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Mapping of brain areas expressing RNA homologous to two different acetylcholine receptor α-subunit cDNAs

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ABSTRACT **We have used an *in situ* RNA·RNA hybridization technique to determine, in the central nervous systems of the mouse and rat, the distribution of RNA homologous to cDNA clones encoding the α subunit of a putative neural nicotinic acetylcholine receptor and the α subunit of the muscle nicotinic acetylcholine receptor. Hybridization of the neural α-subunit probe was strongest in the medial habenula but was also detected consistently in the compact part of the substantia nigra and ventral tegmental area, in the neocortex, and in certain parts of the thalamus and hypothalamus. The *in situ* hybridization technique makes it possible to compile a map of brain regions containing cell bodies expressing RNA coding for a specific receptor type and subsequently to apply the techniques of molecular biology to study these brain receptors.**

Nicotinic acetylcholine receptors are found in both muscle and neural tissue (1–3). Most of our knowledge concerning these receptors comes from studies of those found at the neuromuscular junction (reviewed in ref. 1). The identification, purification and analysis of the muscle acetylcholine receptor has been greatly facilitated by the availability of sources rich in this receptor, such as *Torpedo* electric organ, and by the use of high-affinity α-neurotoxins.

Investigations of putative neural nicotinic acetylcholine receptors have lagged behind their muscle counterpart, due largely to the lack of suitable high-affinity specific ligands. Since α-bungarotoxin binds to the muscle nicotinic receptor, it has been used as a probe to label putative neural nicotinic receptors (4, 5). However, a number of reports indicate that the α-bungarotoxin-binding component found in the central and peripheral nervous systems may be distinct from the functional nicotinic receptor (6–11). In the rat pheochromocytoma cell line PC12, it is clear that α-bungarotoxin does not bind to the nicotinic acetylcholine receptor (11). On the other hand, there are examples in toad, fish, and insect where α-bungarotoxin does bind to a neural nicotinic receptor (reviewed in refs. 5 and 12). Radiolabeled nicotine, acetylcholine, and α-bungarotoxin bind to brain sections or homogenates and have been used to map putative nicotinic receptors. These experiments have resulted in the identification of brain regions likely to contain nicotinic acetylcholine receptors, although the relationship between the acetylcholine- and nicotine-binding components and the α-bungarotoxin binding site is not clear. That α-bungarotoxin binding within the mammalian nervous system can be distinguished from nicotine and acetylcholine binding may indicate heterogeneity in neural nicotinic acetylcholine receptors. Alternatively, the α-bungarotoxin binding site may not correspond to a functional acetylcholine receptor. The use of traditional biochemical techniques to study these molecules has been hampered by the difficulty in identifying and purifying putative neural nicotinic receptors. We have begun investigating neural nicotinic acetylcholine receptors by using the tools of molecular biology.

In recent work, two cDNA clones were isolated that appear to code for α subunits of different acetylcholine receptors. One clone (pMARα15) was isolated from a mouse muscle cell line and codes for the α subunit of skeletal muscle nicotinic acetylcholine receptor (13). The second clone (λPCA48) was obtained from a rat pheochromocytoma cell line, PC12, and codes for a putative neural nicotinic receptor α subunit (14). This clone contains an open reading frame of 1497 nucleotides coding for a protein that is 47% homologous to the α subunit of the mouse muscle nicotinic acetylcholine receptor. Like its muscle counterpart, the neural α-subunit cDNA encodes four hydrophobic sequences thought to form four transmembrane segments and a fifth, amphipathic helix which, in the case of the muscle α subunit, has been proposed to form part of the ion channel (15, 16). The extracellular portion of the muscle α subunit contains four cysteines at positions 128, 142, 192, and 193, which have been proposed to be in the vicinity of the acetylcholine binding site (17). This proposal is supported by the finding that affinity probes label cysteines 192 and 193 (18). The neural α-subunit cDNA also encodes four cysteines at these positions. Based on the sequence homology of the cDNA derived from the PC12 cell line and the cDNA for the muscle α-subunit, it is likely that the PC12 cDNA encodes an α subunit of a neural nicotinic acetylcholine receptor. By hybridizing RNA prepared from these cDNAs to RNA in brain sections, we have begun to map the localization of those cells containing putative nicotinic acetylcholine receptor transcripts in the central nervous system. This is an alternative approach to previous studies that have used either ligands or antibodies as probes for specific receptors. The advantages are that it is possible to identify cell bodies expressing genes coding for the receptor, to subsequently clone the cDNAs coding for this molecule, and to apply the techniques of molecular biology to study its structure and function.

