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A comparison of $\alpha 2$ nicotinic acetylcholine receptor subunit mRNA expression in the central nervous system of rats and mice

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Abstract

The nicotinic acetylcholine receptor (nAChR) $\alpha 2$ subunit was the first neuronal nAChR to be cloned. However, data for the distribution of $\alpha 2$ mRNA in the rodent exists in only a few studies. Therefore we investigated the expression of $\alpha 2$ mRNA in the rat and mouse central nervous systems using non-radioactive in situ hybridization histochemistry. We detected strong hybridization signals in cell bodies located in the internal plexiform layer of the olfactory bulb, the interpeduncular nucleus of the midbrain, the ventral and dorsal tegmental nuclei, the median raphe nucleus of the pons, the ventral part of the medullary reticular nucleus, the ventral horn in the spinal cord of both rats and mice, and in a few Purkinje cells of rats, but not of mice. Cells that moderately express $\alpha 2$ mRNA were localized to the cerebral cortex layers V and VI, the subiculum, the oriens layer of CA1, the medial septum, the diagonal band complex, the substantia innominata, and the amygdala of both animals. They were also located in a few midbrain nuclei of rats, whereas in mice, they were either few or absent in these areas. However, in the upper medulla oblongata, $\alpha 2$ mRNA was expressed in several large neurons of the gigantocellular reticular nucleus and the raphe magnus nucleus of mice, but not of rats. The data obtained show that a similar pattern of $\alpha 2$ mRNA expression exists in both rats and mice, with the exception of a few regions, and provide the basis for cellular level analysis.

Keywords

interpeduncular nucleus; ventral tegmental nucleus; dorsal tegmental nucleus; oriens layer of CA1; internal plexiform layer; in situ hybridization

The nicotinic acetylcholine receptors (nAChRs) in the rat nervous system are a gene family containing $\alpha 2$ (Wada et al., 1988), $\alpha 3$ (Boulter et al., 1986), $\alpha 4$ (Goldman et al., 1987), $\alpha 5$ (Boulter et al., 1990), $\alpha 6$ (Lamar et al., 1990), $\alpha 7$ (Seguela et al., 1993), $\alpha 9$ (Elgoyhen et al., 1994), $\alpha 10$ (Elgoyhen et al., 2001), $\beta 2$ (Deneris et al., 1988), $\beta 3$ (Deneris et al., 1989), and $\beta 4$ (Isenberg and Meyer, 1989; Duvoisin et al., 1989) (see reviews in Role, 1992; Sargent, 1993; Lindstrom et al., 1998; Lukas et al., 1999; McGehee, 1999). In the mouse nervous system, the expression of $\alpha 2$ -6 and $\beta 2$ -3 was identified using in situ hybridization histochemistry techniques with rat cRNA or oligonucleotide probes (Marks et al., 1992; Zoli

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et al., 1998) and mouse α 2-7 and β 2-4 sequences were recently reported (Picciotto et al., 1995; Orr-Urtreger et al., 1995; Watanabe et al., 1998; Kuo, et al., 2002). Combinations of these subunits are thought to form multiple functionally different nAChR subtypes (Luetje and Patrick, 1991), although α 7 and α 9 subunits form functional homooligomers when expressed in *Xenopus* oocytes (Couturier et al., 1990). The different neuronal nAChR subunit genes are expressed in distinct areas of the central and peripheral nervous systems in the adult rat (Wada et al., 1989, 1990; Dineley-Millar and Patrick, 1992; Rust et al., 1994; Le Novère et al., 1996; Flores et al., 1996) and also during development (Zoli et al., 1995).

Data for the distribution of α 2 mRNA in the rodent has been obtained from only a few studies that used radioactive in situ hybridization histochemistry (Picciotto et al., 2001). In the rat, the distribution of α 2 mRNA has been investigated in various regions of the rat central nervous system (CNS) (Wada et al., 1989). In the mouse, positive hybridization for α 2 mRNA was found only on three nuclei which showed strong hybridization: the interpeduncular nucleus, and the ventral, as well as dorsal tegmental nuclei (Marks et al., 1992). Because of relatively high background using full-length cRNA probes, it was especially difficult to detect a small number of scattered cells expressing α 2 mRNA. No detailed study of the distribution of α 2 mRNA in the mouse has yet been performed. It was expected that a similar distribution of α 2 mRNA would be observed in both the rat and mouse CNSs. However, it has been reported that rats and mice do exhibit some differences in their expression of certain nAChR subunits. For example, in the rat hippocampal CA1 field, neurons that weakly expressed the α 5 subunit were detected in the pyramidal cell layer in the adult rat (Wada et al., 1990) and strong expression for the α 5 subunit was only temporarily detected in rats that were in their developmental stages (Winzer-Serhan and Leslie, 2005). In contrast, expression for the α 5 subunit was detected in the CA1 pyramidal cell layer in the adult mouse (Salas et al., 2003). In the same region of the dorsal hippocampal CA1 field, α 4 subunit expression was not detected in rats (Wada et al., 1988, 1989), while moderate hybridization signals were found in mice (Ross et al., 2000; Salas et al., 2003).

Neuronal nAChR subunit transgenic mice provide a new approach for the study of the structure and function of nAChRs in the central and peripheral nervous systems (Picciotto et al., 2001; Byun et al., 2004). Therefore, a precise knowledge of the cellular distribution of the nAChR α 2 subunit is needed. Non-radioactive in situ hybridization histochemistry enables us to obtain a more precise localization of cells, as well as a higher resolution of cell morphology in comparison to the radioactive method. Therefore, in this study we investigated the expression of α 2 mRNA in the rat and mouse CNSs using non-radioactive in situ hybridization histochemistry. A portion of the present study has been published in abstract form (Ishii et al., 2004).

MATERIALS AND METHODS

cRNA probe preparation

Rat probe—The full-length cDNA clone HYP16, coding for rat nAChR α 2 subunit, was kindly provided by Dr. J. Boulter (UCLA, Los Angeles, CA). The 5' terminal EcoRI/BamHI fragment [657 base pairs (bp)] was subcloned into a pGEM-4Z vector (Promega, Madison,

WI) between SP6 and T7 promoter sites. The antisense rat $\alpha 2$ subunit riboprobe was linearized with BamHI and transcribed by T7 RNA polymerase, while the sense riboprobe was linearized with EcoRI, transcribed by SP6 RNA polymerase, and used for the synthesis of digoxigenin-11-UTP labeled RNA probes (Fig. 1). A search of the GenBank database revealed that the sequence of this fragment contains no significant homology to other rat DNA sequences.

