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Distribution of the Vesicular Acetylcholine Transporter (VAChT) in the Central and Peripheral Nervous Systems of the Rat

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Abstract

Expression of the acetylcholine biosynthetic enzyme choline acetyltransferase (ChAT), the vesicular acetylcholine transporter (VAChT), and the high-affinity plasma membrane choline transporter uniquely defines the cholinergic phenotype in the mammalian central (CNS) and peripheral (PNS) nervous systems. The distribution of cells expressing the messenger RNA encoding the recently cloned VAChT in the rat CNS and PNS is described here. The pattern of expression of VAChT mRNA is consistent with anatomical, pharmacological, and histochemical information on the distribution of functional cholinergic neurons in the brain and peripheral tissues of the rat. VAChT mRNA-containing cells are present in brain areas, including neocortex and hypothalamus, in which the existence of cholinergic neurons has been the subject of debate. The demonstration that VAChT is a completely adequate marker for cholinergic neurons should allow the systematic delineation of cholinergic synapses in the rat nervous system when antibodies directed to this protein are available.

Index Entries: Cholinergic, vesicular acetylcholine transporter; VAChT; mRNA; brain; parasympathetic; spinal cord; cerebral cortex; basal forebrain.

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Introduction

Cholinergic neurons subserve motor, vegetative, and cognitive processes throughout the central and peripheral nervous systems (Goldberg and Hanin, 1976; Dun and Perlman, 1985), and therefore, the detailed chemical neuroanatomy of this system is of significance for understanding nervous system function and pathology, including dysautonomic, neuromuscular, and dementing diseases (Perry et al., 1977, 1978; Engel, 1984). Currently, the most reliable marker for cholinergic function has been the presence of the biosynthetic enzyme choline acetyltransferase (ChAT), responsible for the synthesis of acetylcholine. However, the difficulty of developing adequate immunochemical reagents with which to visualize the ChAT protein, its apparently ectopic expression in non-neuronal tissues, and the caveat that synthesis of ACh alone does not adequately describe the cholinergic phenotype all limit the use of ChAT as an unambiguous marker for cholinergic neurons (Wu and Hersh, 1994). The vesicular acetylcholine transporter (VAChT), responsible for ACh accumulation in storage vesicles of cholinergic neurons, has recently been functionally characterized (Erickson et al., 1994), and the cDNA encoding it has been cloned from several species, including nematode (Alfonso et al., 1993), marine ray (Varoqui et al., 1994), rat, and humans (Erickson et al., 1994) In addition to its biosynthesis in the cytosol, ACh accumulation into secretory vesicles mediated by VAChT is required for cholinergic neurotransmission (Alfonso et al., 1993). Therefore, VAChT as well as ChAT expression is required to define the cholinergic neuronal phenotype. A preliminary *in situ* hybridization study of VAChT mRNA expression in the rat indicated that VAChT mRNA was coexpressed in the major brain nuclei reported to contain ChAT mRNA, and that in fact VAChT and ChAT are coexpressed from the same gene locus within the mammalian genome (Erickson et al., 1994).

Here, we describe in detail the distribution and relative abundance of VAChT mRNA in neurons throughout the central and peripheral nervous systems to establish that this mRNA is a reliable cholinergic marker. The demonstration of VAChT mRNA in certain brain regions may help to resolve controversy about the distribution and organization of cholinergic neurons in the central nervous system (CNS).

Materials and Methods

Preparation of Tissues

Brain, spinal cord, sympathetic (superior cervical), sensory (dorsal root, trigeminal), cranial parasympathetic (ciliary, pterygopalatine, submandibular) ganglia, atrial regions containing cardiac ganglia, thyroid glands with intramural thyroid ganglia, pelvic plexus with pelvic ganglia, and intestines (colon, duodenum) were obtained from four male Wistar rats (200–250 g) sacrificed under ether anesthesia. The tissues were rapidly removed and immediately frozen in isopentane cooled to –50°C on dry ice. Frozen serial sections of the brain, of predissected cervical, thoracic, lumbar, and sacral spinal cord segments, of the various sensory, sympathetic, and parasympathetic ganglia, and of colon and

duodenum were cut at 14–16-μm thickness on a cryostat (Reichert Jung), mounted on gelatin-coated slides, and stored at –80°C. Frozen sections were thawed at room temperature for 15 min and processed for *in situ* hybridization as described previously (Schäfer et al., 1993, 1994).