MATERIALS AND METHODS

Muscle Denervation. Mice were anesthetized with methoxyflurane (Metofane) and the sciatic nerve was cut in the left hindlimb. A minimum of 5 days elapsed between denervation and perfusion of animals.

Tissue Preparation. While under ether anesthesia, animals were perfused through the heart as described by Swanson *et al.* (19). Muscle and brains were postfixed overnight at 4°C in 4% paraformaldehyde/0.05% glutaraldehyde/10% sucrose/0.1 M sodium borate buffer, pH 9.5. Tissue was frozen with dry ice and 20-μm-thick sections of brain were cut on a sliding microtome. Alternatively, muscle sections, 8 μm thick, were cut on a cryostat. Sections were mounted on polylysine-coated slides and allowed to dry.

***In Situ* Hybridization.** The method of Cox *et al.* (20) was used for *in situ* hybridization. Before hybridization, tissue sections were digested with proteinase K at 5 μg/ml (brain)

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or 10 μg/ml (muscle). Sections were hybridized with a single-stranded ^{32}P-labeled RNA probe prepared from an SP6 vector containing either a muscle acetylcholine receptor α-subunit cDNA insert (pMARα15) or a putative neural acetylcholine receptor α-subunit insert (λPCA48). These clones contain the entire coding region, with some 5′ and 3′ untranslated sequences (13, 14). Tissue sections were exposed to probe at ≈10^8 cpm/ml, coverslipped, and immersed in a mineral oil bath at 42°C for 14–18 hr. Posthybridization treatments included digestion with RNase A (40 μg/ml) and a final wash in 15 mM NaCl/1.5 mM sodium citrate, pH 7, for 30–60 min at 42°C. Slides were dehydrated in ethanol and exposed to Kodak XAR film at room temperature for 2–7 days.

RNA Analysis. RNA was prepared from the PC12 cell line and from a region of the rat brain containing the habenula. Fifty male Sprague–Dawley rats were anesthesized with ether and decapitated, and brains were removed. After the hypothalamus was removed, a wedge of tissue containing the habenula was cut from the thalamus just above the hypothalamus. The lateral edge of the stria medullaris served as lateral margin, the habenular commissure served as a landmark for the posterior margin, and the anterior margin of the wedge was 2 mm anterior to the commissure. Total RNA was prepared using the guanidine thiocyanate/CsCl procedure (21). Poly(A)$^+$ RNA was isolated from total RNA by oligo(dT)-cellulose chromatography (22). Blot analysis of electrophoretically fractionated RNA and nuclease S1 digestion of heteroduplexes formed between poly(A)$^+$ RNA and the cDNA clone coding for the neural acetylcholine receptor α subunit (λPCA48) were carried out as described (23).

RESULTS

***In Situ* Hybridization to Muscle.** We first tested the specificity of the *in situ* hybridization conditions by using mouse hindlimb muscle and a probe prepared from either the muscle nicotinic acetylcholine receptor α-subunit clone or the neural receptor α-subunit clone. To prepare radioactive probes suitable for *in situ* hybridization, α-subunit cDNA clones were subcloned in pSP65, a plasmid that contains the SP6 promoter adjacent to a multiple cloning site (24). Radiolabeled single-stranded RNA was prepared from these subclones by run-off transcription *in vitro* (24). Initially, we used these probes to detect, by *in situ* hybridization, RNA in mouse lower hindlimb muscle, where a muscle nicotinic acetylcholine receptor is expressed and the levels of mRNA coding for the α subunit are known to increase 50- to 100-fold upon denervation (23, 25). Incubation of sections of innervated and denervated muscle with a probe corresponding to the α subunit of the muscle nicotinic receptor showed, as expected, that muscle denervation results in a large increase in hybridizing material (Fig. 1*A*).