Mouse probe—A full-length mouse $\alpha 2$ cDNA clone was obtained from Open Biosystems (Huntsville, AL). For the synthesis of digoxigenin-11-UTP labeled RNA probes, the XbaI/HindIII fragment (700 bp) in the 3' non-coding sequence was subcloned into a pGEM-3Z vector (Promega) between SP6 and T7 promoter sites (Fig. 1). Based on a search of the GenBank database, the sequence of this fragment contains no significant homology to other mouse DNA sequences.

Tissue preparation—Four young adult rats (Sprague-Dawley, 5-8-week-old, Body Weight 120-215g) and five adult mice (C57BL/6, 3-month-old, Body Weight 28-33g) of either sex were obtained from Charles River Laboratories and were deeply anesthetized by urethane (3g/kg, i.p.). The research protocol was approved by the University Animal Care and Use Committee and followed Federal guidelines. Transcardial perfusions were performed, first with 200 ml or 30 ml 0.9% NaCl for rats and mice, respectively. For in situ hybridization histochemistry, the perfusate was switched to 300 ml of 4% paraformaldehyde in 0.1M sodium phosphate buffer (PB; pH 7.4) for rats, or 50 ml for mice, for 15 minutes at room temperature (RT). Brains and spinal cords were then removed, postfixed with the same fixative for 6 hours and kept in 30% sucrose overnight at 4°C for cryoprotection.

The fixed brains were embedded in Tissue-Tek O.C.T. compound (Sakura Finetek, Torrance, CA), sectioned at 40 or 35- μ m thickness in the coronal, sagittal, or horizontal plane with a cryostat (Leica Instruments, Nussloch, Germany), and then stored in 4% paraformaldehyde in 0.1M PB for 1 week at 4°C. Every other sagittal and horizontal sections and every third coronal section was processed for in situ hybridization histochemistry according to a modification of the method described by Liang et al. (2000) and Tochitani et al. (2001). The remaining sections were stained with thionin for Nissl substances and used for comparison with in situ hybridization histochemical preparations. To avoid RNase contamination as much as possible, all procedures were carried out in new plastic ware.

In situ hybridization histochemistry—Free-floating sections were washed twice in PB (pH 7.4) for 10 minutes at RT on a shaker and then incubated twice in 0.75% glycine in 0.1M PB for 15 minutes. They were washed successively in 0.1M PB for 5 minutes, 0.3% Triton X-100 in 0.1M PB for 5 minutes, and 0.1M PB for 5 minutes. After rinsing with 0.1N HCl in standard saline citrate (SSC) for 20 minutes, they were treated with 1 μ g/ml proteinase K for 30 minutes at 37°C and 0.25% acetic anhydride in 0.1M triethanolamine (TEA) for 10 minutes at RT. After washing with 0.1M PB for 5 minutes and twice in 2xSSC (1xSSC: 150mM NaCl, 15mM sodium citrate; pH7.0) for 20 minutes, they were incubated in a hybridization solution (5xSSC, 2% blocking reagent, 50% (v/v) formamide, and 0.1% N-lauroylsarcosine) without the probe for 60 minutes at 55°C in the dark. They were then

incubated in a hybridization solution containing digoxigenin-labeled sense or antisense riboprobes with a concentration of 1.5µg/ml for 16-20 hours, at 55°C in the dark.

Hybridized sections were washed twice in a solution of $2 \times \text{SSC}$, 50% formamide, 0.1% N-lauroylsarcosine for 20 minutes at 55°C, and subsequently, RNase A buffer (10mM Tris-HCl, 0.5M NaCl, 1 mM EDTA) for 10 minutes at 37°C, 20µg/ml RNase A in RNase A buffer for 30 minutes at 37°C, 2 xSSC, 0.1% N-lauroylsarcosine for 20 minutes at RT, followed by 55°C, 0.2 xSSC, 0.1% N-lauroylsarcosine for 20 minutes at RT and then at 55°C. When we omitted the RNase A treatment, high backgrounds of mRNA expression or nonspecific signals were observed. Hybridization signals were visualized by alkaline phosphatase immunohistochemistry. The sections were washed in 0.1M Tris-HCl buffered saline (TBS; pH 7.5) for 5 minutes, incubated in 1% blocking reagent and 5% normal sheep serum in TBS for 60 minutes at RT, and sheep anti-digoxigenin-alkaline phosphatase (Fab fragments) (Roche Diagnostics GmbH, Mannheim, Germany) in TBS (working dilution 1:500) containing 1% blocking reagent and 5% normal sheep serum for 60 minutes at RT. After washing three times in TBS containing 0.1% Tween 20 for 15 minutes, sections were incubated with a TBS-MG buffer [0.1M Tris-HCl (pH9.5), 0.1M NaCl, 5mM MgCl₂] containing 0.1% Tween 20 for 5 minutes, and successively reacted with a color solution [approximately 2% nitroblue tetrazolium chloride (NBT) / 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) stock solution, Boehringer Mannheim, Germany] in the TBS-MG buffer containing 1mM levamisole (Sigma) for 16-24 hours at RT in the dark. The reaction was stopped by washing twice in TBS for 10 minutes. The sections were mounted on superfrost plus glass slides (Fisher scientific, Tustin, CA), left to dry for about 16 hours, washed in water, dehydrated in graded ethanol, cleared with xylene, and coverslipped by Entellan New mounting media (Electron Microscopy Sciences, Washington, PA).

Light microscopic examination—All images were observed under a microscope (Axioscope, Carl Zeiss, Germany), captured by a digital camera (AxioVersion, Carl Zeiss) using software Open lab version 2.0 (Improvision Inc, Lexington, MA), and digitized through a computer. The sampled images were processed in an off-line computer using Photoshop 6.0 (Adobe, San Jose, CA), and printed on a color printer. The diagrams were drawn from montage photomicrographs.

