern endoplasmic reticulum to cell surface trafficking of the

- nicotinic acetylcholine receptor alpha-Subunit. *J Biol Chem.* 2001; 276:18384–18391. [PubMed: 11279119]
- Khan Z, Koulen P, Rubinstein M, Grandy D, Goldman-Rakic P. An astroglia-linked dopamine D2-receptor action in prefrontal cortex. *Proc Natl Acad Sci USA.* 2001; 98:1964–1969. [PubMed: 11172059]
- Kim O, Ariano M, Namkung Y, Marinec P, Kim E, Han J, Sibley D. D2 dopamine receptor expression and trafficking is regulated through direct interactions with ZIP. *J Neurochem.* 2008; 106:83–95. [PubMed: 18346199]
- Lambe E, Picciotto M, Aghajanian G. Nicotine induces glutamate release from thalamocortical terminals in prefrontal cortex. *Neuropsychopharmacology.* 2003; 28:216–225. [PubMed: 12589374]
- Lapish C, Kroener S, Durstewitz D, Lavin A, Seamans J. The ability of the mesocortical dopamine system to operate in distinct temporal modes. *Psychopharmacology (Berl).* 2007; 191:609–625. [PubMed: 17086392]
- Laruelle M, Frankle W, Narendran R, Kegeles L, Abi-Dargham A. Mechanism of action of antipsychotic drugs: from dopamine D(2) receptor antagonism to glutamate NMDA facilitation. *Clin Ther.* 2005; 27(Suppl A):S16–S24. [PubMed: 16198197]
- LeMoine C, Gaspar P. Subpopulations of cortical GABAergic interneurons differ by their expression of D1 and D2 dopamine receptor subtypes. *Mol Brain Res.* 1998; 58:231–236. [PubMed: 9685656]
- Leranth, C.; Pickel, V.; Heimer, L.; Zaborszky, L. *Neuroanatomical Tract-tracing Methods 2: Recent Progress.* New York: Plenum Publishing, Co.; 1989. Electron microscopic pre-embedding double immunostaining methods; p. 129-172.
- Levin E, Rezvani A. Nicotinic interactions with antipsychotic drugs, models of schizophrenia and impacts on cognitive function. *Biochem Pharmacol.* 2007; 74:1182–1191. [PubMed: 17714691]
- Levin E, Simon B. Nicotinic acetylcholine involvement in cognitive function in animals. *Psychopharmacology (Berl).* 1998; 138:217–230. [PubMed: 9725745]
- Levy R, Aoki C. Alpha7 nicotinic acetylcholine receptors occur at postsynaptic densities of AMPA receptor-positive and-negative excitatory synapses in rat sensory cortex. *J Neurosci.* 2002; 22:5001–5015. [PubMed: 12077196]
- Li A, Guo H, Luo X, Sheng J, Yang S, Yin Y, Zhou J. Apomorphine-induced activation of dopamine receptors modulates FGF-2 expression in astrocytic cultures and promotes survival of dopaminergic neurons. *FASEB J.* 2006; 20:1263–1265. [PubMed: 16636101]
- Livingstone P, Dickinson J, Srinivasan J, Kew J, Wonnacott S. Glutamate-dopamine crosstalk in the rat prefrontal cortex is modulated by Alpha7 nicotinic receptors and potentiated by PNU-120596. *J Mol Neurosci.* 2010; 40:172–176. [PubMed: 19688191]
- Lubin M, Erisir A, Aoki C. Ultrastructural Immunolocalization of the $\alpha 7$ nAChR Subunit in Guinea Pig Medial Prefrontal Cortex. *Ann NY Acad Sci.* 1999; 868:628–632. [PubMed: 10414345]
- Mandeville J, Jenkins B, Kosofsky B, Moskowitz M, Rosen B, Marota J. Regional sensitivity and coupling of BOLD and CBV changes during stimulation of rat brain. *Magn Reson Med.* 2001; 45:443–447. [PubMed: 11241702]
- Mansvelder H, van Aerde K, Couey J, Brussaard A. Nicotinic modulation of neuronal networks: from receptors to cognition. *Psychopharmacol.* 2006; 184:292–305.
- Marota J, Mandeville J, Weisskoff R, Moskowitz M, Rosen B, Kosofsky B. Cocaine activation discriminates dopaminergic projections by temporal response: an fMRI study in rat. *Neuroimage.* 2000; 11:13–23. [PubMed: 10686113]
- Martina M, Bergeron R. D1 and D4 dopaminergic receptor interplay mediates coincident G protein-independent and dependent regulation of glutamate NMDA receptors in the lateral amygdala. *J Neurochem.* 2008; 106:2421–2435. [PubMed: 18662324]
- McLane K, Wu X, Lindstrom J, Conti-Tronconi B. Epitope mapping of polyclonal and monoclonal antibodies against two alpha-bungarotoxin-binding alpha subunits from neuronal nicotinic receptors. *J Neuroimmunol.* 1992; 38:115–128. [PubMed: 1374423]