In contrast, a radiolabeled single-stranded RNA probe made from the neural clone obtained from the PC12 cell line detected little hybridizing material in either innervated or denervated muscle sections (Fig. 1*B*). The small amount of hybridization to the denervated muscle section with the neural α-subunit probe probably represents cross-hybridization of this probe with muscle α-subunit mRNA. This is expected, since under the hybridization and washing conditions employed (T_m 76°C), molecules with only 70% sequence homology will still hybridize (20). DNA sequence analysis of the neural and muscle α-subunit cDNA clones indicates that in the region coding for two of the membrane-spanning regions, homology >70% exists at the nucleotide level (14). This is consistent with previous observations that muscle contains RNA that hybridizes to the cDNA clone encoding the muscle receptor α subunit but hybridizes poorly to the cDNA clone encoding the putative neural acetylcholine receptor α subunit (14). Thus, the *in situ* hybridization method is specific and can distinguish between RNAs coding for these two closely related α-subunit peptides.

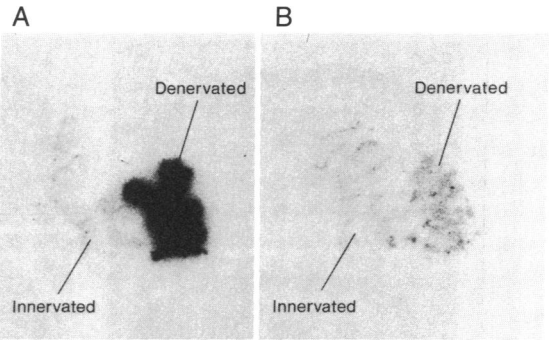

FIG. 1. Autoradiograph illustrating *in situ* hybridization of innervated and denervated muscle sections with the two α-subunit RNA probes. (*A*) Mouse muscle α-subunit probe (pMARα15). (*B*) Rat neural α-subunit probe (λPCA48). Length of exposure to x-ray film was 3 days at room temperature.

***In Situ* Hybridization to Brain.** Having demonstrated the specifity of this method, we tested the ability of the neural and muscle α-subunit probes to detect homologous sequences in the brain. For this, sections through the forebrain of the mouse and rat were probed with radiolabeled single-stranded RNA derived from the neural and muscle α-subunit cDNAs (Fig. 2). We chose initially to analyze mouse brain sections to compare the regional hybridization of our two α-subunit cDNA probes (Fig. 2 *A* and *B*). However, since one of our probes was derived from a rat cell line (PC12), we also performed *in situ* hybridization with rat brain sections (Fig. 2*C*). Similar results were obtained with both mouse and rat brain sections. It is clear that the neural α-subunit probe hybridizes much more strongly to RNA in different regions of the brain than does the muscle α-subunit probe. Most noticeably, in both mouse and rat, the neural α-subunit probe hybridizes with RNA in the medial habenula (MH; Fig. 2 *A* and *C*), although clear hybridization is also seen in the ventral tegmental area (VTA) and adjacent substantia nigra pars compacta (SN). On the other hand, the muscle nicotinic receptor α-subunit probe shows little hybridization to these regions. The small amount of hybridization observed in these regions with the muscle α-subunit probe probably represents hybridization of those sequences encoding the membrane-spanning regions in the muscle probe that are >70% homologous with the neural α-subunit cDNA membrane-spanning sequences. The neural α-subunit probe also hybridizes, although to a lesser extent, to the anteroventral nucleus of the thalamus, the medial geniculate nucleus, and the neocortex (AV, MG, and C; Fig. 2). Other brain regions that show a positive hybridization signal with the neural α-subunit probe, but appear to vary in signal strength from one experiment to another, are the hypothalamus, dentate gyrus, and hippocampus (H, DG, and Hi; Fig. 2). The clearest hybridization in the hypothalamus was centered in the region of the dorsomedial nucleus.