The intensity of hybridization signals was judged relatively by the intensity of the alkaline phosphatase reaction product. Intensities were recorded as no expression (-); a weak signal (+), which is characterized by a loose, puncture-like, faint reaction product in the cytoplasm; a moderate signal (++), between (+) and (+++); a strong signal (+++), characterized by an intense and thick reaction product accumulated in the entire cytoplasm. The density of semiquantitative evaluation was based on the number of nAChR α 2 subunits mRNA expressing cells in each structure: -, not detected; +, a few cells; ++, several cells; +++, many cells; +++++, most cells (Table 1).

Cell size was approximately evaluated by the somatic diameters of nAChR α 2 subunit mRNA expressing cells of rats: small-sized, <10 µm; medium-sized, 10 -20 µm; large-sized, 20 µm<, and those of mice were assigned lower numerical values than those of rats.

Definitions of anatomical terms in the brain were based on different atlases of the rat brain (Paxinos and Watson, 1986) and the mouse brain (Paxinos and Franklin, 1997).

Control experiments—First, the sense-riboprobe did not detect any signals in the rat and mouse CNSs. We present part of these results obtained from rat (Fig. 2C) and mouse (Fig. 2D) main olfactory bulbs of coronal sections. Second, sections pretreated without any probe did not exhibit any significant hybridization signals.

RESULTS

We detected strong hybridization signals in cell bodies of the internal plexiform layer of the olfactory bulb, the interpeduncular nucleus (IP) of the midbrain, the ventral and dorsal tegmental nuclei, the median raphe nucleus of the pons, the ventral part of the medullary reticular nucleus, in several cells located in the ventral part of the ventral horn in the spinal cord of both rats and mice, and in a small number of Purkinje cells of rats, but not mice. Structures containing a high density of $\alpha 2$ mRNA expressing cells were the IP, and the ventral and dorsal tegmental nuclei in both animals. A detailed analysis of the distribution of hybridization signals for each subunit $\alpha 2$ mRNA probe in the rat and mouse brains is summarized in Table 1.

Olfactory bulb and related regions

In the main olfactory bulb of both the rat (Figs. 2A, E) and mouse (Figs. 2B, F), most of the $\alpha 2$ mRNA expressing cells are located in the internal plexiform layer of the olfactory bulb, which is a thin layer inside the mitral cell layer. $\alpha 2$ mRNA expressing cells have multipolar cell bodies and are dispersed throughout the internal plexiform layer, which most likely consists of short-axon cells. A few $\alpha 2$ mRNA expressing cells are also located in the external plexiform and glomerular layers (shown by an arrow in Fig. 2B).

Neocortex

In the cerebral cortex of coronal sections of the rat (Figs. 3A, C) and mouse (Figs. 3B, D), moderate $\alpha 2$ mRNA expression was localized to the scattered neurons of layers V and VI (arrows in Figs. 3A, B). Since these cells are small to medium-sized and oval or fusiform in shape, it is likely that they are nonpyramidal cells.

Hippocampal formation

In the subiculum, $\alpha 2$ mRNA was expressed in medium-sized cells. Moderate hybridization signals were observed in rats (Figs. 6C, 7F) while weak signals were seen in mice (Fig. 4B). In the rat hippocampus, expression was observed in the interneurons of the oriens layer and the alveus of CA1 and CA3: oval or horizontally elongated somata oriented in parallel to the alveus surface, which were mainly oriens-lacunosum moleculare cells (Figs. 4A, C, E). A few $\alpha 2$ mRNA expressing cells were located in the pyramidal cell layer of CA3 (Figs. 4C, E). In the mouse, a few $\alpha 2$ mRNA expressing cells (Figs. 4B, D, F) were localized to the subiculum and interneurons of the oriens layer and the alveus of CA1, but no expression was observed in CA3 (Fig. 4F).

Septum, Basal ganglia, and Amygdala

In the both rat (Figs. 5A, C, E, G) and mouse (Figs. 5B, D, F, H) ventral regions, $\alpha 2$ mRNA was expressed in the medial septal nucleus (Figs. 5A, B), the diagonal band complex (nucleus of vertical and horizontal limb diagonal band)(Figs. 5C, D, 12), the lateral bed nucleus stria terminalis (Fig. 5F, 12), scattered neurons in the region of the substantia innominata (Figs. 5E, F, 12), the ventral pallidum (Fig. 5F, 12), the olfactory tubercle, the magnocellular preoptic nucleus, the lateral zone of the hypothalamus, and in the amygdaloid nuclei (medial nucleus, central nucleus, and basomedial nucleus)(Figs. 6A, B, D). In almost the entire amygdala except for the lateral and basolateral nuclei, $\alpha 2$ mRNA was expressed in the medium to relatively large-sized neurons of round or ovoid shapes. In horizontal sections, these $\alpha 2$ mRNA expressing cells formed a broad band.

Between the entorhinal cortex and the medial amygdaloid nucleus, $\alpha 2$ mRNA was expressed in medium-sized cells of the posteromedial cortical amygdaloid nucleus (Figs. 6C, 7F). This area corresponds to medium-sized cells of layer II in the medial entorhinal area (MEA') as described by Wada et al. (1989). These cells are grouped in clusters (Fig. 6C) known as cell islands (Amaral and Witter, 1995; Insausti et al., 1997).

Brainstem

$\alpha 2$ mRNA was expressed in the rat midbrain (Figs. 7A-F). A few $\alpha 2$ mRNA expressing cells located in the optic nerve layer of the superior colliculus of rats had medium-sized cell bodies that were round or multipolar in shape (Fig. 12 and arrows in Fig. 7A), but these were not seen in mice. Many $\alpha 2$ mRNA expressing cells were scattered around the inferior colliculus (Fig. 7B, 12). They have small to medium-sized cell bodies and their shape is either round, oval, or multipolar. In the periaqueductal gray (Fig. 7C), the nucleus of the brachium of the inferior colliculus (Fig. 7D), and the deep mesencephalic reticular nucleus (Fig. 7E), there are many $\alpha 2$ mRNA expressing cells in the rat, but either none or a few $\alpha 2$ mRNA expressing cells in the mouse. The deep mesencephalic reticular nucleus of rats corresponds to the subcuneiform part of the central tegmental field as described by Wada et al. (1989).

