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Alpha4 containing nicotinic receptors are positioned to mediate postsynaptic effects on serotonin neurons in the rat dorsal raphe nucleus

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Abstract

Nicotinic acetylcholine receptors containing the alpha4 and beta2 subunits constitute the most abundant high-affinity binding site of nicotine in the brain and are critical for the addictive qualities of nicotine. Serotonin neurotransmission is thought to be an important contributor to nicotine addiction. Therefore in this study it was examined how alpha4-containing receptors are positioned to modulate the function of serotonin neurons using ultrastructural analysis of immunolabeling for the alpha4 receptor subunit in the dorsal raphe nucleus (DR), a primary source of forebrain serotonin in the rat. Of 150 profiles labeled for the alpha4 subunit, 140 or 93% consisted of either soma or dendrites, these were often small-caliber (distal) dendrites <1.5 μ m in diameter (63/150 or 42%). The majority (107/150 or 71%) of profiles containing labeling for alpha4 were dually labeled for the synthetic enzyme for serotonin, tryptophan hydroxylase (TPH). Within dendrites immunogold labeling for alpha4 was present on the plasma membrane or near postsynaptic densities. However, labeling for alpha4 was commonly localized to the cytoplasmic compartment often associated with smooth endoplasmic reticulum, plausibly representing receptors in transit to or from the plasma membrane. Previous studies have suggested that nicotine presynaptically regulates activity onto serotonin neurons, however alpha4 immunolabeling was detected in only 10 axons in the DR or 7% of profiles sampled. This finding suggest that alpha4 containing receptors are minor contributors to presynaptic regulation of synaptic activity onto serotonin neurons, but rather alpha4 containing receptors are positioned to influence serotonin neurons directly at postsynaptic sites.

Keywords

acetylcholine; nicotine; anxiety; 5-HT-1A; addiction

The dorsal raphe nucleus (DR) is one of the primary sources of serotonin (5-HT) in the forebrain. Cholinergic modulation of the DR likely influences several physiological functions including sleep cycle (Portas et al., 2000), anxiety and pain sensitivity. In addition, the actions of nicotine in the DR may contribute to nicotine addiction. Acute nicotine administration modifies 5-HT release in the forebrain (Toth et al., 1992, Ribeiro et al., 1993, Summers et al., 1996). Chronic nicotine administration produces alterations in 5-HT receptors, transporters and brain metabolism of 5-HT in both humans and animal models (Benwell and Balfour,

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1979, Benwell and Balfour, 1982, Benwell et al., 1990, Rasmussen and Czachura, 1997, Kenny et al., 2001). Moreover, smoking is more common in individuals with depression, a disorder associated with modified 5-HT neurotransmission (Breslau et al., 1993, Covey et al., 1998, Diwan et al., 1998, Balfour and Ridley, 2000, Picciotto et al., 2002, Paperwalla et al., 2004, Rohde et al., 2004).

In vitro electrophysiological studies have suggested nicotinic ligands have at least two mechanisms of action within the DR. These include presynaptic effects to stimulate the release of norepinephrine and perhaps other neurotransmitters (Li et al., 1998, Chang et al., 2004). In addition nicotinic agonists elicit a 5-HT release and subsequent activation of 5-HT-1A receptors through an unknown mechanism (Li et al., 1998, Mihailescu et al., 2002). Evidence suggests these effects result in a complex modulation of DR network activity. That is, *in vivo* acute nicotine administration appears to result in oscillatory activation of 5-HT and GABAergic neurotransmission within the DR (Mihailescu et al., 2002) and topographically specific patterns of increases and decreases in forebrain 5-HT (Toth et al., 1992, Ribeiro et al., 1993, Summers et al., 1996).

Although several receptors may participate in the effects of cholinergic agonists in the DR, receptors containing the alpha4 subunit may be a primary site of action. The alpha4 receptor subunit together with beta2, comprise the primary high-affinity binding site of nicotine in the brain (Picciotto et al., 1995, Zoli et al., 1998). Together these receptors are critical for the addictive qualities of nicotine (Picciotto et al., 1998). Serotonin neurons in the DR express mRNA and protein for the alpha4 subunit (Bitner et al., 2000, Cucchiaro and Commons, 2003, Cucchiaro et al., 2005). Moreover local administration of the alpha4 selective ligand epibatidine in the DR has pronounced behavioral effects (Cucchiaro et al., 2005).