Hybridization to both the neural and muscle α-subunit probes is observed at low levels in the dentate gyrus, hippocampus, and neocortex (DG, Hi, and C; Fig. 2). This hybridization signal may reflect the presence of transcripts with sequence homology to both of these probes or it may reflect nonspecific binding. The level of nonspecific binding was assessed by preparing a probe identical in sequence to the endogenous RNA encoding the neural α-subunit (the "sense" strand). This probe cannot hybridize to the RNA encoding the α-subunit, so any observed hybridization must be nonspecific. When this probe is hybridized with brain

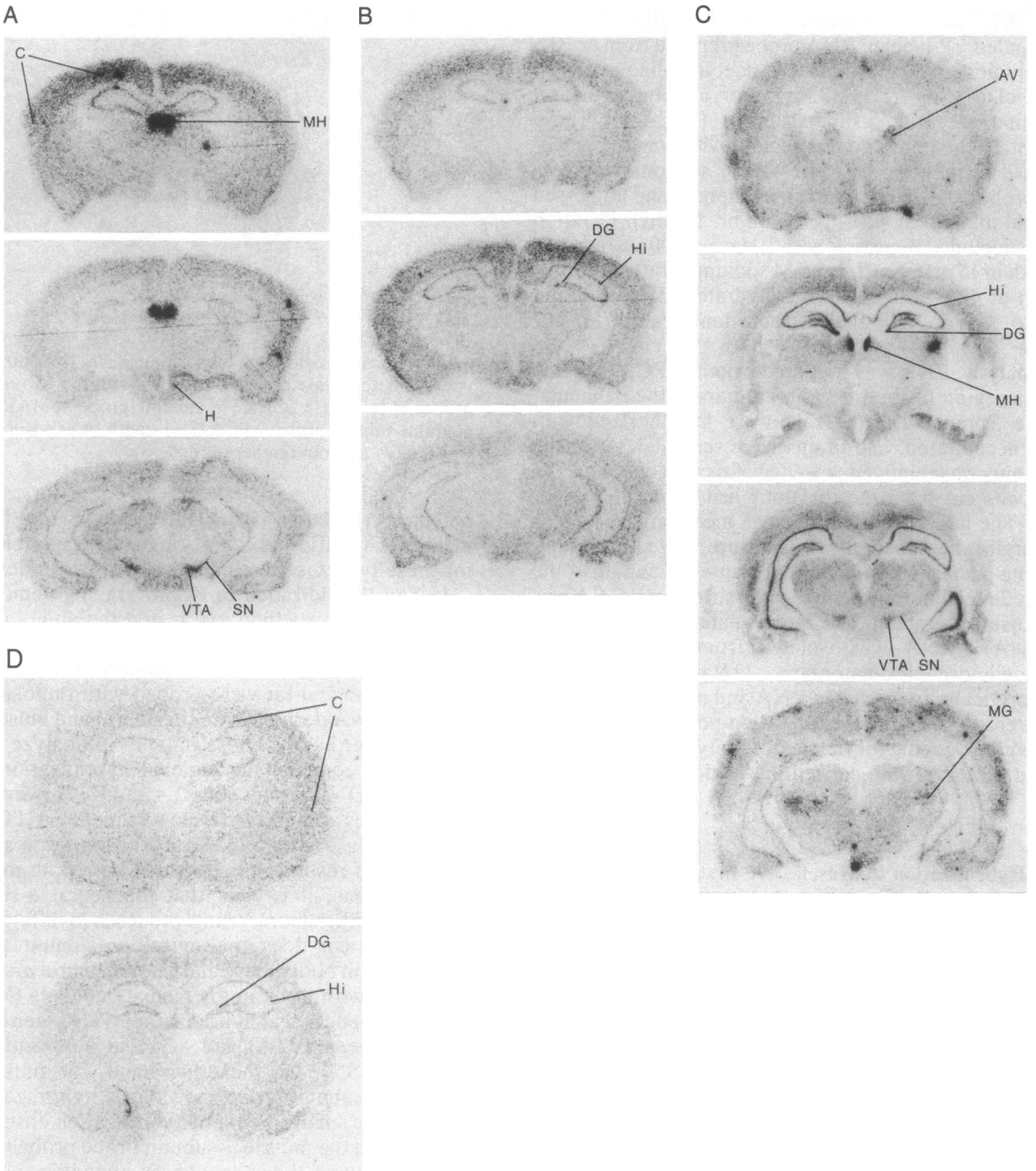

FIG. 2. *In situ* hybridization of mouse (*A*, *B*, and *D*) and rat (*C*) brain sections with the rat neural α-subunit probe λPCA48 (*A* and *C*) and the mouse muscle α-subunit probe pMARα15 (*B*). *D* shows *in situ* hybridization of mouse brain sections with a sense-strand probe prepared from the rat neural α-subunit clone to detect nonspecific binding. AV, anteroventral nucleus of the thalamus; C, neocortex; DG, dentate gyrus; H, hypothalamus; Hi, hippocampus; MG, medial geniculate nucleus; MH, medial habenula; SN, substantia nigra pars compacta; VTA, ventral tegmental area. Mouse and rat brain sections were exposed to x-ray film for 4 days at room temperature.

sections, little hybridization to the neocortex is observed, although a significant signal is found in the dentate gyrus and hippocampus (C, DG, and Hi; Fig. 2*D*). Therefore, the signal in the dentate gyrus and hippocampus is, at least in part, due to nonspecific hybridization. However, hybridization in the neocortex probably represents the presence of transcripts homologous to both α-subunit probes.