Additionally, $\alpha 2$ mRNA was localized to the IP in the midbrain and the ventral and dorsal tegmental nuclei and the median raphe nucleus in the pons (Figs. 8A-F). $\alpha 2$ mRNA expression was most intensely distributed in the rostral subnucleus of the interpeduncular nucleus (IP-R) which consists of small, closely spaced cells. In the rostral region of the IP, the IP-R occupied almost the entire dorsoventral extent of the IP (Figs. 8C, D). At a level halfway through the length of the IP, the IP-R occupied the dorsal half of the IP and the central subnucleus (IP-C), and the intermediate subnucleus (IP-I) occupied the ventral half of the IP (Figs. 8E, F). The IP-C consists of small to medium-sized neurons which are more sparsely distributed than neurons in the IP-R. In the other subnuclei of the IP, $\alpha 2$ mRNA expressing cells were observed. In the median raphe nucleus, dorsocaudal region of IP, $\alpha 2$ mRNA expressing cells were located in a band between the IP and the ventral and dorsal tegmental nuclei (Figs. 8A-D). These cells were medium to large-sized cells, elongated in the rostroventral to caudodorsal direction. In the ventral and dorsal tegmental nuclei, $\alpha 2$ mRNA expressing cells were seen in a large, loosely aggregated cluster extending from the

caudal midbrain into the rostral pontine tegmentum (Figs. 8A, B). These cells were found in a region extending from the dorsal part of the IP, in a caudodorsal direction through the midbrain reticular formation, into the area surrounding the superior cerebellar peduncle. In sagittal sections, $\alpha 2$ mRNA expressing cells formed an intense band (Figs. 8A-D, 12).

In the ventral region of the caudal pons, $\alpha 2$ mRNA was localized to several small-sized cells of the superior olivary complex, especially the periolivary nucleus in both animal species (Figs. 9E, F, 12).

In the upper medulla oblongata, $\alpha 2$ mRNA was scattered in several large neurons of the gigantocellular reticular nucleus (Gi), including areas such as the gigantocellular field, the dorsal paragigantocellular reticular field, the alpha part of the gigantocellular reticular field, and the ventral part of the gigantocellular reticular field, as well as the raphe magnus nucleus of mice (Fig. 9D), but not of rats (Fig. 9C). $\alpha 2$ mRNA was also located in dorsal neurons of the cuneate nucleus in both animals (Figs. 10A, B). In the lower medulla oblongata (Figs. 10C-F), $\alpha 2$ mRNA was localized to several cells in the ventral part of the medullary reticular nucleus (Figs. 10C, D). These cells were small to medium-sized and multipolar or fusiform in shape. Their distribution extended as far as the ventral part of the ventral horn in the cervical cord of both rats and mice.

Small numbers of $\alpha 2$ mRNA expressing cells or weakly expressing cells were seen in several nuclei in the brainstem: the peripeduncular nucleus, the ventral tegmental area, the pedunculopontine tegmental nucleus, the ventral nucleus of the lateral lemniscus, the parabrachial nucleus, the laterodorsal tegmental nucleus, the spinal trigeminal nucleus, the vestibular nucleus, the linear nucleus medulla, and the parvicellular reticular nucleus.

Cerebellum

In the cerebellum, intensely-labeled cells were localized to a small number of Purkinje cells of rats (Fig. 9A). However, $\alpha 2$ mRNA expression in mice Purkinje cells was either weak or absent (Fig. 9B). We examined five mice, one of which exhibited weak expression, while the other four showed no $\alpha 2$ mRNA expression.

Spinal cord

In the spinal cord of both the rat (Figs. 11A, C) and the mouse (Figs. 11B, D), $\alpha 2$ mRNA expression was localized to several cells in the ventral part of the ventral horn in the cervical cord (Figs. 11A-D). Many of these cells are located in lamina VII and also in laminae VIII and IX (Molander and Grant, 1995). These cells were small to medium-sized and multipolar or fusiform in shape, but they were not large motoneurons. Therefore they most likely consist of Renshaw cells (Fyffe, 1990; Carr et al., 1998). Nissl-stained sections of the same regions in the rat (Fig. 11E) and mouse (Fig. 11F) spinal cord were obtained. When compared to these sections, it is clear that $\alpha 2$ mRNA expressing cells are small to medium-sized cells. Arrows in Figure 11A indicate $\alpha 2$ mRNA expressing cells located in the dorsal regions of the ventral horn. The more caudal regions of the spinal cord were not examined.

DISCUSSION

nAChR probe specificity and sensitivity

To make the cRNA probes, we used a rat $\alpha 2$ cDNA fragment (657 bp) and a mouse $\alpha 2$ cDNA fragment (700 bp). We confirmed that the sequences of these fragments contain no significant homology to other rat or mouse DNA sequences, respectively, by searching the GenBank database (Fig.1). We tested mouse probes that had been subjected to alkaline hydrolysis, using the method of Cox et al. (1984) to yield products with sizes of 50-200-bp before hybridization. However, we could not distinguish any differences in the results obtained by probes that were or were not subjected to alkaline hydrolysis. Hence in this experiment, we omitted the alkaline hydrolysis step. Hybridization signals were detected in the substantia nigra and ventral tegmental area using the long rat $\alpha 2$ probe from the HYP16 clone which may likely cross-react with rat $\alpha 4$ mRNA (Azam et al., 2002). In the present study, only a few $\alpha 2$ mRNA expressing cells were detected in the neurons of the ventral tegmental area surrounding the IP (Figs. 8E, F), but not in the substantia nigra. These findings resemble those of a previous study using the C183 clone containing noncoding regions of the cDNA (Wada et al., 1989), and the results obtained from using the shorter rat $\alpha 2$ probe (from the HYP16 clone) to reduce cross-reactivity with $\alpha 4$ mRNA (Azam et al., 2002). The shorter riboprobe prepared from cDNA showed a more restricted expression (Liang et al., 2000; Azam et al., 2002). Our findings confirm the results of studies that employed PCR methods to show the presence of $\alpha 2$ subunit mRNA in dopaminergic neurons of the ventral tegmental area, but not in the substantia nigra (Charpentier et al., 1998). These results show that our $\alpha 2$ cRNA probes have high specificity and do not cross-react with any other nAChR subunits.