Even though the alpha4 subunit has been localized to the DR, it remains poorly understood if this receptor subunit is positioned either pre- or post-synaptically to mediate the effects of nicotinic agonists on 5-HT neurons in the raphe. Understanding the mechanism of action of this receptor on DR neurons will likely be relevant to the mechanisms through which cholinergic ligands influence brain serotonin relevant for sleep cycle, anxiety, addictive behavior, pain sensitivity as well as other interrelated functions of these neurochemicals. Therefore in this study the subcellular distribution of alpha4 receptor subunits with respect to 5-HT neurons in the DR using dual immunolabeling was examined with light and electron microscopic analyses.

Experimental Procedures

Tissue preparation

Animal procedures were approved by the Institutional Animal Care and Use Committee of The Children's Hospital, Boston and conformed to NIH guidelines. All efforts were made to minimize the number of rats necessary to produce reliable scientific data and experiments were designed to reduce any distress experienced. The rats were housed 2–3 per cage on a 12-h light schedule in a temperature controlled (20 °C) colony room and received standard rat chow and water. Rats were anesthetized with sodium pentobarbital (80–120 mg/kg) and transcardially perfused through the ascending aorta with 300 ml of 4% paraformaldehyde in 0.1 M phosphate buffer (PB; pH 7.4) for immunofluorescence or with 50 ml of 3.75% acrolein followed by 200 ml of 2% paraformaldehyde in 0.1 M PB (pH 7.4) for electron microscopy. Brains were then removed and post fixed in 4% paraformaldehyde overnight at 4°C.

For immunofluorescence, brain tissue was equilibrated in 25% sucrose and 35 um-thick sections were cut using a freezing microtome. In some cases, sections were stored in a storage solution (30% sucrose, 30% ethylene glycol in PB) at –80 °C until usage. For immunolabeling,

sections were incubated in a mixture of alpha4 receptor subunit antisera raised in goat (Santa Cruz Biotech, 1:250 dilution) and either antisera raised against tryptophan hydroxylase (TPH; Protos Biotech, 1:1000 dilution) or 5-HT (Immunostar, 1:10,000 dilution) both of which were raised in rabbit. In some cases to confirm the immunolabeling for alpha4 was consistent with previous descriptions, immunolabeling for alpha4 was determined in the substantia nigra with respect to that of tyrosine hydroxylase (TH), detected using a mouse monoclonal antibody from Immunostar diluted 1:1000. The alpha4 receptor antiserum was raised against a peptide corresponding to a portion of the intracellular domain of the rat alpha4 subunit and affinity purified. The antiserum to TPH produces immunolabeling of neurons with a distribution consistent with identification of 5-HT but not catecholamine containing cells (unpublished observation). Incubation time was 18 h at room temperature or 48 h at 4 °C on a rotary shaker. Sections were then washed in PBS and incubated in a secondary antisera prepared in 0.1 % BSA and 0.25% Triton X-100 in PBS for 2 h. Secondary antisera diluted 1:200 were raised in donkey and conjugated to CY3 (Jackson ImmunoResearch) and Alexa 488 (Molecular Probes) and had minimal cross-reactivity to other relevant species. Usually Alexa 488 was used to immunolabel alpha4 while other markers were detected with CY3 conjugated antisera, although this was occasionally switched to ensure the localization was not dependent on the method of detection. Following incubation with the secondary antisera, the tissue sections were washed thoroughly in PBS and mounted on slides and allowed to dry. Coverslips were applied using a glycerol based mounting media. Sections were visualized using a Zeiss microscope equipped with epi-fluorescence illumination or a Zeiss LSM510 Meta confocal microscope. Images were captured digitally, pseudocolored and merged using Adobe Photoshop software or the Zeiss confocal software. Light microscopy figures were assembled using Adobe Photoshop.

For electron microscopy, after post-fixation forty to fifty micrometer thick sections through the DR were cut using a Vibratome. Sections were placed in 1% sodium borohydride in 0.1 M PB for 30 min to remove reactive aldehydes. Sections were stored at -80 °C in storage solution until use. Primary antisera of alpha4 and TPH were diluted as described for light microscopy using the same buffer but without the addition of triton X-100. TPH was used for electron microscopy because of the limited immuno-detection of 5-HT in acrolein-fixed tissue. After incubation with the primary antisera, tissue sections were rinsed in PBS and processed for immunogold labeling of alpha4 and immunoperoxidase labeling of TPH. For this, sections were incubated in a biotinylated donkey anti-goat IgG (1:400; Jackson ImmunoResearch) for 60 min followed by rinses in PBS. This was followed by an incubation with streptavidin nanogold (Nanoprobes Inc.). For all incubations and washes, sections were continuously agitated with a rotary shaker. Nanogold particles were subsequently enhanced using a Goldenhance kit (Nanoprobes) for 7 minutes, which is similar to a silver enhancement process but produces particles resistant to etching by osmium tetroxide.