To determine in a more direct way whether the hippocampus contains RNAs that specifically hybridize to the two α-subunit probes, we have isolated the hippocampal region from rat brains and prepared poly(A)$^+$ RNA. When this RNA was size-fractionated in agarose gels and transfer blots were hybridized with the α-subunit cDNA probes, distinct RNA species were detected under low-stringency conditions [5× SSPE (0.9 M NaCl/50 mM sodium phosphate, pH 7.4/5 mM EDTA), 65°C] (14). This result indicates that there are specific transcripts in the hippocampus that are homologous to both the muscle and the neural α-subunit cDNA probes that are not detected by *in situ* hybridization, probably due to the background of nonspecific binding.

RNA Analysis. To confirm that the strong *in situ* hybridization signal observed in the habenula reflects the presence of RNA complementary to our cDNA probe, we prepared poly(A)$^+$ RNA from a region of the thalamus containing the habenula. This RNA, along with poly(A)$^+$ RNA isolated from the PC12 cell line, was size-fractionated in denaturing formaldehyde/agarose gels and transferred to GeneScreen-*Plus* (New England Nuclear). RNA blots were probed with the neural α-subunit cDNA, radiolabeled by nick-translation (26). Two transcripts were detected in both the PC12 cell line and the area of the thalamus containing the habenula (Fig. 3*A*).

Nuclease S1 protection experiments were performed to determine whether the RNA in the habenula hybridizing to