- Mechawar N, Cozzari C, Descarries L. Cholinergic innervation in adult rat cerebral cortex: a quantitative immunocytochemical description. *J Comp Neurol.* 2000; 428:305–318. [PubMed: 11064369]
- Millar NS, Harkness PC. Assembly and trafficking of nicotinic acetylcholine receptors (Review). *Mol Membr Biol.* 2008; 25:279–292. [PubMed: 18446614]
- Missale C, Nash S, Robinson S, Jaber M, Caron M. Dopamine receptors: from structure to function. *Physiol Rev.* 1998; 78:189–225. [PubMed: 9457173]
- Muly EC III, Szigeti K, Goldman-Rakic PS. D1 receptor in interneurons of macaque prefrontal cortex: distribution and subcellular localization. *J Neurosci.* 1998; 18:10553–10565. [PubMed: 9852592]
- Negyessy L, Goldman-Rakic P. Subcellular localization of the dopamine D2 receptor and coexistence with the calcium-binding protein neuronal calcium sensor-1 in the primate prefrontal cortex. *J Comp Neurol.* 2005; 488:464–475. [PubMed: 15973684]
- Oikawa H, Nakamichi N, Kambe Y, Ogura M, Yoneda Y. An increase in intracellular free calcium ions by nicotinic acetylcholine receptors in a single cultured rat cortical astrocyte. *J Neurosci Res.* 2005; 79:535–544. [PubMed: 15635597]
- Olinicy A, Harris J, Johnson L, Pender V, Kongs S, Allensworth D, Ellis J, Zerbe G, Leonard S, Stevens K, Stevens J, Martin L, Adler L, Soti F, Kem W, Freedman R. Proof-of-concept trial of an alpha7 nicotinic agonist in schizophrenia. *Arch Gen Psychiatry.* 2006; 63:630–638. [PubMed: 16754836]
- Parpura V, Haydon P. Physiological astrocytic calcium levels stimulate glutamate release to modulate adjacent neurons. *Proc Natl Acad Sci USA.* 2000; 97:8629–8634. [PubMed: 10900020]
- Parri R, Crunelli V. An astrocyte bridge from synapse to blood flow. *Nat Neurosci.* 2003; 6:5–6. [PubMed: 12494240]
- Pasti L, Volterra A, Pozzan T, Carmignoto G. Intracellular calcium oscillations in astrocytes: A highly plastic, bidirectional form of communication between neurons and astrocytes in situ. *J Neurosci.* 1997; 17:7817–7830. [PubMed: 9315902]
- Patti L, Raiteri L, Grilli M, Zappettini S, Bonanno G, Marchi M. Evidence that alpha7 nicotinic receptor modulates glutamate release from mouse neocortical gliosomes. *NeurochemInt.* 2007; 51:1–7.
- Paxinos, G.; Watson, C. The rat brain in stereotaxic coordinates. San Diego: Academic Press; 1998.
- Perea G, Araque A. Glial calcium signaling and neuron-glia communication. *Cell Calcium.* 2005; 38:375–382. [PubMed: 16105683]
- Peters, A.; Palay, S.; Webster, H. The fine structure of the nervous system. New York: Oxford University Press; 1991. p. 1-494.
- Pickel V, Chan J, Kearn C, Mackie K. Targeting dopamine D2 and cannabinoid-1 (CB1) receptors in rat nucleus accumbens. *J Comp Neurol.* 2006; 495:299–313. [PubMed: 16440297]
- Pickel V, Douglas J, Chan J, Gamp P, Bunnett N. Neurokinin 1 receptor distribution in cholinergic neurons and targets of substance P terminals in the rat nucleus accumbens. *J Comp Neurol.* 2000; 423:500–511. [PubMed: 10870089]
- Pinto A, Sesack S. Ultrastructural analysis of prefrontal cortical inputs to the rat amygdala: spatial relationships to presumed dopamine axons and D1 and D2 receptors. *Brain Struct Funct.* 2008; 213:159–175. [PubMed: 18340460]
- Piper RC, Katzmann DJ. Biogenesis and function of multivesicular bodies. *Annu Rev Cell Dev Biol.* 2007; 23:519–547. [PubMed: 17506697]
- Porter J, Cauli B, Tsuzuki K, Lambolez B, Rossier J, Audinat E. Selective excitation of subtypes of neocortical interneurons by nicotinic receptors. *J Neurosci.* 1999; 19:5228–5235. [PubMed: 10377334]
- Porter LL, Rizzo E, Hornung JP. Dopamine affects parvalbumin expression during cortical development in vitro. *J Neurosci.* 1999; 19:8990–9003. [PubMed: 10516317]
- Pratt J, Winchester C, Egerton A, Cochran S, Morris B. Modelling prefrontal cortex deficits in schizophrenia: implications for treatment. *Br J Pharmacol.* 2008; 153:S465–S470. [PubMed: 18311160]