For immunoperoxidase localization of TPH, sections were rinsed with PBS and then incubated with a peroxidase-conjugated horse anti-rabbit antiserum (Vector Labs, Burlingame, CA) for 2 hours. Peroxidase labeling was visualized using the VECTOR SG substrate (Vector Labs), which produces an electron dense reaction product similar to diaminobenzidine (Zhou and Grofova, 1995). Usually tissue was processed through immunogold and gold enhancement before VECTOR SG, but in some cases the reverse sequence was used with equivalent results. Tissue sections were then incubated in 2% osmium tetroxide (Electron Microscopy Sciences) in 0.1 M PB for 30 min, dehydrated in an ascending series of ethanols and flat embedded between two sheets of plastic using EMBED 812 (Electron Microscopy Sciences). Areas of the DR representing the midline portions where 5-HT neurons are densest were selected using a light microscope. These areas were mounted on EMBED 812 chunks using super glue and trimmed to the appropriate size using a razor blade. Thin sections of approximately 50–100 nm in thickness were cut off the surface of the embedded section with a diamond knife using

a Leica Ultracut. Sections were collected on copper mesh grids and some were counterstained stained with 5% uranyl acetate followed by Reynold's lead citrate. Sections were examined with an electron microscope and photographs were taken using digital cameras supplied by AMT. Figures were assembled and adjusted for brightness and contrast in Adobe Photoshop. In some cases uneven brightness produced by ridges in the diamond knife was reduced using the "burn" and "dodge" tools. Several particles that were in every image taken with a specific digital camera and therefore resembled dust grains were removed using the "rubber-stamp" tool.

Controls and Data analysis

To evaluate cross-reactivity of the primary antisera by secondary antisera, some sections were processed for dual labeling with omission of one of the primary antisera. These sections produced labeling consistent with each label individually. In addition, in some cases the sequence of labeling or the method of detection was varied to ensure the results remained consistent. For quantitative analysis, tissue sections from rats with good preservation of ultrastructural morphology and with both markers clearly apparent were used. At least 10 grids containing 5–10 ultrathin sections each were collected from at least 3 plastic embedded sections from each rat. All profiles containing immunoperoxidase labeling and immunogold labeling in the same fields of at least 11,000X magnification were photographed and later classified.

Selective immunogold labeled profiles were identified by the presence, in single thin sections, of at least three times the number of immunogold particles within a cellular compartment than in adjacent compartments. The cellular elements were identified based on the description of Peters and colleagues (Peters et al., 1991, Peters and Palay, 1996). Asymmetric synapses were characterized by the close apposition of pre- and postsynaptic structures with a pronounced density on the postsynaptic membrane. Symmetric synapses were defined by the close apposition of membranes with equal electron density associated with both pre- and postsynaptic membranes with a minimal postsynaptic density. Appositions were close contacts between neural elements, but the morphology of the synapse could not be clearly defined. Dendritic shafts had a rounded or ovoid shape and were divided into small caliber (<1.5 microns measured at their longest dimension), or large caliber (>1.5 micron diameter).

Results

Using immunofluorescence, the distribution of alpha4 was examined first in an area where it has previously been well described, the substantia nigra pars compacta, with respect to tyrosine hydroxylase (TH, Fig. 1) to confirm labeling specificity. Consistent with previous descriptions that report that more than 99% of TH cells contain alpha4 (Arroyo-Jimenez, et al., 1999; Nashmi et al., 2007), a high level of co-incident labeling was observed. However there was also labeling for alpha4 present in non-TH cells, consistent with the presence of alpha4 within some interneurons in the substantia nigra (Nashmi et al., 2007). Subsequently, the distribution in the DR was examined. At the light microscopic level, the distribution between alpha4 was similar when colocalized with either TPH or 5-HT. Specifically, alpha4 labeling was present over 5-HT or TPH labeled cell soma where it appeared diffuse when examined with conventional fluorescence or confocal microscopy (Fig. 2). Axons labeled for 5-HT were not detectably dually labeled for alpha4 at this level of analysis. Alpha4 was also present in specific areas of the neuropil devoid of detectable 5-HT or TPH labeling as well, suggesting a potential localization to a population of non-5-HT containing cells.

At the ultrastructural level, immunolabeling for alpha4 was sometimes detected in soma (9 of 150 sampled profiles) usually in those also containing immunolabeling for TPH (8 of 9). Perisomatic labeling for alpha4 was largely associated with rough endoplasmic reticulum (Fig. 3). The most common type of profile to contain alpha4 labeling however were dendrites (Figs.