In rats, $\alpha 2$ mRNA expressing cells were located in the midbrain nuclei (Figs. 7A-F), CA3 of the hippocampal formation (Figs. 4C, E), and cerebellar Purkinje cells (Figs. 9A), whereas in mice, they were either few or absent in these areas (Figs. 4F, 9B). Therefore, there may be the possibility that the mouse cRNA probe has a lower sensitivity than the rat cRNA probe. However, in the upper medulla oblongata, $\alpha 2$ mRNA was expressed in several large neurons of the gigantocellular reticular nucleus and the raphe magnus nucleus of mice (Fig. 9D), but not of rats (Fig. 9C). Thus, it is most likely that both cRNA probes used in this study have high sensitivity.

$\alpha 2$ mRNA was not detected in the adult rat mesencephalon using a oligodeoxynucleotide probe (Le Novere et al., 1996). Although in situ hybridization using an oligodeoxynucleotide probe minimizes cross-recognition of different members of the nAChR subunit family, this method is less sensitive than cRNA probe-based in situ hybridization. Thus, the oligodeoxynucleotide probe can only label specific regions of cells containing large quantities of mRNA. In regions of the brain consisting of various cell types, in situ hybridization with oligodeoxynucleotide probes can only detect the expression of mRNA at a regional level, but not at a cellular level.

Association between the distribution of nAChR α 2 subunit and choline acetyltransferase immunoreactivity in the CNS

By determining the distribution of α 2 mRNA in the CNS, we can potentially reveal possible functions of α 2-containing nAChRs. In order to work, all functional receptors need to receive acetylcholine (ACh) from cholinergic fibers. Therefore, we explored the association between the distribution of α 2 mRNA and the distribution of immunoreactivity for choline acetyltransferase (ChAT) in the CNS.

Olfactory bulb and related regions—In the internal plexiform layer of the olfactory bulb, ChAT immunopositive fibers lie in a layer richly targeted by centrifugal inputs from the nucleus of the diagonal band (Shipley et al., 1996). The internal plexiform layer also contains a rich plexus of Cholesystokinin-containing axons and terminals derived from tufted cells (Liu and Shipley, 1994), which synapse onto the dendrites of GABA-ergic granule cells. α 2 expressing cells (most likely short axon cells) may modulate this tufted-granule cell system.

Neocortex—The predominant type of nonpyramidal cell in the rodent cortex is local plexus neurons, which are probably inhibitory in function (Peter and Saint Marie, 1984). Cholinergic innervation of the cerebrum was observed immunohistochemically throughout all cortical layers in the rat (Ichikawa and Hirata, 1986), which revealed that a trilaminar pattern with cholinergic processes can be seen in the mouse (Kitt et al., 1994).

Hippocampal formation—In the hippocampus, α 2 mRNA expressing cells are interneurons consisting of various cell types, which are classified according to their axon arbors. Many cells are oriens-lacunosum molecule cells, while some are oriens-bistratified cells, oriens-trilaminar cells, hilar perforant path-associated cells, and hippocampo-septal cells (Jinno and Kosaka, 2002; Gulyas et al., 2003). ChAT immunopositive punctuate structures were present in all layers of the hippocampus and dentate gyrus. A moderately high density of ChAT positive profiles was found in the portion of the oriens layer immediately adjacent to the pyramidal cell layer, and in the stratum lacunosum-moleculare (Houser et al., 1983; Ichikawa and Hirata, 1986).

Septum, Basal ganglia, and Amygdala— α 2 mRNA was expressed in neurons of ventral regions including the medial septal nucleus, the diagonal band complex, the magnocellular preoptic nucleus, and both dorsal and ventral divisions of the substantia innominata (Grove, 1988). These nuclei correspond to those exhibiting immunoreactivity for ChAT (Houser et al., 1983; Armstrong et al., 1983; Mesulam et al., 1983; Ichikawa et al., 1997). However, it remains to be ascertained whether ChAT immunopositive cells do express α 2 mRNA. In this study, α 2 mRNA expression was observed throughout the medial amygdala (Krettek and Price, 1977; Alheid et al., 1995), although results obtained by Wada et al. (1989) showed α 2 mRNA expression only in the anterior part of the medial amygdaloid nucleus. Its expression was detected in the medium to relative large-sized neurons, which are scattered in the medial, basomedial, and central amygdaloid nuclei. These cells may be associated with major neural inputs and outputs of the three functional systems: the accessory olfactory system, main olfactory system, and autonomic system

respectively (Swanson and Petrovich, 1998). Cholinergic innervation of the amygdala was observed immunohistochemically in the lateral and basolateral nuclei, but was not observed in any other nuclei in the rat (Ichikawa and Hirata, 1986; Ichikawa et al., 1997). Therefore, $\alpha 2$ mRNA-containing neurons in the amygdala may have functional nAChRs consisting of the $\alpha 2$ subunit, in the vicinity of their axon terminals.

Brainstem—In the superior colliculus, $\alpha 2$ mRNA expressing cells are most likely interneurons, which may include wide-field vertical cells that project to deeper layers of the superior colliculus. An extensive network of cholinergic fibers is present in the intermediate gray of the deeper superior colliculus, and a low number of ChAT-immunoreactive fibers and terminals are contained in the optic nerve layer (Tan and Harvey, 1989).

In the IP, rostral and lateral IP cells project mainly to the dorsal tegmental region, the laterodorsal tegmental nucleus, and the median raphe nucleus (Groenewegen et al., 1986). A cluster of ChAT-immunoreactive fibers derive from the medial habenular nucleus through the fasciculus retroflexus (Houser et al., 1983; Ichikawa and Hirata, 1986).

In the pons and medulla oblongata, ChAT-immunoreactive neurons are present in both the pontomesencephalic tegmentum, including the pedunculopontine tegmental nucleus and the laterodorsal tegmental nucleus (Mesulam et al., 1983), and the medullary reticular formation which consists of a group of gigantocellular reticular nuclei and midline raphe nuclei (Jones, 1990). There is a dense plexus of ChAT-immunoreactive varicose fibers throughout the entire brainstem reticular formation (Jones, 1990).

