Immunohistochemical localization of a neuronal nicotinic acetylcholine receptor in mammalian brain

(monoclonal antibodies/substantia nigra/medial habenula/interpeduncular nucleus/raphe)

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ABSTRACT A monoclonal antibody generated against purified acetylcholine receptor from *Torpedo* electric organ was used to immunohistochemically localize a neuronal nicotinic acetylcholine receptor. Regions of the rat brain stained with this antibody paralleled those areas of the brain exhibiting [³H]nicotine binding sites and corresponded to areas in which mRNAs encoding for α subunits of the neuronal nicotinic acetylcholine receptor are present. Thus, the anteroventral thalamus, cortex, hippocampus, medial habenula, interpeduncular nucleus, and substantia nigra/ventral tegmental area exhibited significant immunoreactivity. Neurons of the medial habenula and substantia nigra were densely stained, and processes were prominently delineated. Furthermore, in the projection areas of the medial habenula (interpeduncular nucleus and median raphe) axons were strongly immunoreactive and were distributed to distinct subdivisions of the target sites. The present data suggest that there are several discrete neuronal systems in which nicotinic acetylcholine receptors have functional importance. These immunohistochemical studies delineate at the single-cell level the localization within the mammalian central nervous system of certain nicotinic acetylcholine receptors.

Although acetylcholine exerts some of its effects within the central nervous system (CNS) through interaction with nicotinic acetylcholine receptors (nAChRs), the identity, localization, and functional significance of these receptors have yet to be clearly resolved. The demonstration that curare-mimetic neurotoxins derived from snake venom, such as α -bungarotoxin (α -BGTX), produce neuromuscular blockade by binding to the muscle form of the nAChR greatly facilitated the characterization of this receptor. While α -BGTX binding sites are also present within the CNS, these binding sites appear to be distinct from the neuronal nAChR (1). Lack of a reliable probe for neuronal nAChR, in addition to relatively low levels of nAChR within the CNS, has hampered biochemical identification and characterization of CNS nAChR and has prevented its localization to single neurons.

Recent studies using a cDNA clone encoding the α -subunit of the mouse muscle nAChR have suggested that a family of genes is related to the muscle nAChR (2, 3). The screening of genomic libraries prepared from rat neural tissue with a cDNA clone to the muscle nAChR has identified at least two α -subunit-related genes (α_3 , α_4) as members of this family (3). Furthermore, studies utilizing *in situ* hybridization histochemistry (*isHH*) indicate that different CNS regions vary considerably in the expression of mRNA that cross-hybridizes with the α_3 and α_4 cDNAs. These studies offer evidence that different nAChR subtypes exist within the CNS and

identify the brain areas in which these gene products are expressed.

Immunohistochemical localization of receptors or other antigens offers greater anatomical resolution than do current autoradiographic binding techniques and, in contrast to *isHH* methods, also allows the determination of both the sites of receptor synthesis and the final location of receptors in nerve terminals. Thus, immunohistochemistry represents a powerful technique complementary to autoradiographic binding methods and *isHH*. We now report the immunohistochemical localization within the rat CNS of an antigenic determinant recognized by a monoclonal antibody (designated mAb 35.74) generated against purified *Torpedo* electric organ nAChR.

METHODS

Generation and Characterization of Monoclonal Antibodies.

Monoclonal antibodies were produced against affinity-purified nAChR from *Torpedo* electric organ membranes using C57 mice and were tested against mAb 35 (American Type Culture Collection) in binding competition studies; mAb 35 has previously been demonstrated to bind to the main immunogenic region (MIR) of the nAChR (4, 5). One of our antibodies, mAb 35.74, is an IgG1 and binds to *Torpedo* membrane-bound nAChR, to intact mouse muscle cells (unpublished work), and to *Drosophila* neural tissue (6). Because we find that mAb 35.74 completely blocks the binding of the well-characterized rat mAb 35 to the purified *Torpedo* nAChR, the antigenic determinant on *Torpedo* nAChR recognized by mAb 35.74 must coincide with, or lie close to, the MIR on the α subunit (7). On the basis of these competition experiments to be described in detail elsewhere, mAb 35.74 was selected for use in immunohistochemical studies.