4–5) representing 131 (87%) of 150 labeled profiles. These were fairly evenly divided between large or > 1.5 μm in diameter ($n=68$) and small diameter ($n=63$) dendrites. The majority of dendrites labeled for alpha4 (98/131; 75%) also contained labeling for TPH and equally represented both large and small caliber dendrites. In a random sampling of fields from the photographed sample, TPH labeled dendrites accounted for about 40% of the total dendrites in the neuropil (71/191); therefore alpha4 appeared to be preferentially located in TPH-containing vs. unlabeled dendrites.

Within dendrites, immunogold grains for alpha4 were sometimes found at or near synaptic contacts (Fig. 4, 5). These were typically asymmetric in morphology, consistent with descriptions of both cholinergic and glutamatergic axons in the DRN. The postsynaptic density in these cases was usually only occasionally labeled, rather labeling appeared either at the edge immediately lateral to an asymmetric synapse, or subsynaptically. Additional immunogold grains were found over the plasma membrane at sites that were not detectably near a synaptic junction.

Additional examination of immunolabeling for alpha4 within dendrites revealed that when labeling was present with the cytoplasmic compartment, often gold grains were associated with saccules of smooth endoplasmic reticulum typically 100 nm or less in diameter (Fig. 5). In some cases, labeled smooth endoplasmic reticulum appeared to be associated with electron dense surrounding structures (5C), although a distinct clathrin-like coat was not resolved. In one cases, labeled endoplasmic reticulum appeared contiguous with the plasma membrane (5D), possibly representing a trafficking intermediate. In addition, occasionally endoplasmic reticulum labeled for alpha4 was within close proximity to synaptic contacts (5E).

The detection of alpha4 immunolabeling within axons occurred with a low frequency (10 or 7% of 150 labeled profiles) (Fig. 6). This represented about 1% (10/939) of axons that appeared in the sampled photographed fields. Four axons immunolabeled for alpha4 formed a detectable synaptic contact and in each case these were asymmetric in morphology. Whereas boutons labeled for alpha4 uniformly lacked detectable labeling for TPH, a single large myelinated axon contained both immunolabeling for alpha4 and TPH (Fig. 6B).

Discussion

Immunolabeling for the alpha4 nicotinic receptor subunit is largely found in dendrites in the DR, and often in dendrites that contained immunolabeling indicative of serotonergic neurons. Within dendrites some immunolabeling was present on the plasma membrane at or near synaptic contacts, however most labeling was localized to the cytoplasmic compartment where it could reflect receptors in transit to or from the plasma membrane. Few examples of alpha4 localized to axon terminals were detected. These findings suggest that nicotinic receptors containing the alpha4 subunit are positioned to primarily mediate postsynaptic effects directly on 5HT neurons in the DRN.

Methodological considerations

Immunohistochemical studies rely on the specificity and sensitivity of antisera, and as such are subject to both false-negative and false-positive results. Previously we have shown that the antisera used in this study to detect alpha4 subunit produces highly convergent labeling with two other antisera that are raised to distinct portions of the receptor subunit protein (Cucchiari and Commons, 2003). The distribution of alpha4 labeling was consistent with the pattern previously described by us and others and with in situ hybridization patterns (Bitner et al., 2000, Cucchiari and Commons, 2003). Moreover, labeling of the alpha4 in the substantia nigra was examined, and the distribution was identical to previous observations, including those that do not rely on immunohistochemical detection methods (Nashmi et al., 2007) Arroyo-Jimenez,

et al., 1999). Taken together, these observations suggest specific labeling for the alpha4 receptor subunit. To identify serotonin neurons an antiserum raised against TPH was used, which identifies dendrites with high sensitivity. Since co-localization studies depend on detection of two antigens simultaneously, they are more highly subject to underestimation due to limitations of detection. Therefore estimates of co-localization generally represent an underestimation of coexistence.

Alpha4 immunolabeling was most commonly localized to dendrites, many of which contained immunolabeling indicative of serotonergic neurons. This, in conjunction with previous studies that showed direct innervation of serotonergic dendrites by cholinergic axons (Wang et al., 2000), strongly supports the conclusion that cholinergic ligands can have direct effects on serotonin neurons. However, the details of those effects remain poorly understood. Previous studies using an *in vitro* slice preparation showed that nicotinic agonists produce a release of 5-HT and subsequent activation of 5-HT-1A receptors in (Li et al., 1998). This effect did not appear to be presynaptic, such that it was action-potential independent and synaptic vesicle release-independent. However, activation of 5-HT-1A receptors produced by nicotine was not accompanied by a detectable inward current, which would be expected for a postsynaptic effect. Although the mechanism of interaction between nicotinic and 5-HT-1A receptors remains to be resolved, the common localization of alpha4 subunit to small-caliber (distal) dendrites may contribute to the difficulty of detecting postsynaptic effects of these there receptors because they are located at electrotonically distant sites.