Cerebellum—Cholinergic innervation of the rat cerebellum was observed immunohistochemically as thin varicose fibers, which were closely associated with the Purkinje cell layer and also found in the molecular layer (Ojima et al., 1989).

Spinal cord— $\alpha 2$ mRNA expressing cells most likely consist of Renshaw cells which receive cholinergic contacts (Houser et al., 1983; Alvarez et al., 1999).

Several conclusions can be drawn from these data. First, we clarified the distribution of nAChR $\alpha 2$ subunit mRNA in the rodent CNS at a cellular level, using non-radioactive in situ hybridization histochemistry and summarized these findings in Table 1 and Figure 12. Similar patterns of $\alpha 2$ mRNA distribution were observed in the rat and the mouse (black dots in Fig. 12), with the exception of a few regions. In rats, moderately expressing $\alpha 2$ mRNA cells were located in the hippocampal formation (Figs. 4A, C, E) and the midbrain nuclei (Figs. 7A-F) including the superior colliculus, the inferior colliculus, the nucleus brachium inferior colliculus, the periaqueductal gray, and the deep mesencephalic reticular nucleus, whereas in mice, they were few or absent in these areas (stars in Fig. 12). Likewise, there were a few strongly expressing $\alpha 2$ mRNA cerebellar Purkinje cells in rats, whereas in mice, they were either weak or absent (Figs. 9A, B and stars in Fig. 12). However, in the upper medulla oblongata of mice, $\alpha 2$ mRNA was expressed in several large neurons of the gigantocellular reticular nucleus and the raphe magnus nucleus of mice, but this was not observed in rats (Figs. 9C, D and open circles in Fig. 12).

Second, these results largely correspond to a previous study of $\alpha 2$ mRNA distribution in rats, using radioactive in situ hybridization histochemistry (Wada et al., 1989). However, we also detected $\alpha 2$ mRNA expression in the ventral and dorsal tegmental nuclei in the rat, as had been observed in mice (Marks et al., 1992), and $\alpha 2$ mRNA expression was observed not only in the anterior part of the medial amygdaloid nucleus, but also in other amygdaloid nuclei.

Third, based on the localization of $\alpha 2$ mRNA expressing cells, some neurons may have common functions as output modulators of their structures through cholinergic innervations. These neurons could be short axon cells in the internal plexiform layer of the olfactory bulb, nonpyramidal cells in the layers V and VI of the neocortex, hippocampal oriens-lacunosum molecule cells, several cells in the ventral part of the medullary reticular nucleus, several small to medium-sized cells in the ventral horn of the spinal cord, as well as optic nerve layer neurons in the superior colliculus of rats, and several small-sized cells in the periolivary nucleus of the superior olivary complex.

Fourth, some $\alpha 2$ mRNA expressing nuclei, namely the medial septal nucleus, diagonal band complex, substantia innominata, magnocellular preoptic nucleus, and pontine tegmental nuclei, correspond to those nuclei exhibiting immunoreactivity for ChAT (Houser et al., 1983; Armstrong et al., 1983; Mesulam et al., 1983; Ichikawa et al., 1997). If $\alpha 2$ mRNA expressing cells show ChAT immunopositivity, functional nAChRs that include the $\alpha 2$ subunit could perhaps function as autoreceptors, or as an ACh signal system that receives ACh from other ChAT axon terminals and releases ACh to other cells.

Finally, many of the other $\alpha 2$ expressing cells, such as the IP, the median raphe nucleus, the ventral and dorsal tegmental nuclei, Purkinje cells (rats), gigantocellular reticular nucleus and the raphe magnus nucleus (mice), are projection neurons.

Although neither the subunit composition, nor the pharmacological and biological properties of $\alpha 2$ subunit-containing nAChRs have been determined, it is very likely different combinations of nAChR subtypes may produce different receptor functions. Because $\alpha 4$, $\alpha 7$, and $\beta 2$ subunits are expressed in many neurons (Wada et al., 1989; Seguela et al., 1993), it is difficult to clarify their specific functions. However, by studying nAChR subtypes like $\alpha 2$ which have a relatively restricted distribution, it may be possible to determine certain specific receptor functions. Dual in situ hybridization histochemistry may potentially resolve the composition of nAChR subtypes. If two nAChRs subunit mRNAs are coexpressed in the same cell, they can possibly form a functional nAChR channel that is characterized by a particular function.

Presynaptic or postsynaptic nicotinic ACh receptors

Presynaptic and preterminal axonal locations of nicotinic receptors enhance neurotransmitter release (Lena et al., 1993) and postsynaptic nicotinic receptors mediate a small minority of fast excitatory transmission (see reviews in Wonnacott, 1997; Dani, 2001). Although we discussed the association between the distribution of the $\alpha 2$ subunit and ChAT immunoreactivity in the CNS, there is generally some distance between the region of $\alpha 2$ mRNA expressing cell-bodies and the projection areas. However, we do not know whether

α 2 subunit protein exists in the presynaptic and preterminal axonal or postsynaptic locations. At present, we only know the localization of the putative nAChR α subunits including the α 2 subunit, based on the distribution of [3 H] acetylcholine and [3 H] nicotine binding sites (Clark et al., 1985). Unfortunately, a specific antibody recognizing the nAChR α 2 subunit protein has yet to be developed, although the antibodies for other nAChR subunits have been used in many studies (Swanson et al., 1987; Whiting and Lindstrom, 1988; Schoepfer et al., 1990; Hill et al., 1993; Dominguez del Toro et al., 1994; Rogers et al., 1998). Until a specific antibody for α 2 subunit protein is produced, determining whether α 2 subunit protein exist in presynaptic or postsynaptic locations, or both, will remain a difficult task.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.
Regions unique to the rat and mouse neuronal nAChR $\alpha 2$ sequences.

Fig. 2.

Photomicrographs showing hybridization with nAChR $\alpha 2$ subunit antisense-riboprobes in the rat (A) and mouse (B), sense-riboprobes in the rat (C) and mouse (D), and Nissl-stained sections of the same regions in the rat (E) and mouse (F) main olfactory bulb of coronal sections. A and B: Most of the $\alpha 2$ mRNA expressing cells are located in the internal plexiform layer of the olfactory bulb (IPL), which is a thin layer inside the mitral cell layer (MCL). Arrow shows an $\alpha 2$ mRNA expressing cell around the border of glomerular (GGL) and external plexiform (EPL) layers. C and D: Sense-riboprobe did not detect any signals. Scale bar = 100 μ m.