Immunohistochemistry. Adult male Sprague–Dawley rats were anesthetized with chloral hydrate and transcardially perfused with saline followed by (i) 4% (wt/vol) paraformaldehyde in 0.1 M sodium phosphate buffer, pH 7.4; (ii) 2% paraformaldehyde in phosphate buffer; or (iii) 4% paraformaldehyde in 0.1 M sodium acetate, pH 6.5, followed by 4% paraformaldehyde/0.05% glutaraldehyde in 0.05 M sodium borate, pH 9.5. The brains were postfixed for 6–8 hr at 4°C, transferred to 0.1 M phosphate buffer containing 30% su-

Abbreviations: α -BGTX, α -bungarotoxin; CNS, central nervous system; DR, dorsal raphe; *isHH*, *in situ* hybridization histochemistry; IPN, interpeduncular nucleus; MIR, main immunogenic region; MHb, medial habenula; MR, median raphe; [³H]Nic, [³H]nicotine; nAChR, nicotinic acetylcholine receptor; SN, substantia nigra; VTA, ventral tegmental area; mAb 35.74, monoclonal antibody 35.74.

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crose, pH 7.4, for 36–40 hr, and frozen sections were cut. Sections were either immediately processed by means of an immunoperoxidase method, or stored at -20°C in 0.1 M phosphate buffer containing 30% sucrose and 30% ethylene glycol (8).

Free-floating sections were washed in 0.05 M Tris-buffered saline (TBS) followed by incubation in methanolic peroxide for 10 min. Sections were then thoroughly washed in TBS, incubated for 30 min in TBS containing 2% normal horse serum and 0.2% Triton X-100, and incubated overnight at room temperature in the hybridoma culture supernatants containing the monoclonal antibodies. Sections were subsequently processed by the avidin–biotin immunoperoxidase method.

RESULTS

Immunohistochemical staining with mAb 35.74 revealed a distinct and heterogeneous pattern of cross-reactivity throughout the rat CNS. The pattern of immunoreactivity was strikingly similar to the distribution of nicotine binding sites (9–12). Furthermore, densely stained perikarya, in which labeling appeared cytoplasmic, were observed only in those areas reported by *isHH* to contain mRNA transcripts encoding neuronal nAChRs (3, 13).

The mAb 35.74 staining was not observed under control conditions: staining was not seen in the absence of the mAb nor following incubation in parent myeloma culture supernatant lacking the mAb IgG. Furthermore, we examined the staining pattern obtained when another mAb of the IgG1 class was used instead of mAb 35.74; this mAb (43.37) is directed against an epitope of the *Torpedo* nAChR distinct from the MIR (6, 7). Immunostaining with mAb 43.37 did not label the areas stained using mAb 35.74, indicating that the immunoreactivity obtained with mAb 35.74 cannot simply be due to binding sites that recognize mouse IgG. Although in most brain regions variability in staining intensity was not apparent, in some sites (e.g., the anterior thalamic nuclei) staining appeared to be quite susceptible to very minor variations in perfusion or processing protocols. Staining with mAb 35.74 was optimal using the pH shift perfusion protocol; addition of Triton X-100 also enhanced immunostaining. The weak or variable staining appears to represent specific mAb 35.74 immunoreactivity and may reflect low densities of antigen only marginally detectable.

Telencephalon. Staining with mAb 35.74 revealed immunoreactive neurons in a laminar pattern throughout the cortical mantle. Immunoreactive perikarya were seen in layer V and in layers II and III in most cortical regions. Immunostained cell bodies were also present in layer VI, particularly in the cingulate cortices. Staining of layer V neurons was most prominent in the parietal and temporal cortices, where the immunoreactive product was distributed on the soma and along apical dendrites, resulting in a punctate appearance. The immunoreactivity of deep layer cortical neurons was of sufficient density in some cases to suggest cytoplasmic rather than surface staining (Fig. 1).