In addition to localization to serotonergic dendrites, alpha4 was sometimes detected on non-5-HT labeled dendrites. Although it is possible that TPH can be under-detected in dendrites particularly in small-caliber dendrites, there is the possibility that alpha4 maybe present on non-5-HT cells within the DR. Indeed from light microscopic observations, there appears to be some immunolabeling of non 5-HT cells, albeit at a lower density. Previous *in vivo* electrophysiological studies have reported that nicotine produces oscillatory activation of serotonin and GABAergic neurotransmission within the DR (Mihailescu et al., 2002). Taken together these results emphasize that nicotine acting at alpha4 containing receptors may not only have direct effects on 5-HT neurons, but also indirect effects and therefore influence network activity in the DR.

When immunolabeling was associated with the plasma membrane in dendrites, it was often at or near the postsynaptic density of asymmetric synapses. Labeling of the postsynaptic density itself maybe under-detected because the structure can be refractory to pre-embedding labeling methods (Nusser et al., 1995). However, this localization is in line with previous studies that also reported localization of alpha4 immunolabeling at asymmetric synaptic contacts in the cortex (Whiting and Lindstrom, 1988; Schroder, 1989; Okuda 1993, (Nakayama et al., 1997) in the substantia nigra (Sorenson et al., 1998) and the ventral tegmental area (Arroyo-Jimenez et al., 1999). Cholinergic axons form asymmetric-type contacts on 5-HT neurons (Wang et al., 2000), and therefore these receptors may mediate synaptic transmission at these sites. However, there remains the possibility that alpha4 containing receptors may be located at non-cholinergic synapses. This is suggested by the observation that the alpha7 nicotinic receptor subunit is often found at synaptic contacts in the cortex that are also enriched in glutamate receptors (Levy and Aoki, 2002), consistent with a modulatory interaction between cholinergic and glutamatergic neurotransmission. Previously we have shown that glutamatergic axons in the DR form similar asymmetric-type synapses as cholinergic axons do (Commons et al., 2005).

When alpha4 receptor subunit labeling was detected in dendrites, a small fraction of labeling was present on the plasma membrane. This is thought to reflect functional sites of action for ligands available in the intracellular space. In addition, substantial labeling was found within the cytoplasm where it was sometimes associated with membranous structures such as smooth

endoplasmic reticulum. This may reflect receptor in transit to or from the plasma membrane. Previous studies have shown that chronic nicotine administration produces an up-regulation of high affinity nicotine binding sites (Ksir et al., 1985, Schwartz and Kellar, 1985, Collins et al., 1989), and that this is mediated by post-translational mechanisms (Marks et al., 1992, Peng et al., 1994). This suggests the possibility that the immunolabeling present in the cytoplasm of dendrites in the DR has the capacity to transfer to the plasma membrane under the appropriate conditions, for example after exposure to chronic nicotine administration. In addition, there is a growing literature suggesting that ionotropic receptors may rapidly shuttle in and out of the cytoplasmic surface and thereby contribute to plasticity of synaptic signaling (reviewed by (Nicoll et al., 2006)).

Although nicotinic agonists often have presynaptic effects in the central nervous system, the localization of alpha4 immunolabeling to axons terminals within the DR was a fairly rare occurrence. However, previous studies have suggested that other receptor subtypes could contribute to the presynaptic effects of nicotinic ligands in the DR. Specifically presynaptic activation of norepinephrine release in the DR may be mediated by alpha7 containing receptors (Li et al., 1998). In addition to alpha7 and alpha4, there are several other nicotinic receptor subunits identified which could contribute to presynaptic effects in the DR. The finding of myelinated axons with alpha4 labeling however raises the possibility of presynaptic function of this receptor in areas that receive innervation of the DR.

Endogenous and exogenous cholinergic agonists in the DR may function to influence forebrain serotonin during the sleep-wake cycle, and perhaps influence behaviors where both serotonin and nicotinic agonists are known to modulate such as feeding behavior, anxiety state, and pain sensitivity. In addition, nicotinic effects in the DR may have an important influence on the establishment and maintenance of nicotine addiction. The current study shows that alpha4 nicotinic receptors are overwhelmingly present on serotonergic dendrites in the DR suggesting they could mediate a direct effect on 5-HT neurons. This could be related to the poorly understood mechanism through which nicotinic agonists produce a 5-HT-1A receptor activation within the DR. 5-HT-1A receptors have been implicated in the anxiolytic effect of nicotine, the development of behavioral sensitization and in cholinergic modulation of serotonin in sleep. Further elucidation of the role of the alpha4 containing receptors in these effects will be important for understanding the mechanisms underlying interactions between cholinergic and serotonergic neurotransmission.