Probe Synthesis

A pcDNAI/Amp transcription vector (Invitrogen) containing a rat VAChT cDNA fragment (nt. 1790–2881) (Erickson et al., 1994) was linearized with the restriction endonucleases *Hind*III and *Xba*I to generate riboprobes in sense (SP6 polymerase-catalyzed transcription) and antisense (T7 polymerase-catalyzed transcription) orientation, respectively. In vitro transcription using ^{35}S-UTP as radioactive label (NEN) was performed as described (Melton et al., 1984). To increase tissue penetration of probes, the generated single-stranded riboprobes were subjected to limited alkaline hydrolysis as described (Angerer et al., 1987).

In Situ Hybridization

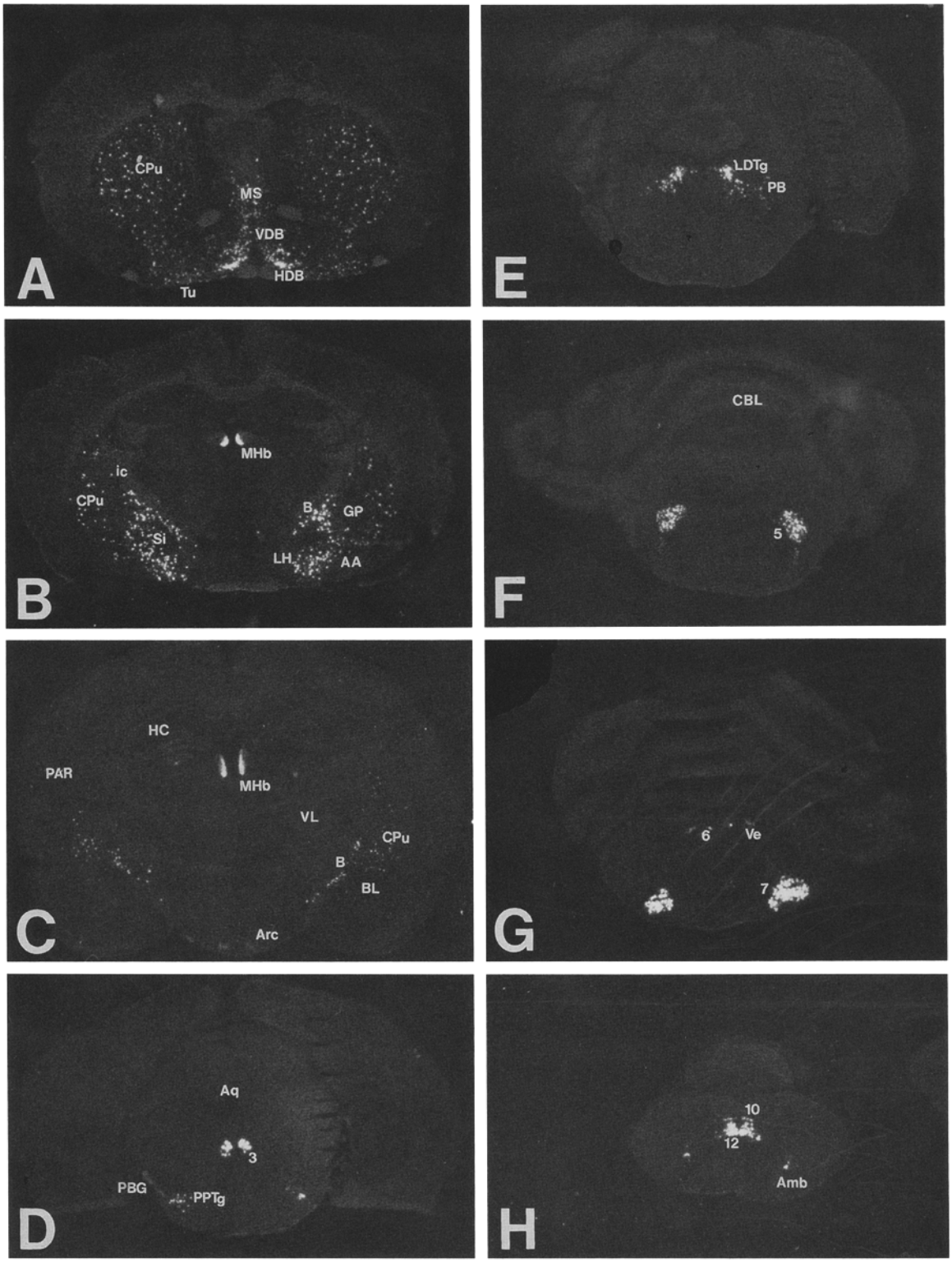
Briefly, the tissues were fixed on slides in 4% phosphate-buffered formaldehyde for 60 min at room temperature and then washed three times in 50 m*M* PBS, pH 7.4, for 10 min each. Slides were briefly rinsed in distilled water, transferred to 0.1*M* triethanolamine, pH 8.0, and incubated in the same solution containing 0.25% v/v acetic anhydride for 10 min under rapid stirring. Sections were quickly rinsed in 2X SSC, dehydrated in 50 and 70% ethanol, and air-dried.