- Prou D, Gu WJ, Le Crom S, Vincent JD, Salamero J, Vernier P. Intracellular retention of the two isoforms of the D(2) dopamine receptor promotes endoplasmic reticulum disruption. *J Cell Sci.* 2001; 114:3517–3527. [PubMed: 11682611]
- Reuss B, Unsicker K. Atypical neuroleptic drugs downregulate dopamine sensitivity in rat cortical and striatal astrocytes. *Mol-Cell Neurosci.* 2001; 18:197–209. [PubMed: 11520180]
- Rezvani K, Teng Y, De Biasi M. The ubiquitin-proteasome system regulates the stability of neuronal nicotinic acetylcholine receptors. *J Mol Neurosci.* 2010; 40:177–184. [PubMed: 19693707]
- Robbins T. From arousal to cognition: the integrative position of the prefrontal cortex. *Prog Brain Res.* 2000; 126:469–483. [PubMed: 11105663]
- Roghani A, Feldman J, Kohan S, Shirzadi A, Gundersen C, Brecha N, Edwards R. Molecular cloning of a putative vesicular transporter for acetylcholine. *Proc Natl Acad Sci USA.* 1994; 91:10620–10624. [PubMed: 7938002]
- Rotaru D, Barrionuevo G, Sesack S. Mediodorsal thalamic afferents to layer III of the rat prefrontal cortex: synaptic relationships to subclasses of interneurons. *J Comp Neurol.* 2005; 26:220–238. [PubMed: 16082676]
- Sato A, Sato Y, Uchida S. Activation of the intracerebral cholinergic nerve fibers originating in the basal forebrain increases regional cerebral blood flow in the rat's cortex and hippocampus. *Neurosci Lett.* 2004; 361:90–93. [PubMed: 15135901]
- Schmued LC, Stowers CC, Scallet AC, Xu L. Fluoro-Jade C results in ultra high resolution and contrast labeling of degenerating neurons. *Brain Res.* 2005; 1035:24–31. [PubMed: 15713273]
- Schoepfer R, Conroy W, Whiting P, Gore M, Lindstrom J. Brain alpha-bungarotoxin binding protein cDNAs and MAbs reveal subtypes of this branch of the ligand-gated ion channel gene superfamily. *Neuron.* 1990; 5:35–48. [PubMed: 2369519]
- Serova L, Sabban E. Involvement of alpha 7 nicotinic acetylcholine receptors in gene expression of dopamine biosynthetic enzymes in rat brain. *J Pharm Exp Ther.* 2002; 303:896–903.
- Sesack S, Hawrylak V, Melchitzky D, Lewis D. Dopamine innervation of a subclass of local circuit neurons in monkey prefrontal cortex: ultrastructural analysis of tyrosine hydroxylase and parvalbumin immunoreactive structures. *Cereb Cortex.* 1998; 8:614–622. [PubMed: 9823482]
- Severance E, Yolken R. Novel alpha7 nicotinic receptor isoforms and deficient cholinergic transcription in schizophrenia. *Genes Brain Behav.* 2008; 7:37–45. [PubMed: 17504249]
- Sharma G, Vijayaraghavan S. Nicotinic cholinergic signaling in hippocampal astrocytes involves calcium-induced calcium release from intracellular stores. *Proc Natl Acad Sci USA.* 2001; 98:4148–4153. [PubMed: 11259680]
- Simard M, Nedergaard M. The neurobiology of glia in the context of water and ion homeostasis. *Neuroscience.* 2004; 129:877–896. [PubMed: 15561405]
- Smit AB, Syed NI, Schaap D, van Minnen J, Klumperman J, Kits KS, Lodder H, van der Schors RC, van Elk R, Sorgedrager B, Brejc K, Sixma TK, Geraerts WPM. A glia-derived acetylcholine-binding protein that modulates synaptic transmission. *Nature.* 2001; 411:261–268. [PubMed: 11357121]
- Stigliani S, Zappettini S, Raiteri L, Passalacqua M, Melloni E, Venturi C, Tacchetti C, Diaspro A, Usai C, Bonanno G. Glia re-sealed particles freshly prepared from adult rat brain are competent for exocytotic release of glutamate. *J Neurochem.* 2006; 96:656–668. [PubMed: 16405496]
- Sun X, Zhao Y, Wolf M. Dopamine receptor stimulation modulates AMPA receptor synaptic insertion in prefrontal cortex neurons. *J Neurosci.* 2005; 25:7342–7351. [PubMed: 16093384]
- Svingos A, Colago E, Pickel V. Vesicular acetylcholine transporter in the rat nucleus accumbens shell: subcellular distribution and association with mu-opioid receptors. *Synapse.* 2001; 40:184–192. [PubMed: 11304756]
- Toga A, Thompson P, Mori S, Amunts K, Zilles K. Towards multimodal atlases of the human brain. *Nat Rev Neurosci.* 2006; 7:952–966. [PubMed: 17115077]
- Torrealba F, Müller C. Ultrastructure of glutamate and GABA immunoreactive axon terminals of the rat nucleus tractus solitarius, with a note on infralimbic cortex afferents. *Brain Res.* 1999; 820:20–30. [PubMed: 10023027]