Fig. 3.

Photomicrographs showing hybridization with nAChR α 2 subunit probes in the rat (A) and mouse (B) and Nissl-stained sections of the same regions in the rat (C) and mouse (D) cerebral cortex of coronal sections. A and B: Moderate α 2 mRNA expression is localized to the scattered neurons of layers V and VI (arrows). Scale bar = 100 μ m.

Fig. 4.

Photomicrographs showing hybridization with nAChR $\alpha 2$ subunit probes in the rat (A, C) and mouse (B, D) hippocampal formation of coronal sections. A and C: Interneurons of the oriens layer (Or) and the alveus (alv) of CA1 and CA3 have oval or elongated somata, and are mainly oriens-lacunosum moleculare (O-LM) cells. B: Weak $\alpha 2$ mRNA expression is localized to the subiculum and interneurons of the oriens layer and the alveus of CA1 in the mouse. D: After in situ hybridization histochemistry was carried out, the section was mounted on a slide temporarily with 0.1M Tris-HCl buffered saline and photographed. Compared with Figure 4B, $\alpha 2$ mRNA expression is much more distinct. E and F: The boxes on the diagrams of rat (E) and mouse (F) hippocampus-coronal sections show the approximate location of the areas photomicrographed (A-D). Black dots show the distribution of $\alpha 2$ mRNA expressing cells. Scale bars = 100 μ m in A (also applies to C), B (also applies to D).

Fig. 5.

Photomicrographs showing hybridization with nAChR α 2 subunit probes in the rat (A, C, E) and mouse (B, D, F) ventral regions. α 2 mRNA is expressed in the neurons of ventral regions; A and B: medial septal nucleus (MS), C and D: diagonal band complex (VDB and HDB), E and F: substantia innominata (SI) and ventral pallidum (VP). G and H: Nissl-stained sections of the same regions of Figures 5E and F in the rat (G) and mouse (H), respectively. Coronal sections (A, C, E, and G). Horizontal section (B). Sagittal sections (D, F, and H). Scale bar = 100 μ m.

Fig. 6.

Photomicrographs showing hybridization with nAChR $\alpha 2$ subunit probes in the rat (A, C) and mouse (B) amygdaloid nuclei of coronal sections. A and B: $\alpha 2$ mRNA is expressed in the neurons of amygdaloid nuclei (Me, Ce, and BM). C: Between the entorhinal cortex and the medial amygdaloid nucleus, $\alpha 2$ mRNA is expressed in medium-sized cells of the posteromedial cortical amygdaloid nucleus (PMCo). This cluster consists of individual cells expressing $\alpha 2$ mRNA, although the outlines of these cells are not clearly defined. Several $\alpha 2$ mRNA expressing cells are localized to the subiculum (S): medium-sized cells (Fig. 7F). D: The box on the diagram of the rat amygdala- coronal section shows the approximate location of the areas photomicrographed. Black dots show the distribution of $\alpha 2$ mRNA expressing cells in rats (A). Same regions photomicrographed in mice (B). Scale bars = 100 μm in A (also applies to B), C.

Fig. 7.

Photomicrographs showing hybridization with nAChR $\alpha 2$ subunit probes in the rat (A - E) midbrain. A: A few $\alpha 2$ mRNA expressing cells located in the optic nerve layer of the superior colliculus (SC) have medium-sized cell bodies and are round or multipolar in shape (arrows). B: Many $\alpha 2$ mRNA expressing cells are scattered around the inferior colliculus (IC). C, D, and E: Several $\alpha 2$ mRNA expressing cells are located in the periaqueductal gray (PAG), the nucleus brachium inferior colliculus (BIC), and the deep mesencephalic reticular nucleus (DpMe), respectively. F: The boxes on the diagram of the rat midbrain- coronal section show the approximate location of the areas photomicrographed (Figs. 6C, 7C, D, E). Black dots show the distribution of $\alpha 2$ mRNA expressing cells in rats. Sagittal sections (A and B). Coronal sections (C-E). Scale bar = 100 μ m.

Fig. 8.

Photomicrographs showing hybridization with nAChR $\alpha 2$ subunit probes in the rat (A, C, E) and mouse (B, D, F) midbrain and pons. In the midbrain, $\alpha 2$ mRNA is localized to the interpeduncular nucleus (IP), and in the pons, ventral (VTg) and dorsal (DTg) tegmental nuclei and the median raphe nucleus (MnR). A and B: In the ventral and dorsal tegmentum, $\alpha 2$ mRNA expressing cells are seen in a large, loosely aggregated cluster extending from the caudal midbrain into the rostral pontine tegmentum. C-F: $\alpha 2$ mRNA expression is most intensely distributed in the rostral subnucleus of the IP (IP-R). These cells are found in a region extending from the dorsal part of the IP, in a caudodorsal direction through the midbrain reticular formation into the area surrounding the superior cerebellar peduncle. They form an intense band in sagittal sections. Sagittal sections (A-D). Coronal sections (E and F). Scale bars = 100 μ m in A (also applies to C, E), B (also applies to D, F).

Fig. 9.

Photomicrographs showing hybridization with nAChR α 2 subunit probes in the rat (A, C, E) and mouse (B, D, F). A and B: In the cerebellum, intense α 2 mRNA expression is localized to a small number of Purkinje cells of rats in the Purkinje cell layer (A), whereas α 2 mRNA expression in mice Purkinje cells is absent (B). C and D: In the upper medulla oblongata, α 2 mRNA is scattered in several large neurons of the gigantocellular reticular nucleus (Gi) and the raphe magnus nucleus (RMg) in mice (D), but not in rats (C). Arrows (D) indicate α 2 mRNA expressing cells in the RMg. E and F: In the ventral region of the caudal pons, α 2 mRNA is localized to several small-sized cells of the superior olivary complex (SOC), especially the periolivary nucleus. Sagittal sections (A, B, E, and F). Coronal sections (C and D). Scale bars = 100 μ m in A (also applies to B), C (also applies to D-F).

Fig. 10.