Staining of the striatum was marked by a diffuse neuropil with weak-to-moderate staining of occasional large perikarya, particularly in the dorsal and lateral striatum (Fig. 2). In contrast, weak staining of the ventromedial striatum, nucleus accumbens, and olfactory tubercle was seen.

Immunohistochemical staining of the regions of the magnocellular cholinergic cell groups of the basal forebrain (e.g., medial septum–diagonal band complex) was very weak. Staining of the basolateral nucleus of the amygdala was weak and variable.

Staining intensity of hippocampal neurons was also variable. Immunoreactive neurons in the pyramidal cell layer were seen and were most frequent in fields CA3–4. Densely

FIG. 1. (A) Thalamus stained for mAb 35.74. The anteroventral thalamus (AV) is strongly immunoreactive (calibration bar, 1 mm). (B) Immunoreactive neurons in the hippocampus. Densely stained neurons can be seen in the stratum oriens (SO) and in the pyramidal cell layer (SP) (150 μm). (C) Staining of layer V neurons in the temporal cortex. Arrow indicates a densely labeled neuron (150 μm).

stained neurons were occasionally seen in the stratum oriens (Fig. 1) and were frequently seen in the molecular layer of the dentate gyrus.

Diencephalon. Neurons of the ventral portion of the medial habenula (MHb) were densely stained by mAb 35.74 (Fig. 2). These were of small size, and the staining appeared to be cytoplasmic. Staining was not observed in the lateral habenula.

Distinct thalamic labeling was observed (Fig. 1). Strong labeling of the neuropil and perikarya in the anteroventral nucleus was seen, as was staining of moderate-to-strong density in the anteromedial and anterior paraventricular thalamic nuclei. Perikarya were stained in the interanteromedial region. Scattered cells in the thalamic reticular nucleus were moderately stained. Cells in the ventroposteromedial and ventroposterolateral nuclei were stained; no significant immunoreactivity in the ventrolateral thalamic nucleus was observed. Immunoreactive neurons in the zona incerta and subthalamic nucleus were seen. Except for densely stained cells in the supraoptic nucleus of the hypothalamus, there was very little specific staining of hypothalamic nuclei.

Mesencephalon. Many neurons within the pars compacta of the substantia nigra (SN), ventral tegmental area (VTA), and the retrorubral field were intensely stained (Fig. 2). The staining of these midbrain cells was cytoplasmic in appearance, and dendrites were clearly labeled. Little staining of the zona reticulata was seen.

The interpeduncular nucleus (IPN) was prominently stained by mAb 35.74 (Fig. 2). Intense fiber staining was present in the

FIG. 2. (A) Low-power photomicrograph of densely labeled neurons in the ventral mesencephalon (calibration bar, 500 μ m). (B) Densely labeled neurons of the SN/VTA in the area of the medial terminal nucleus (mtn) (bar, 150 μ m). (C) Neurons of the pars compacta (pc) of the SN labeled with mAb 35.74. The dendrites of these cells can be clearly visualized (bar, 75 μ m). (D) Staining with mAb 35.74 of the striatum (CP) and cortex (CTX). A moderate staining of the striatal neuropil can be observed; some scattered neurons in the lateral aspects of the striatum (arrows) can also be seen. Pyramidal cells in the deep layers of the cortex are present (bar, 1 mm). (E) Staining with mAb 35.74 of the habenula. Staining is present in the ventral aspects of the MHb, but is not observed in the lateral habenula (LHb) (bar, 500 μ m). (F) Neurons of the MHb stained with mAb 35.74. Small neurons are present in the ventral and lateral MHb. Arrow indicates an immunoreactive neuron and stained dendrite (bar, 75 μ m). (G) Staining of the IPN. Note that staining is confined to the central and apical subnuclei and that staining is not seen in the lateral subnuclei. Also stained are neurons in the caudal aspects of the VTA and neurons of the retrorubral field (bar, 500 μ m). (H) Fiber staining in the MR. Staining is not observed dorsally in the DR. A dense plexus of fibers is visible in the ventral MR (bar, 500 μ m).

rostral subnucleus and in the ventral and caudal aspects of the central subnucleus. The more rostral central and intermediate subnuclei exhibited less intense fiber staining; staining of the lateral and dorsolateral subnuclei was relatively weak. Scattered immunoreactive cells were seen in the caudal and intermediate subnuclei and extended dorsally into the apical subnucleus.