- Towart L, Alves S, Znamensky V, Hayashi S, McEwen B, Milner T. Subcellular relationships between cholinergic terminals and estrogen receptor-alpha in the dorsal hippocampus. *J Comp Neurol*. 2003; 463:390–401. [PubMed: 12836175]
- Trantham-Davidson H, Kroner S, Seamans JK. Dopamine modulation of prefrontal cortex interneurons occurs independently of DARPP-32. *Cereb Cortex*. 2008; 18:951–958. [PubMed: 17693396]
- Turner T. Nicotine enhancement of dopamine release by a calcium-dependent increase in the size of the readily releasable pool of synaptic vesicles. *J Neurosci*. 2004; 24:11328–11336. [PubMed: 15601939]
- Uchida H, Graff-Guerrero A, Mulsant BH, Pollock BG, Mamo DC. Long-term stability of measuring D2 receptors in schizophrenia patients treated with antipsychotics. *Schizophr Res*. 2009; 109:130–133. [PubMed: 19179049]
- Uchida S, Kagitani F, Nakayama H, Sato A. Effect of stimulation of nicotinic cholinergic receptors on cortical cerebral blood flow and changes in the effect during aging in anesthetized rats. *Neurosci Lett*. 1997; 228:203–206. [PubMed: 9218643]
- Uteshev VV, Meyer EM, Papke RL. Regulation of neuronal function by choline and 4OH-GTS-21 through alpha 7 nicotinic receptors. *J Neurophysiol*. 2003; 89:1797–1806. [PubMed: 12611953]
- Van De Werd H, Rajkowska G, Evers P, Uylings H. Cytoarchitectonic and chemoarchitectonic characterization of the prefrontal cortical areas in the mouse. *Brain Struct Funct*. 2010; 214:339–353. [PubMed: 20221886]
- Wanamaker CP, Green WN. Endoplasmic reticulum chaperones stabilize nicotinic receptor subunits and regulate receptor assembly. *J Biol Chem*. 2007; 282:31113–31123. [PubMed: 17728248]
- Wang B, Liao W, Chang C, Wang S. Facilitation of glutamate release by nicotine involves the activation of a Ca²⁺/calmodulin signaling pathway in rat prefrontal cortex nerve terminals. *Synapse*. 2006; 59:491–501. [PubMed: 16565963]
- Wang H, Cuzon V, Pickel V. Postnatal development of muopioid receptors in the rat caudate-putamen nucleus parallels asymmetric synapse formation. *Neuroscience*. 2003; 118:695–708. [PubMed: 12710977]
- Wang Y, Goldman-Rakic P. D2 receptor regulation of synaptic burst firing in prefrontal cortical pyramidal neurons. *Proc Natl Acad Sci USA*. 2004; 101:5093–5098. [PubMed: 15051874]
- Zaborszky L, Gaykema R, Swanson D, Cullinan W. Cortical input to the basal forebrain. *Neuroscience*. 1997; 79:1051–1078. [PubMed: 9219967]