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Abbreviations

DR	Dorsal raphe
TPH	Tryptophan hydroxylase
PB	Phosphate buffer
PBS	Phosphate buffered saline
BSA	

Bovine serum albumin

5-HT

Serotonin

References

- Arroyo-Jimenez MM, Bourgeois JP, Marubio LM, Le Sourd AM, Ottersen OP, Rinvik E, Fairen A, Changeux JP. Ultrastructural localization of the $\alpha 4$ -subunit of the neuronal acetylcholine nicotinic receptor in the rat substantia nigra. *J Neurosci* 1999;19:6475–6487. [PubMed: 10414976]
- Balfour DJ, Ridley DL. The effects of nicotine on neural pathways implicated in depression: a factor in nicotine addiction? *Pharmacol Biochem Behav* 2000;66:79–85. [PubMed: 10837846]
- Benwell ME, Balfour DJ. Effects of nicotine administration and its withdrawal on plasma corticosterone and brain 5-hydroxyindoles. *Psychopharmacology (Berl)* 1979;63:7–11. [PubMed: 112625]
- Benwell ME, Balfour DJ. The effects of nicotine administration on 5-HT uptake and biosynthesis in rat brain. *Eur J Pharmacol* 1982;84:71–77. [PubMed: 7140821]
- Benwell ME, Balfour DJ, Anderson JM. Smoking-associated changes in the serotonergic systems of discrete regions of human brain. *Psychopharmacology (Berl)* 1990;102:68–72. [PubMed: 1697418]
- Bitner RS, Nikkel AL, Curzon P, Donnelly-Roberts DL, Puttfarcken PS, Namovic M, Jacobs IC, Meyer MD, Decker MW. Reduced nicotinic receptor-mediated antinociception following in vivo antisense knock-down in rat. *Brain Res* 2000;871:66–74. [PubMed: 10882784]
- Breslau N, Kilbey MM, Andreski P. Nicotine dependence and major depression. New evidence from a prospective investigation. *Arch Gen Psychiatry* 1993;50:31–35. [PubMed: 8422219]
- Chang, BW.; Mitchum, R.; Nakamura, L.; Madonia, M.; Vezina, P.; McGehee, D. Nicotinic modulation of dorsal raphe neurons that project to nucleus accumbens. 2004 Abstract Viewer/Itinerary Planner. Washington, DC: Society for Neuroscience; 2004. Program No. 48.25, Online
- Collins AC, Marks MJ, Pauly JR. Differential effect of chronic nicotine treatment on nicotinic receptor numbers in various brain regions of mice. *J Subst Abuse* 1989;1:273–286.
- Commons KG, Beck SG, Bey VW. Two populations of glutamatergic axons in the rat dorsal raphe nucleus defined by the vesicular glutamate transporters 1 and 2. *Eur J Neurosci* 2005;21:1577–1586. [PubMed: 15845085]
- Covey LS, Glassman AH, Stetner F. Cigarette smoking and major depression. *J Addict Dis* 1998;17:35–46. [PubMed: 9549601]
- Cucchiari G, Chaijale N, Commons KG. The dorsal raphe nucleus as a site of action of the antinociceptive and behavioral effects of the $\alpha 4$ nicotinic receptor agonist epibatidine. *J Pharmacol Exp Ther* 2005;313:389–394. [PubMed: 15608080]
- Cucchiari G, Commons KG. $\alpha 4$ nicotinic acetylcholine receptor subunit links cholinergic to brainstem monoaminergic neurotransmission. *Synapse* 2003;49:195–205. [PubMed: 12774304]
- Diwan A, Castine M, Pomerleau CS, Meador-Woodruff JH, Dalack GW. Differential prevalence of cigarette smoking in patients with schizophrenic vs mood disorders. *Schizophr Res* 1998;33:113–118. [PubMed: 9783351]
- Kenny PJ, File SE, Rattray M. Nicotine regulates 5-HT(1A) receptor gene expression in the cerebral cortex and dorsal hippocampus. *Eur J Neurosci* 2001;13:1267–1271. [PubMed: 11285026]
- Ksir C, Hakan R, Hall DP Jr, Kellar KJ. Exposure to nicotine enhances the behavioral stimulant effect of nicotine and increases binding of [3H]acetylcholine to nicotinic receptors. *Neuropharmacology* 1985;24:527–531. [PubMed: 4022267]
- Levy RB, Aoki C. $\alpha 7$ 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 X, Rainnie DG, McCarley RW, Greene RW. Presynaptic nicotinic receptors facilitate monoaminergic transmission. *J Neurosci* 1998;18:1904–1912. [PubMed: 9465015]
- Marks MJ, Pauly JR, Gross SD, Deneris ES, Hermans-Borgmeyer I, Heinemann SF, Collins AC. Nicotine binding and nicotinic receptor subunit RNA after chronic nicotine treatment. *J Neurosci* 1992;12:2765–2784. [PubMed: 1613557]