Intense fiber staining and some staining of small cells in the median raphe (MR) were seen (Fig. 2). Neurons of the MR exhibiting mAb 35.74 staining were embedded in a dense plexus of fibers. Immunoreactive fibers were seen only in the most caudal aspects of the dorsal raphe (DR). Neurons of the medial geniculate body were moderately stained; light-to-moderate staining of neurons in the lateral geniculate nucleus was observed. Scattered neurons in the dorsal nucleus of the lateral lemniscus were seen. Lightly stained neurons in the ventral tegmental nucleus of Gudden were also observed. The superior colliculus was not stained with mAb 35.74 to any significant degree.

Rhombencephalon. Diffuse, but moderately dense, staining of the cuneiform nucleus was seen with mAb 35.74. Immunoreac-

tivity appeared to be distributed in a continuous column from the pedunculopontine tegmental nucleus (where moderate cellular staining was also present) to the cuneiform nucleus and then ventrolaterally to extend to the parabrachial nucleus. Cells in the dorsal tegmental nucleus were variably and lightly stained, whereas there was no significant staining in the laterodorsal tegmental nucleus. A densely stained plexus of fibers in the most caudal aspects of the DR was seen.

Neurons in the ventral nucleus of the lateral lemniscus were moderately stained as were neurons of the trapezoid body. The trigeminal tract at all levels was stained with moderate-to-strong intensity. Neurons of the nucleus ambiguus were moderately to strongly stained, and light-to-moderate diffuse staining of the inferior olivary complex was observed. Neurons of the lateral reticular nucleus were stained with moderate intensity.

DISCUSSION

The pattern of immunostaining obtained with mAb 35.74 strikingly parallels the distribution of nAChR as revealed by autoradiographic localization of [3 H]nicotine ([3 H]Nic) or

[³H]acetylcholine binding sites (9–12). Dense mAb 35.74 immunostaining was seen in the MHb, IPN, anterior nuclear group of the thalamus, hippocampus, VTA/SN, and cerebral cortex. The positive correlation in the two staining patterns suggests that central nAChRs contain an epitope similar to the MIR. Additional evidence that mAb 35.74 recognizes a central nAChR is derived from the observation that those regions in which neurons exhibit dense cytoplasmic mAb 35.74 immunoreactivity correspond to those sites where a positive hybridization signal is seen following *isHH* with a cDNA probe to the α_3 gene of a neuronal nAChR (2, 3, 13). The anatomical data suggesting that mAb 35.74 recognizes a neuronal nAChR are also supported by biochemical data (unpublished work) indicating that mAb 35.74 competitively inhibits the binding of mAb 35, an antibody directed against the MIR of the nAChR (4, 5); mAb 35 cross-reacts with a neuronal antigen in chick ciliary neurons (14) and in the chick CNS (15). Consistent with this overlap in epitope specificity is the observation that mAb 35.74 results in dense staining of rat supraoptic nucleus neurons, which are also strongly labeled by mAb 35 (16). Because only one mAb has been used in our study, the possibility that similar epitopes on different proteins are being recognized by mAb 35.74 cannot be excluded. An establishment of identity for all the mAb 35.74 binding sites will depend upon future studies with additional mAbs to different epitopes. The present data suggest, however, that mAb 35.74 does not recognize the α -BGTX binding site in the rat CNS, because there is a marked discrepancy between staining with mAb 35.74 and the pattern of labeling seen in autoradiographic binding studies using ¹²⁵I-labeled α -BGTX. Although ¹²⁵I-labeled α -BGTX binds to muscle nAChR, in the CNS it appears to label a site distinct from neuronal nAChRs as revealed by ³H-labeled agonist binding (11, 17–19).