Hybridization mix (20 μL) containing 50,000 dpm/μL of (^{35}S)-labeled RNA probes in hybridization buffer (3X SSC/50 m*M* $NaPO_4$/10 m*M* dithiothreitol/1X Denhardt's solution/0.25 mg/mL yeast tRNA/10% dextran sulfate and 50% formamide) was applied to each section. Sections were coverslipped and sealed with rubber cement. After hybridization for 16 h in the oven at 55°C, coverslips were removed in 2X SSC. Sections were treated with 20 μg/mL RNase A and 1 U/mL RNase T1 in RNase buffer (10 m*M* Tris, pH 8.0, 0.5*M* NaCl, 1 m*M* EDTA) for 60 min at 37°C. Successive washes were performed at room temperature in 2, 1, 0.5, and 0.2X SSC for 10 min each and in 0.2X SSC at 60°C for 60 min. The tissue was then dehydrated and exposed to Kodak XAR X-ray film for 24–96 h. For microscopic analysis, sections were dipped in Kodak NTB2 nuclear emulsion and developed following exposure times of 3 wk. As negative controls, tissues were treated with RNase A prior to hybridization or probed with a sense strand probe. Developed sections were stained with hematoxylin and eosin, and analyzed and photographed in bright or dark field in a Zeiss Axiophot light microscope.

Results

Distribution Pattern and Signal Intensities of VAChT mRNA in Fore- and Hindbrain

The analysis of serial coronal brain sections hybridized with the VAChT antisense cRNA probe revealed a limited and regiospecific distribution of VAChT mRNA in the fore- and hindbrain. This is shown in a series of X-ray autoradiograms (Fig. 1A–H) and in dark- and bright-field micrographs of selected brain areas (Figs. 2–6), and is summarized in Table 1. Hybridiz-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ation with the sense strand probe (Fig. 2B), or with the antisense strand probe following RNAse pretreatment of the sections (data not shown) yielded a low and uniform background with no concentration over cell bodies even after prolonged autoradiographic exposure.

The hybridization signal for VAChT mRNA was found to be specifically distributed within the well-accepted classical cholinergic regions of the brain, and in addition, in some, but not all brain regions in which the presence of cholinergic neurons has previously been inferred from the existence of cells that are immunoreactive with ChAT antibodies. In the forebrain, VAChT mRNA was present in the medial septum, the nuclei of the ventral and horizontal limbs of the diagonal band, the olfactory tubercle (Figs. 1A and 2A), the caudate-putamen (Figs. 1A–C and 3A), the basal nucleus of Meynert (Figs. 1B,C and 4A,C), the ventral pallidum (Fig. 2A), the Islands of Calleja (Fig. 3C), lateral hypothalamic area (Fig. 1B), and the medial habenular nuclei (Figs. 1B,C and 3D). In the hindbrain, all somatomotor and preganglionic parasympathetic nuclei of the cranial nerves (Figs. 1D,F–H and 6A–D) and the pedunculopontine tegmental nuclei (Fig. 1D), the lateral dorsal tegmental nucleus, and the parabrachial nuclei (Fig. 1E) were labeled.