- Mihailescu S, Guzman-Marin R, Dominguez Mdel C, Drucker-Colin R. Mechanisms of nicotine actions on dorsal raphe serotonergic neurons. *Eur J Pharmacol* 2002;452:77–82. [PubMed: 12323387]
- Nakayama H, Shioda S, Nakajo S, Ueno S, Nakashima T, Nakai Y. Immunocytochemical localization of nicotinic acetylcholine receptor in the rat cerebellar cortex. *Neurosci Res* 1997;29:233–239. [PubMed: 9436649]
- Nashmi R, Xiao C, Deshpande P, McKinney S, Grady SR, Whiteaker P, Huang Q, McClure-Begley T, Lindstrom JM, Labarca C, Collins AC, Marks MJ, Lester HA. Chronic nicotine cell specifically upregulates functional $\alpha 4^*$ nicotinic receptors: basis for both tolerance in midbrain and enhanced long-term potentiation in perforant path. *J Neurosci* 2007;27:8202–8218. [PubMed: 17670967]
- Nicoll RA, Tomita S, Brecht DS. Auxiliary subunits assist AMPA-type glutamate receptors. *Science* 2006;311:1253–1256. [PubMed: 16513974]
- Nusser Z, Roberts JD, Baude A, Richards JG, Sieghart W, Somogyi P. Immunocytochemical localization of the $\alpha 1$ and $\beta 2/3$ subunits of the GABAA receptor in relation to specific GABAergic synapses in the dentate gyrus. *Eur J Neurosci* 1995;7:630–646. [PubMed: 7620614]
- Paperwalla KN, Levin TT, Weiner J, Saravay SM. Smoking and depression. *Med Clin North Am* 2004;88:1483–1494, x–xi. [PubMed: 15464109]
- Peng X, Gerzanich V, Anand R, Whiting PJ, Lindstrom J. Nicotine-induced increase in neuronal nicotinic receptors results from a decrease in the rate of receptor turnover. *Mol Pharmacol* 1994;46:523–530. [PubMed: 7935334]
- Peters A, Palay SL. The morphology of synapses. *J Neurocytol* 1996;25:687–700. [PubMed: 9023718]
- Peters, A.; Palay, SL.; Webster, Hd. The Fine Structure of the Nervous System. Oxford University Press; New York: 1991.
- Picciotto MR, Brunzell DH, Caldarone BJ. Effect of nicotine and nicotinic receptors on anxiety and depression. *Neuroreport* 2002;13:1097–1106. [PubMed: 12151749]
- Picciotto MR, Zoli M, Lena C, Bessis A, Lallemand Y, Le Novère N, Vincent P, Pich EM, Brulet P, Changeux JP. Abnormal avoidance learning in mice lacking functional high-affinity nicotine receptor in the brain. *Nature* 1995;374:65–67. [PubMed: 7870173]
- Portas CM, Bjorvatn B, Ursin R. Serotonin and the sleep/wake cycle: special emphasis on microdialysis studies. *Prog Neurobiol* 2000;60:13–35. [PubMed: 10622375]
- Rasmussen K, Czachura JF. Nicotine withdrawal leads to increased sensitivity of serotonergic neurons to the 5-HT_{1A} agonist 8-OH-DPAT. *Psychopharmacology (Berl)* 1997;133:343–346. [PubMed: 9372532]
- Ribeiro EB, Bettiker RL, Bogdanov M, Wurtman RJ. Effects of systemic nicotine on serotonin release in rat brain. *Brain Res* 1993;621:311–318. [PubMed: 8242344]
- Rohde P, Kahler CW, Lewinsohn PM, Brown RA. Psychiatric disorders, familial factors, and cigarette smoking: II. Associations with progression to daily smoking. *Nicotine Tob Res* 2004;6:119–132. [PubMed: 14982696]
- Schwartz RD, Kellar KJ. In vivo regulation of [3H]acetylcholine recognition sites in brain by nicotinic cholinergic drugs. *J Neurochem* 1985;45:427–433. [PubMed: 4009168]
- Sorenson EM, Shiroyama T, Kitai ST. Postsynaptic nicotinic receptors on dopaminergic neurons in the substantia nigra pars compacta of the rat. *Neuroscience* 1998;87:659–673. [PubMed: 9758232]
- Summers KL, Lippiello P, Giacobini E. A microdialysis study of the effects of the nicotinic agonist RJR-2403 on cortical release of acetylcholine and biogenic amines. *Neurochem Res* 1996;21:1181–1186. [PubMed: 8923478]
- Toth E, Sershen H, Hashim A, Vizi ES, Lajtha A. Effect of nicotine on extracellular levels of neurotransmitters assessed by microdialysis in various brain regions: role of glutamic acid. *Neurochem Res* 1992;17:265–271. [PubMed: 1352387]
- Wang QP, Guan JL, Shioda S. Synaptic contacts between serotonergic and cholinergic neurons in the rat dorsal raphe nucleus and laterodorsal tegmental nucleus. *Neuroscience* 2000;97:553–563. [PubMed: 10828537]
- Zhou M, Grofova I. The use of peroxidase substrate Vector VIP in electron microscopic single and double antigen localization. *J Neurosci Methods* 1995;62:149–158. [PubMed: 8750097]
- Zoli M, Lena C, Picciotto MR, Changeux JP. Identification of four classes of brain nicotinic receptors using $\beta 2$ mutant mice. *J Neurosci* 1998;18:4461–4472. [PubMed: 9614223]