The MHb and its efferent projections were densely stained using mAb 35.74; the MHb has a very high density of [³H]Nic binding sites (9–12). In addition, this region also strongly hybridizes to a cDNA probe (α_3) for a neuronal nAChR (3, 13), consistent with the suggestion that neurons in the ventral aspects of the MHb represent the origin of the cholinergic habenulopeduncular system (20–23). Immunohistochemical examination revealed small densely stained neurons in the ventral MHb. In addition, the entire efferent projection system of the MHb was strongly stained, including axons within the IPN, MR, and caudal DR, consistent with anterograde tracer data indicating that the MHb projects to the IPN and MR but innervates only the most caudal aspects of the DR (24). Furthermore, mAb 35.74 staining of axons in the IPN was restricted to those subnuclei in which [³H]Nic binding sites are present (18). These data therefore suggest that neurons of the MHb synthesize a nAChR, which is then transported to axon terminals within the IPN and MR. These results agree with the hypothesis of Goldman *et al.* (3), suggesting that the α_3 gene encodes a component of a presynaptic nAChR.

A second area exhibiting dense cytoplasmic labeling was the continuum of neurons extending from the VTA to the SN to the retrorubral field of the mesencephalon. These neurons correspond in both position and morphology to the dopamine neurons of the A10, A9, and A8 cell groups of the ventral midbrain. Autoradiographic studies indicate a moderate density of [³H]Nic binding sites in the SN and VTA. In contrast, there is a relatively low density of nicotine binding sites in the striatum and nucleus accumbens, telencephalic projection sites of the midbrain dopamine neurons, despite considerable evidence indicating that presynaptic nAChRs are located on dopamine axons innervating the striatum (25, 26). *isHH* with the α_3 cDNA probe has revealed a strong hybridization signal in the VTA/SN (3, 13). The present immunohistochemical data are therefore in agreement with both autoradiographic

and *isHH* findings and suggest that certain neurons in the VTA/SN/retrorubral field may synthesize a nAChR. The observation that immunostaining of the striatum and nucleus accumbens was relatively weak is consistent with autoradiographic localization of [³H]Nic binding sites (9–12) and may suggest that relatively little nAChR is axonally transported to the striatum.

Cortical neurons, especially those in the deep layers, were stained with mAb 35.74. In most cases, labeling appeared to be confined to the neuronal surface, but in some deep layer cortical neurons the staining appeared to be cytoplasmic. These findings are consistent with *isHH* localization of a nAChR subtype within the cerebral cortex (3, 13) and with the moderate density of [³H]Nic binding sites observed in the deep layers of the cortex (10–12). The densely stained cortical neurons do not appear to correspond to the choline acetyltransferase-positive perikarya in rat cerebral cortex, which are predominantly situated in the superficial layers (27).

The anterior nuclear group of the thalamus was densely, albeit somewhat variably, stained by mAb 35.74. Although neurons of the anteroventral nucleus were clearly labeled, the staining did not appear to be cytoplasmic. This observation is consistent with the suggestion of Goldman *et al.* (3) that the α_4 gene is expressed to a greater degree than the α_3 gene in the thalamus. The pattern of staining in the thalamus was consistent with both the distribution of thalamic [³H]Nic binding sites (9–12) and the observed cholinergic innervation of the thalamus (28, 29). Because Goldman *et al.* (3) propose that the α_4 gene encodes a postsynaptic, rather than a presynaptic, nAChR, the localization of mAb 35.74 immunoreactivity in both the thalamus and the IPN suggests that mAb 35.74 may recognize the α_3 gene product, a putative presynaptic nAChR.

In certain regions [³H]Nic binding sites do not positively correlate with mAb 35.74 staining. The superior colliculus, which exhibits a high density of [³H]Nic binding sites, does not exhibit significant mAb 35.74 staining. While the reason for this discrepancy is not clear, it is interesting that the superior colliculus is one of the few areas in which both considerable ³H-labeled cholinergic agonist binding and ¹²⁵I-labeled α -BGTX binding exist and may suggest that mAb 35.74 does not recognize a protein that may bind both [³H]Nic and ¹²⁵I-labeled α -BGTX.