In addition, positive hybridization signals for VAChT mRNA were observed over neurons of the neocortex, the piriform cortex, the arcuate nucleus, and the parabigeminal nucleus, whereas the hippocampal formation exhibited no signal.

In the neocortex, a very few, mainly small neurons were present, with greatest abundance in lamina 2 and 3. This is shown for the parietal cortex in Fig. 3B. The VAChT mRNA levels in cortical neurons appeared to be much lower than in striatal cholinergic neurons (Fig. 3A), in the scattered VAChT-positive neurons in the Islands of Calleja (Fig. 3C), or in the basal nucleus of Meynert (Fig. 4A,B). There were no apparent differences in the expression of VAChT mRNA among frontal, parietal, and occipital cortex.

The hypothalamic expression of VAChT mRNA was not restricted to the lateral hypothalamic area (Fig. 1B), but was also seen in arcuate nucleus (Figs. 1C and 4C,D), where a major subpopulation of neurons exhibited a signal, although at much lower

Fig. 1. *(opposite page)* Distribution of VAChT mRNA in adult rat brain. Dark-field autoradiograms of coronal sections in rostral (**A**) to caudal (**H**) orientation show a selective distribution of hybridization signals in classical cholinergic regions after hybridization with a ^{35}S-labeled antisense cRNA probe. Exposure times of X-ray films were 48 h. AA, anterior amygdaloid area; Amb, ambiguus nucleus; Arc, arcuate hypothalamic nucleus; Aq, aqueduct; B, basal nucleus of Meynert; BL, basolateral nucleus of amygdala; CBL, cerebellum; CPu, caudate-putamen; GP, globus pallidus; HC, hippocampal formation; HDB, nucleus of the diagonal band, horizontal limb; ic, intemal capsule; LDTg, laterodorsal tegmental nucleus; LH, lateral hypothalamic area; ME, median eminence; MHb, medial habenular nucleus; MS, medial septal nucleus; PAR, parietal cortex; PB, parabrachial nucleus; PBG, parabigeminal nucleus; PPTg, pedunculopontine nucleus; Si, substantia innominata; Tu, olfactory tubercle; VDB, nucleus of the diagonal band, vertical limb; Ve, vestibular nucleus; VL, ventrolateral thalamus; 3, oculomotor nucleus; 6, abducens nucleus; 7, facial motor nucleus; 10, dorsal motor nucleus vagus; 12, hypoglossal nucleus.