Photomicrographs showing hybridization with nAChR α 2 subunit probes in the rat (A, C) and mouse (B, D) and Nissl-stained sections of the same regions in the rat (E) and mouse (F) medulla oblongata. A and B: Weak α 2 mRNA expression is located in dorsal neurons of the cuneate nucleus (Cu) of both animals. C and D: In the lower medulla oblongata, α 2 mRNA is localized to several cells in the ventral part of the medullary reticular nucleus (MdV). These cells are small to medium-sized and multipolar or fusiform in shape. Arrows (C) indicate α 2 mRNA expressing cells in the ventral part of MdV. Sagittal sections (A, B). Adjacent coronal sections (C, E) and horizontal sections (D, F). Scale bars = 100 μ m in A (also applies to B), C (also applies to D - F).

Fig. 11.

Photomicrographs showing hybridization with nAChR α 2 subunit probes in the rat (A, C) and mouse (B, D) spinal cord and Nissl-stained sections of the same regions in the rat (E) and mouse (F) spinal cord. A and B: α 2 mRNA expression is localized to several small to medium-sized cells of the ventral horn (VH). Arrows (A) indicate α 2 mRNA expressing cells in the dorsal regions of the ventral horn. Sagittal sections (A, B) and coronal sections (C - F). Scale bar = 100 μ m.

Fig. 12.

Schematic diagram of the distribution of nAChR $\alpha 2$ subunit mRNA in the rat and mouse central nervous systems. Black dots show the common distribution of $\alpha 2$ mRNA expressing cells in rats and mice. Stars show area of exclusive $\alpha 2$ mRNA expression in rats. Open circles show the exclusive distribution of $\alpha 2$ mRNA in mice.

TABLE 1

Distribution of nAChR $\alpha 2$ subunits mRNA in Rats and Mice CNS Structures as Revealed by In situ hybridization histochemistry.

Brain regions	Structure/ Cells	Rat		Mouse	
		Intensity	Density	Intensity	Density
Olfactory regions	Olfactory bulb,				
	Internal plexiform layer	+++	++	+++	++
	Other layers	+++	+	+++	+
	Olfactory tubercle	++	+	++	+
Neocortex	Layer V and VI	++	++	++	++
	Other layers	–	–	–	–
Hippocampal formation	Presubiculum	+	+	–	–
	Subiculum	++	++	+	+
	Field CA1,				
	Oriens layer and alveus	++	++	+	+
	Other layers	–	–	–	–
	Field CA3,				
	Oriens layer and alveus	++	++	–	–
	Other layers	++	+	–	–
Septum	Medial septal nucleus	++	++	++	++
	Diagonal band,				
	Nucleus of vertical limb	++	++	++	++
	Nucleus of horizontal limb	++	++	++	++
	Bed nucleus stria terminalis,				
	Lateral region	++	++	++	++
	Ventral pallidum	++	++	++	++
	Accumbens nucleus	–	–	–	–
Basal ganglia	Caudate-putamen (Striatum)	–	–	–	–
	Globus pallidus	–	–	–	–
	Substantia innominata	++	++	++	++
	Magnocellular preoptic nucleus	++	+	++	+
	Subthalamic nucleus	–	–	–	–
	Substantia nigra	–	–	–	–
	Medial nucleus	++	++	++	++
	Central nucleus	++	+	++	+
Amygdala	Basomedial nucleus	++	+	++	+
	Anterior amygdaloid area	++	+	++	+
	Posteromedial cortical nucleus	+	+++	–	–
	Others	–	–	–	–
Thalamus		–	–	–	–
Hypothalamus	Lateral hypothalamic area	++	++	++	++
	Lateral preoptic area	++	+	++	+

Brain regions	Structure/ Cells	Rat		Mouse	
		Intensity	Density	Intensity	Density
Midbrain	Superior colliculus, LayerIII	++	++	—	—
	Other layers	—	—	—	—
	Periaqueductal gray	++	++	+	+
	Peripenduncular nucleus	+	+	—	—
	Inferior colliculus	++	+++	++	+
	Nucleus brachium inf. colliculus	++	++	+	+
	Deep mesencephalic reticular n.	++	++	++	+
	Ventral tegmental area	++	+	++	+
	Interpeduncular nucleus,				
	Rostral subnucleus	+++	++++	+++	++++
	Apical subnucleus	+++	++	+++	++
	Dorsomedial subnucleus	++	++	++	++
	Lateral subnucleus	+++	+++ / ++	+++ / ++	+/++
	Intermediate subnucleus	++	++	++	++
	Central subnucleus	++	++	++	++
Pons	Pontine nuclei	—	—	—	—
	Pedunculopontine tegmental n.	++	+	+	+
	Nucleus of lateral lemniscus	++	++	+	+
	Parabrachial nucleus	+	++	+	+
	Laterodorsal tegmental nucleus	+	++	+	++
	Ventral tegmental nucleus	+++	+++	+++	+++
	Dorsal tegmental nucleus	+++	+++	+++	+++
	Median raphe nucleus	+++	++	+++	++
	Paramedian raphe nucleus	+++	+	+++	+
	Pontine reticular nucleus,				
	Oral and caudal parts	++	+	++	+
	Superior olivary complex	++	++	++	++
	Spinal trigeminal nucleus	++	++	+/++	++
Medulla oblongata	Vestibular nucleus	+	+	+	+
	Cuneate nucleus	+/++	++	+/++	++
	Raphe magnus nucleus	—	—	++	++
	Gigantocellular reticular nucleus	+ or —	+ or —	++	++
	Linear nucleus medulla	+	+	+	+
	Parvicellular reticular nucleus	+	+	+	+
	Medullary reticular nucleus,				
Cerebellum	Ventral part	+++	++	+++	++
	Purkinje cell layer/Purkinje cells	+++	+	+ or —	+ or —
	Others	—	—	—	—
Spinal cord	Ventral horn,				
	Medium to small-sized cells	+++	++	+++	++

Intensity of hybridization signals: —, not detected; +, weak signal; ++, moderate signal; +++, strong signal.

Density of nAChR $\alpha 2$ subunits mRNA expressing cells in each structure: –, not detected; +, a few cells; ++, several cells; +++, many cells; +++, most cells.