Figure 1.

Immunofluorescence detection of alpha4 receptor subunit was consistent with previous descriptions in the substantia nigra pars compacta, supporting specificity of labeling. A. A very high level of coincidence between tyrosine hydroxylase (TH, red, panel B) containing dopaminergic cells and alpha4 immunolabeling (green, panel C). The same examples of dually labeled cells are pointed out with arrows in each panel. However, there was some labeling that appeared to correspond to non-TH labeled cells, potentially interneurons (crossed arrow). Bar = 50 μ m.

Figure 2.

A. A single optical section of immunofluorescence labeling for the alpha4 receptor subunit with respect to 5-HT neurons in the DR as visualized using confocal microscopy. 5-HT neurons (red, panel B) contain diffuse perisomatic labeling for alpha4 (green, panel C; arrows indicate examples of dually labeled cells). Some labeling is evident in dendritic processes (single arrowhead) although it is difficult to ascertain if axons contain labeling. In addition, alpha4 is evident in non-5-HT-labeled areas (double arrowhead). Bar = 50 μ m.

Figure 3.
At the ultrastructural level, perisomatic immunogold labeling for alpha4 (arrows) appears primarily associated with rough endoplasmic reticulum (arrow heads). Bar = 500 nm.

Figure 4.

Immunogold labeling for alpha4 is present in dendrites of serotonergic neurons labeled for TPH with immunoperoxidase. A. Two axons contact (double arrowheads) a small dendrite labeled for both alpha4 and TPH. Some immunogold grains for alpha4 are located on the plasma membrane of the dendrite lateral to the synaptic zones (arrows) while additional grains are located in the cytoplasm. B. In another dually labeled dendrite, immunogold grains for alpha4 (arrows) appear next to a synapse (arrowheads). C. A distal (small caliber) dendritic processes dually labeled for alpha4 and TPH, immunogold grains for alpha4 are apposed to astrocytic contact (arrows). D. At the top of the field a dendrite is dually labeled for alpha 4 and TPH (compare gray values in circle labeled +TPH), while the lower dendrite is singly labeled for

alpha 4 (-TPH). Both dendrites receive synaptic contacts in the plain of section (double arrowheads).

Figure 5.

Within dendrites, alpha 4 is often localized to smooth endoplasmic reticulum. A. A lower magnification of the dendrite shown at higher magnification in B. B. In many instances immunogold grains for alpha 4 are associated with endoplasmic reticulum. C. Another dendrite with grains on endoplasmic reticulum (arrows). In one case, gold grains appear associated with dense coating (arrowheads). D. Boxed region shown at higher magnification in inset shows immunogold grains associated with membrane that appears contiguous with the plasma membrane (arrowheads), possibly representing a trafficking intermediate. E. Boxed region shown at higher magnification in inset shows gold grains for alpha4 on a portion of endoplasmic reticulum (arrow) just below a synaptic contact (s).

Figure 6.

In some instances alpha 4 was detected in axons. A. An axon with gold grains for alpha4 (axon) synapses (arrow) on an unlabeled dendrite, while a nearby dually labeled dendrite (dl-dend) is separated from the axon by an astrocytic sheath (a, arrowheads). B. A large myelinated axon (m, arrows) contains labeling for both TPH and alpha4.