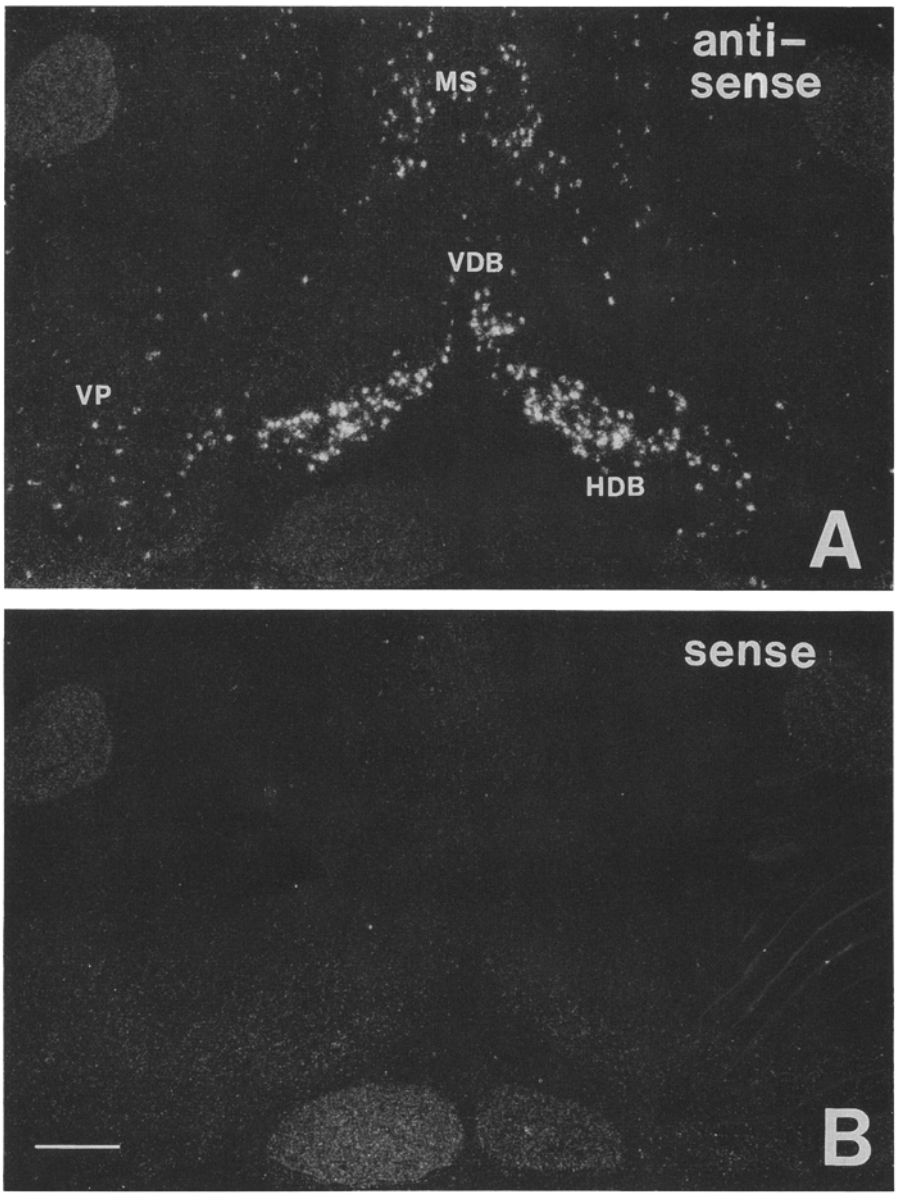


Fig. 2. Dark-field micrograph depicting the localization of VAChT mRNA in the basal forebrain. (**A**) After hybridization with a VAChT antisense cRNA probe, numerous cells are positively labeled in the well-known cholinergic regions of the medial septal nucleus (MS), the nuclei of the vertical and horizontal limbs of the diagonal band (VDB, HDB), and the ventral pallidum (VP). (**B**) Hybridization with a VAChT probe in sense strand orientation yields only background labeling. Exposure times of emulsion-coated slides were 21 d. Scale bar 400 μm.

levels than neurons of the basal nucleus of Meynert (Fig. 4A,B). Levels of VAChT mRNA in the parabigeminal nucleus (Fig. 5) were intermediate between those in the basal forebrain and in the arcuate nucleus (Table 1).

Distribution of VAChT mRNA in the Spinal Cord

Throughout the cervicothoracic and lumbosacral spinal cord, VAChT mRNA was concentrated in motoneurons of the ventral horn (Figs. 7 and 8C) and in the thoracolumbar preganglionic sympathetic neurons of the intermediolateral cell column (Figs. 7B and 8B). As a rule, a few scattered neurons showing a positive VAChT signal were present in lamina 10 throughout all spinal cord levels (Fig. 8A). VAChT mRNA-containing neurons in the dorsal horn were extremely rare. The hybridization signal for neurons in the intermediomedial cell column was faint (not shown).