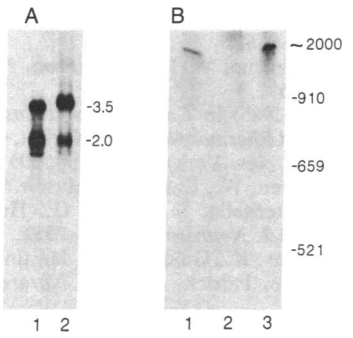

FIG. 3. (*A*) Blot hybridization analysis. Poly(A)$^+$ RNA from either the PC12 cell line (lane 1) or a region of the brain containing the habenula (lane 2) was size-fractionated by electrophoresis in denaturing agarose gels, transferred to GeneScreen*Plus*, and hybridized with ^{32}P-labeled λPCA48 insert. Sizes of major hybridizing species are given in kilobases. (*B*) Nuclease S1 analysis. λPCA48 was subcloned in M13 vectors mp18 and mp19. Single-stranded DNA was prepared and used to form heteroduplexes with poly(A)$^+$ RNA isolated from the PC12 cell line (lane 1) and a region of the brain containing the habenula (lane 3). Lane 2 represents a control where RNA was omitted. Hybridization reaction mixtures were incubated with nuclease S1 and those molecules surviving digestion were fractionated in denaturing acrylamide gels and electroblotted to GeneScreen*Plus*. Heteroduplexes surviving digestion were visualized by hybridization with ^{32}P-labeled λPCA48. Blots were exposed to x-ray film with an intensifying screen for 18 hr at −70°C. pBR322 restriction fragments run in a parallel lane served as size markers (lengths in base pairs at right).

the neural α-subunit probe was identical to the cDNA encoding the neuronal α-subunit cloned from the PC12 cell line. When heteroduplexes were formed between neural α-subunit cDNA and RNA isolated from either the PC12 cell line or the region of the brain containing the habenula, complete protection of the cDNA clone from nuclease S1 digestion was observed (Fig. 3*B*). This method would detect differences of just a few bases between the mRNA and the cDNA. This result is consistent with the idea that the medial habenula expresses the same gene that is expressed in the PC12 cell line, and it suggests that the hybridization we see in the brain sections reflects the presence of this RNA species.

DISCUSSION

We have used *in situ* hybridization to map the distribution of cells expressing RNA with homology to cDNA clones encoding acetylcholine receptor α subunits. The specificity and reliability of this method was demonstrated by applying the technique to sections of skeletal muscle before and after denervation. As expected, denervation resulted in a large increase in the amount of *in situ* hybridization with the muscle cDNA probe and little hybridization with the neural cDNA probe. The small amount of hybridization seen when the neural cDNA probe was used can be explained by the fact that short regions of the probes are about 70% homologous to each other (see *Results*).

When the muscle and neural cDNA clones were used to probe brain sections for hybridizing RNA, a very different result was seen. A number of brain regions showed very strong hybridization to the neural cDNA probe, whereas there was much less detectable hybridization to the muscle cDNA probe. For one such region, the medial habenula, nuclease S1 analysis showed that the signal observed upon *in situ* hybridization is due to the expression of mRNA corresponding to the neural cDNA. RNA transcripts isolated from this brain region that hybridize to the neural α-subunit cDNA are of a similar size as those transcripts obtained from the PC12 cell line. These results indicate that at least part of the signal observed upon *in situ* hybridization of neural α-subunit probe with the medial habenula is the result of expression of the gene that encodes RNA corresponding to the neural cDNA. Thus, this gene is expressed both in the habenula and in the PC12 cell line (and, by inference, in the chromaffin cell, since the PC12 cell line is thought to be derived from the rat chromaffin cell). Thus, under our hybridization conditions it is possible to distinguish between these two related mRNA species, which are 47% homologous at the protein level but have regions that are about 70% homologous at the nucleotide level (14).

Boulter *et al.* (14) proposed that the cDNA isolated from the neuronal cell line PC12 codes for a neural nicotinic acetylcholine receptor α subunit. The data presented here are consistent with this proposal. However, since this cDNA was derived from the PC12 cell line, which expresses an α-bungarotoxin-binding component in addition to a nicotinic receptor (27), it is possible that this cDNA hybridizes to RNA coding for the α-bungarotoxin-binding component found in PC12 cells and in the brain. The relationship between the α-bungarotoxin-binding site and neural nicotinic acetylcholine receptor is not clear, but it has been shown that, in some cases, they have a different regional distribution consistent with the idea that they are different molecules (6, 27).