Fig. 1.

Photomicrographs showing peroxidase immunoreactivity for the $\alpha 7$ nAChR (A) and the D_2R (B) in the mouse. In A, a dark precipitous reaction product is seen in the cytoplasm of somata (black arrows) having a laminar distribution. In B, intensely labeled somata (small black arrows) often give rise to apical dendrites oriented toward the brain surface, whereas a more lightly labeled soma (white block arrow) appears to have multiple short processes typical of an interneuron. m, medial; v, ventral relative to the cortical surface. Scale bars = 50 μ M.

Fig. 2.

Electron micrographs showing the somatodendritic colocalization of $\alpha 7$ nAChR immunoperoxidase (white block arrows) and D_2 R immunogold (small arrows) in rodent PFC. **A,B:** The $\alpha 7$ nAChR-ir and D_2 R-ir are seen in the cytoplasm of a large soma ($\alpha 7$ nAChR+ D_2 R-som) and a proximal dendrite ($\alpha 7$ nAChR+ D_2 R-de) of a neuron whose somatic stacks of packed rough endoplasmic reticulum (rer) are typical of cortical pyramidal cells. The dual labeling is prominently associated with the endoplasmic reticulum (er) and other endomembranes including an endosomal/multivesicular body (mvb) in the dendrite. **C,D:** The $\alpha 7$ nAChR-ir and D_2 R-ir are dispersed throughout the cytoplasmic compartment in somata ($\alpha 7$ nAChR+ D_2 R-som) recipient to multiple unlabeled axon terminals (U-te). In D, the D_2 R-immunogold also is located along membranes of the endoplasmic reticulum (er) and on the plasmalemmal surface of a rudimentary spine (Sp). Nu, nucleus; Go, Golgi lamellae. Scale bars = 500 nm.

Fig. 3.