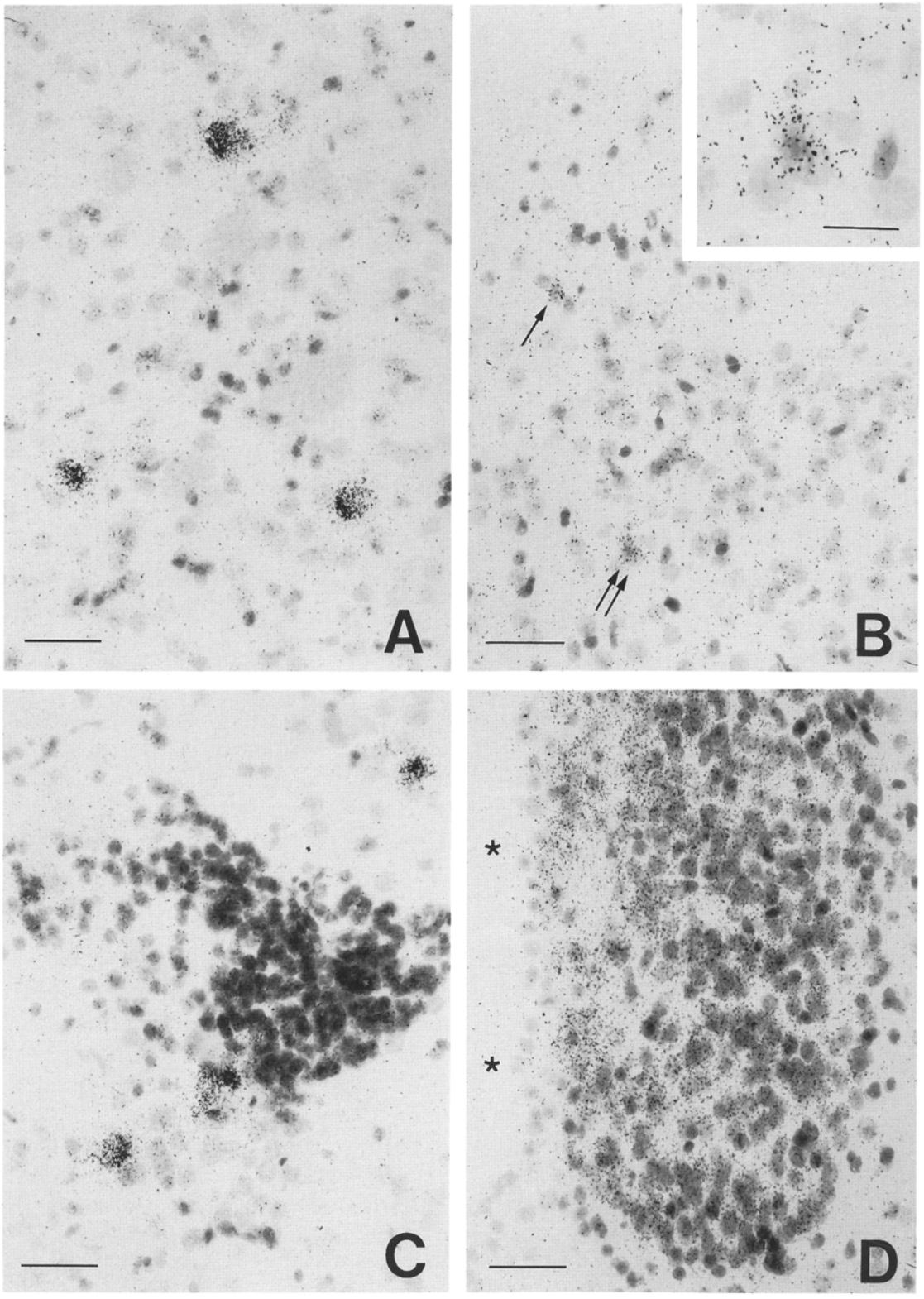
Comparison of VAChT mRNA Levels and Cellular Patterns in Different Regions of the Brain and in the Spinal Cord

The levels and cellular expression patterns of VAChT mRNA exhibited major regional differences in fore- and hindbrain and spinal cord areas as summarized in Table 1. The following categories of expression levels could be recognized.

Highest levels of VAChT mRNA were seen in somatic motoneurons of the brainstem and spinal cord (Figs. 1D,F–H and 6A,B) followed by projection-type neurons of various regions, which also exhibited relatively high levels. These included the nucleus accumbens, basal nucleus of Meynert, ventral pallidum, Islands of Calleja, anterior amygdala, medial septum, the vertical and horizontal limb of the diagonal band, the medial preoptic area, the magnocellular preoptic nucleus, and the lateral hypothalamic area. Moderate to high levels occurred in the ambiguus nucleus, the dorsal nucleus of the vagal nerve, the thoracolumbar intermediolateral cell columns, the pontine tegmental regions, and the medial habenula. Low levels of expression were seen in the parabigeminal nucleus and even lower levels in the neocortex, arcuate nucleus, lateral septum, Edinger Westphal nucleus, sacral intermediomedial cell columns, and dorsal horn neurons (Table 1).