The present anatomical data confirm and extend previous reports of the distribution of the nAChR in the mammalian CNS. Those regions of the brain that possess [³H]Nic binding sites were stained with mAb 35.74 (10–12). Furthermore, the distribution of densely stained neurons parallels the distribution of the mRNA encoding for an α -like subunit of the putative presynaptic nAChR (3, 13). In particular, in two regions of the rat brain that exhibit a very strong hybridization signal to the α_3 gene probe, the MHb and the VTA/SN, dense staining of neurons and their processes was seen. Such apparently cytoplasmic staining is suggestive of cells in which nAChR is being synthesized, consistent with a large intracellular pool of a nAChR (30).

Also consistent with the suggestion that those neurons that exhibit intense cytoplasmic labeling represent sites of nAChR synthesis is the observation that axons within the projection fields of the densely stained neurons are stained with mAb 35.74. Thus, fibers within discrete subnuclei of the IPN and the MR, the major terminal fields of the MHb, are strongly stained with mAb 35.74. Such terminal staining would be expected for a presynaptic nAChR. Although the localization of mAb 35.74 staining agrees with the hypothesis that the α_3 gene encodes for a presynaptic nAChR (3), this receptor may not necessarily correspond to a cholinergic autoreceptor.

The present data offer several clues as to the functional as well as anatomical organization of central cholinergic sys-

tems that operate through nAChR sites. The clear restriction of mAb 35.74 staining to those subnuclei of the IPN that exhibit [³H]Nic binding sites (18) suggests that the cholinergic innervation of these regions of the IPN is derived from neurons of the MHB, which in turn exhibit dense mAb 35.74 staining. Conversely, the cholinergic innervation of the lateral subnuclei of the IPN, which possess muscarinic binding sites (18), may be derived from other cholinergic neurons that do not exhibit mAb 35.74 staining, such as the cells of the laterodorsal tegmental nucleus. Recent tract-tracing experiments suggest that such a dual innervation pattern of the IPN may be present (31). Whereas the contention that the MHB provides a cholinergic innervation of the IPN is still debatable (20–23, 32–34), the present data localizing nAChR to the habenulopeduncular system are not discordant with the hypothesis that MHB neurons contribute to the cholinergic innervation of the IPN.

The densely labeled cells of the SN/VTA appear most likely to be dopamine neurons. Although the presynaptic regulation of dopamine release from nigrostriatal axons is well characterized (35), the regulation of dopamine neurons through a nAChR at the level of the cell body is less well understood. The presence of mAb 35.74 staining of the soma and dendrites of SN/VTA neurons suggests that acetylcholine regulates the activity of certain dopamine neurons at the level of the cell bodies, as well as via an impulse-independent process. Although cholinergic neurons are not present in the SN, cholinergic axons innervate the pars compacta (36, 37). The cholinergic innervation of the SN appears to be derived from the pedunculopontine tegmental nucleus (29), although this may not represent the only source of cholinergic afferents (38, 39). Recent data suggest that nicotine and acetylcholine may act directly to excite the dopaminergic neurons of the SN (40, 41). Given the relatively sparse cholinergic innervation of the SN/VTA, it will be of interest to see if distinct subpopulations of dopamine neurons are differentially regulated by nAChR mechanisms.

The present immunohistochemical data suggest that the nAChR is heterogeneously distributed within the rat CNS, representing several distinct nicotinic neuronal systems. The existence of precisely demarcated subsystems within discrete central sites suggests that the nAChR may functionally regulate restricted neurons within a given area, such as has been recently demonstrated within the cerebellum (42). The precise regional localization of the nAChR recognized by mAb 35.74 may offer insights into central systems in which cholinergic mechanisms operate through distinct nicotinic receptors. It may prove possible to define other central nAChRs, as predicted from molecular biology studies, using the appropriate monoclonal antibodies (43).

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