Subcellular distribution of $\alpha 7$ nAChR immunoperoxidase (block arrow) and/or D₂R immunogold (small arrows) in dendritic shafts and spines of the rodent PFC. **A:** The $\alpha 7$ nAChR-ir is localized in aggregates near accumulations of D₂R-ir in a longitudinally sectioned dendritic shaft ($\alpha 7$ nAChR+D₂R-de), whereas a nearby dendritic shaft shows exclusively D₂R-ir (D₂-de). **B:** A diffuse distribution of $\alpha 7$ nAChR-ir is seen throughout the cytoplasm of a transversely sectioned dendritic shaft ($\alpha 7$ nAChR+D₂R-de) in which some of the immunogold D₂R is located beneath a symmetric synapse. Sparse D₂R-ir also is evident in the presynaptic terminal and an apposed glial process (D₂-g). **C,D:** Peroxidase $\alpha 7$ nAChR-ir is seen in small dendrites ($\alpha 7$ -de) in which the respective labeling has either a diffuse or more aggregated (block arrows) distribution. Both dendrites receive asymmetric excitatory-type synapses (curved arrow), as does another unlabeled dendritic spine (U-sp). In labeled dendrites (D₂R-de), the D₂R-immunogold is seen beneath a symmetric synapse (chevron) in **E** and in the cytoplasm near an asymmetric synapse (curved arrow) in **F**. In **G**, D₂R immunogold has a respective pre- and post-synaptic distribution in an axon terminal (D₂-te) and spine (D₂-sp). **H:** The $\alpha 7$ nAChR-ir is seen in a small axon terminal ($\alpha 7$ -te) that forms an asymmetric synapse with a dendritic spine containing D₂R immunogold (D₂-sp). In **I, J**, the respective micrographs illustrate the distribution of $\alpha 7$ nAChR-ir (white arrows)

in dendritic spines that also contain D₂R-ir ($\alpha 7$ +D₂-sp). Unlabeled terminals, U-te; Asymmetric synapse, curved arrow. M, mitochondrion. Scale bars = 500 nm.

Fig. 4.

Pre- and postsynaptic distribution of D₂R immunogold (small arrows) and α7nAChR immunoperoxidase labeling in the PFC. **A:** The D₂R-ir is located within an axon terminal (D₂-te) that forms a symmetric synapse with a dendritic profile containing α7nAChR-immunoperoxidase labeling (block arrow). **B:** D₂R-ir is seen in an axon terminal that forms an asymmetric synapse with a dendritic spine containing α7nAChR-ir (α7-sp). **C:** Immunoperoxidase labeling for the α7nAChR is seen in small axon terminal (α7-te) showing what appears to be a symmetric synapse with a small D₂R-labeled dendrite (D₂-de). **(D,** The respective micrographs illustrate nonsynaptic axon terminals containing D₂R-immunogold and α7nAChR-immunoperoxidase labeling are colocalized in a small non-synaptic axon terminal (α7+D₂-te). Unlabeled terminals, U-te; symmetric synapse, chevron; asymmetric synapse, curved arrow. Scale bars = 500 nm.

Fig. 5.

Confocal fluorescence images showing the localization of the $\alpha 7$ nAChR and D₂R in astrocytes in the PFC of GFAP-GFP mice (A–F) and the D₂R-ir in D₂-transfected HEK cells (G–I). In A and D, the native GFP (green) is seen in many astrocytes and their filamentous processes. In B and E, the respective Cy5 immunolabeling (red) for the $\alpha 7$ nAChR and the D₂R is observed in many neuronal soma and thick as well as filamentous processes some of which are shown in the merged images (C and F) to express GFAP-GFP. White arrows identify the astrocytic processes that are dually labeled in the merged images. In G, a cluster of HEK cells can be identified by the presence of DAPI staining in their nuclei. Only two (arrows) of these cells show D₂R-ir (H), and these are the same ones having D₂R transfection as indicated by the FLAG tagged D₂RL isoform in I. Scale bars = 50 μ m in A–F and 15 μ m in G–I. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Fig. 6.

Electron micrographs showing the location of $\alpha 7$ nAChR-ir in perisynaptic glial processes in PFC tissue processed for dual labeling of $\alpha 7$ nAChR and either VACHT or D₂R. **A:** Immunoperoxidase reaction for $\alpha 7$ nAChR is seen in a glial process ($\alpha 7$ -g) contacting unlabeled axon terminals that form asymmetric synapses with dendritic spines that are either unlabeled (u-sp) or express postsynaptic immunoperoxidase $\alpha 7$ nAChR-ir ($\alpha 7$ -sp). **B:** Immunogold labeling (small arrows) for $\alpha 7$ nAChR is located in a glial process ($\alpha 7$ -g) in contact with an axon terminal that contains dense immunoperoxidase VACHT-ir (VACHT-te). The labeled glial process is near several unlabeled axon terminals, some of which form asymmetric synapses. **C,D:** Micrographs respectively show the perisynaptic glial $\alpha 7$ nAChR immunoperoxidase labeling alone ($\alpha 7$ -g) or together ($\alpha 7 + D_2$ -g) with D₂R-immunogold (small arrows). **E:** A perisynaptic astrocytic process ($\alpha 7 + D_2$ -g) contains both immunoperoxidase $\alpha 7$ nAChR-ir and D₂R-immunogold (arrows). The apposing unlabeled axon terminal forms an asymmetric synapse with an unlabeled dendrite (U-de). Unlabeled terminals= U-te; Asymmetric synapse, curved arrow. Scale bars = 500 nm.