The medial habenula shows very strong hybridization to the neural α-subunit cDNA probe (MH; Fig. 2). One expects that this RNA is transcribed in neurons and codes for a protein localized in their cell bodies and dendrites, and/or in their axonal projections. Pharmacological studies indicate that the medial habenula contains nicotinic acetylcholine receptors and binds little α-bungarotoxin (4, 6). The medial habenula receives a cholinergic input, which appears to arise at least partly in the nucleus of the diagonal band, via the stria medullaris (28, 29). In addition, fibers from certain neurons in the medial habenula appear to be cholinergic (30), and their axons contribute to the fasciculus retroflexus, which terminates in the interpeduncular nucleus (31) where the amount of α-bungarotoxin binding is controversial (4, 6) although acetylcholine binding is high (6). Therefore, the RNA hybridizing to the neural α-subunit probe could reasonably encode either the nicotinic receptor seen in the medial habenula and interpeduncular nucleus or the α-bungarotoxin-binding component reported in the interpeduncular nucleus. One could presumably distinguish between these possibilities by use of antibodies directed against the protein that the neural α-subunit clone encodes and by functional studies.

The probe prepared from the neural α-subunit cDNA also showed clear hybridization in the substantia nigra pars compacta (SN; Fig. 2), which gives rise to the dopaminergic nigrostriatal projection, and in the adjacent ventral tegmental area (VTA; Fig. 2), which gives rise to the dopaminergic mesolimbic projection. Again, the hybridizing species could encode a protein found in the substantia nigra pars compacta and ventral tegmental area or in their associated projections. In this case, little α-bungarotoxin binding is found in the substantia nigra (4, 6). However, in the caudate putamen, which is innervated by axons from the substantia nigra, the level of α-bungarotoxin binding has been reported to be low in the rat (6) but high in the mouse (4). The axonal projections from the substantia nigra pars compacta to the striatum are known to both release dopamine and contain presynaptic nicotinic acetylcholine receptors (2, 32, 33). Nicotine and acetylcholine appear to act on the dopaminergic neurons and terminals, resulting in a stimulation of dopamine release and turnover in the striatum (2, 32). Therefore, the RNA hybridizing to the neural α-subunit probe may encode the α subunit found on the nigrostriatal and mesolimbic dopaminergic neurons and/or the α-bungarotoxin-binding component ob-

served in the projection of the substantia nigra to the caudate putamen.

Hybridization with the neural α-subunit probe is also observed in the neocortex and the anteroventral nucleus of the thalamus (C, AV; Fig. 2). Both of these regions bind nicotine and acetylcholine (6). The anteroventral nucleus of the thalamus appears to receive a direct cholinergic projection from the dorsolateral tegmental nucleus (34), suggesting that it contains cholinergic receptors. Likewise, the neocortex receives a cholinergic input from the magnocellular nuclei of the basal forebrain (30, 35, 36) and may contain cholinergic interneurons as well (30).

Our results illustrate the potential of *in situ* hybridization for mapping cell bodies expressing RNA coding for specific neurotransmitter receptors. Numerous studies have relied on the binding of radiolabeled agonist or antagonist to identify and localize neural nicotinic receptors. These methods suffer from a failure to distinguish between receptor molecules that may be different but interact with the same ligand. Nucleic acid hybridization is a specific and sensitive method that can distinguish between similar molecules and therefore provides a sensitive probe for mapping the distribution of cells that synthesize particular neurotransmitter receptors in the central nervous system. The cloned cDNAs can also be used to deduce the primary structure of the receptor and to study mechanisms underlying the regulation of receptor genes. Once the primary structure is known, it is possible to generate antibodies to map the location of receptor proteins by standard histochemical techniques (37). Further, the availability of cDNA clones makes it possible to express the receptor in cell types easily accessible to the techniques of electrophysiology and biochemistry to determine the function and structure of receptors in the central nervous system.

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