Fig. 7.

Subcellular distribution of $\alpha 7$ nAChR-ir in perivascular astrocytic glia in rodent PFC. **A:** The $\alpha 7$ nAChR-immunoperoxidase labeling (white arrows) is seen in a glial process ($\alpha 7$ -g) that contacts the endothelial basal membrane (bm) of a microvessel. The block arrow points to a higher magnification of the labeled process in the boxed region. **B:** An $\alpha 7$ nAChR immunogold (arrows)-labeled perivascular glial process ($\alpha 7$ -g) is contacted by an axon terminal showing VACHT-immunoperoxidase labeling (VACHT-te). **C,D:** Dual $\alpha 7$ nAChR-immunoperoxidase (white block arrow) and D_2 R-immunogold (small arrows) labeling is seen in perivascular glial process ($\alpha 7+D_2$ -g). In **D**, the D_2 R-ir is localized near an astrocytic

gap junction (GJ). This labeled glial process is also contacted by axon terminals forming asymmetric synapses with dendritic spines that are unlabeled (U-sp) or containing $\alpha 7$ AChR-immuno-peroxidase labeling ($\alpha 7$ -sp). Endothelial cell, end; endothelial nucleus, nu; unlabeled terminals, U-te; asymmetric synapse, curved arrow; Scale bars = 500 nm.

Fig. 8.

Schematic diagram showing the subcellular neuronal and glial distribution of the $\alpha 7$ nAChRs and their relationship to the D_2 R in the rodent PFC. The $\alpha 7$ nAChR (stars) and D_2 R (crescents) are located on the plasma membrane and in the cytoplasm of an interneuron, a pyramidal cell, and an astrocyte. Excitatory-type axon terminals [at (+)] contact the dendritic shaft of the interneuron and the dendritic spines of the pyramidal cell, whose dendritic shaft is also contacted by an inhibitory-type axon terminal [at (-)]. The astrocyte is depicted as having both perisynaptic (A) and perivascular (B) associations as well as contacts (C) with axonal processes containing VAcHT-labeled synaptic vesicles (black circles). In B, the basement membrane = black dashed line.

TABLE I
Percentage of all α 7nAChR and/or D₂R immunolabeled profiles identified as neuronal and glial processes in rodent PFC

Profile	Somata and dendritic shafts		Dendritic spines		Axon terminals		Glial processes	
	Mouse	Rat	Mouse	Rat	Mouse	Rat	Mouse	Rat
α 7nAChR	10%	6%	39%	58%	3%	3.7%	58%	69%
D ₂ R	54%	74%	44%	42%	93%	96%	13%	7%
α 7nAChR/D ₂ R	36%	20%	17%	0%	4%	0.2%	29%	24%
Total ^a	429	399	80	38	360	541	155	287

^aTotal number of profiles seen in sections through the middle layers of the PFC. The numbers are those observed in a tissue area of 4,582 μm^2 (four mice) and 4,739 μm^2 (three rats).

TABLE IISynaptic and vascular associations of $\alpha 7$ nAChR and/or D₂R labeled glial profiles in rat PFC

	$\alpha 7$ nAChR	D ₂ R	$\alpha 7$ nAChR/D ₂ R
BM associated	18%	4%	46%
ES associated	37%	8%	36%
Other locations	45%	88%	18%
Total	114	27	11

N = 5, 2818 μm^2 tissue, BM, basement membrane; ES, excitatory synapse.