Distribution of VAChT mRNA and Comparison of mRNA Levels in the Peripheral Nervous System

As summarized in Table 2, hybridization signals for VAChT mRNA were found in cranial parasympathetic ganglia (Figs. 9A and 10A,B), in intramural ganglia of the heart and the thyroid gland (Fig. 10C,D) and in ganglia of the pelvic plexus (not shown). In addition, VAChT mRNA was found in the enteric nervous system (Fig. 11; Table 2). In the cranial parasympathetic and intramural ganglia, it appeared that all neurons were positive, whereas in pelvic ganglia and myenteric plexus neurons, the majority of neurons, but not all of them, exhibited a positive hybridization signal (Table 2). The highest labeling intensity was seen in the submandibular and pterygopalatine ganglia. A somewhat less intense signal was present in ciliary, pelvic, and thyroid ganglia. The levels of VAChT mRNA in the myenteric plexus were relatively low.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A specific hybridization signal was absent from sensory dorsal root and trigeminal ganglia (Table 2). In sympathetic superior cervical ganglia, single cells that exhibited a low hybridization signal were detected only occasionally (Table 2).

Discussion

Distribution and Abundance of VAChT mRNA in CNS

The distribution of cholinergic neurons in the peripheral and central nervous systems has been primarily recognized on the basis of histochemical and immunohistochemical investigations analyzing the enzymes participating in ACh biosynthesis (ChAT) and catabolism (acetylcholinesterase; AChE) (Armstrong et al., 1983; Eckenstein and Sofroniew, 1983; Eckenstein and Thoenen, 1983; Houser et al., 1983; Levey et al., 1983; Barber et al., 1984; Eckenstein and Baughman, 1984; Levey et al., 1984; Wainer et al., 1984; Woolf et al., 1984; Houser et al., 1985; Paxinos and Butcher, 1985; Sofroniew et al., 1985; Woolf and Butcher, 1985, 1986; Tago et al., 1987; Chedotal et al., 1994) although AChE is apparently present on cholinoceptive as well as cholinergic neurons in the CNS (Butcher and Woolf, 1984) More recently, *in situ* hybridization with radiolabeled and nonradiolabeled probes for ChAT has been employed to identify cholinergic neurons and to map their distribution in the rat CNS (Cavicchioli et al., 1991; Ibanez et al., 1991a; Butcher et al., 1992, 1993; Oh et al., 1992; Lauterborn et al., 1993; Mori et al., 1993). Analysis of the distribution of VAChT mRNA in the central nervous system allows confirmation that previously demonstrated ChAT-positive cell groups are indeed functionally cholinergic, i.e., capable of storage of synthesized ACh into vesicular packets for exocytotic secretion required for neurotransmission (Whittaker, 1988).

The distribution of cholinergic neurons in the CNS identified by VAChT *in situ* hybridization was found to conform essentially to previous immunocytochemical data (Table 1), with some exceptions as discussed in the next section. In general, the levels as well as the distribution of VAChT mRNA in major known cholinergic cell groups in the CNS are consistent with previous reports of ChAT mRNA localization and relative abundance in these cell groups. Thus, VAChT mRNA is present at high levels in cholinergic projection neurons in the basal forebrain and habenulae, cranial and spinal motoneurons, and striatal interneurons, and moderate to low in the parabigeminal, ventrolateral septal,

Fig. 3. *(opposite page)* Bright-field micrographs demonstrating the distribution pattern of and labeling intensity for VAChT mRNA in several cholinergic forebrain areas. (**A**) In caudate-putamen, scattered neurons of medium to large size resembling striatal cholinergic neurons express VAChT mRNA at relatively high levels. (**B**) In contrast, single neurons exhibiting extremely low hybridization signals were present in lamina II (single arrow) and lamina III (double arrow) of parietal cortex. The insert demonstrates the lamina III neuron at higher magnification. (**C**) VAChT mRNA containing neurons showing a similar labeling intensity and cell size as neurons in the striatum (A) are present within and in juxtaposition to an Island of Calleja. (**D**) In the medial habenular nucleus, virtually all neurons, which are densely packed and of small size, express moderate to high levels of VAChT mRNA. Asterisks indicate third ventricle. Exposure times 21 d. Scale bars 50 μm.

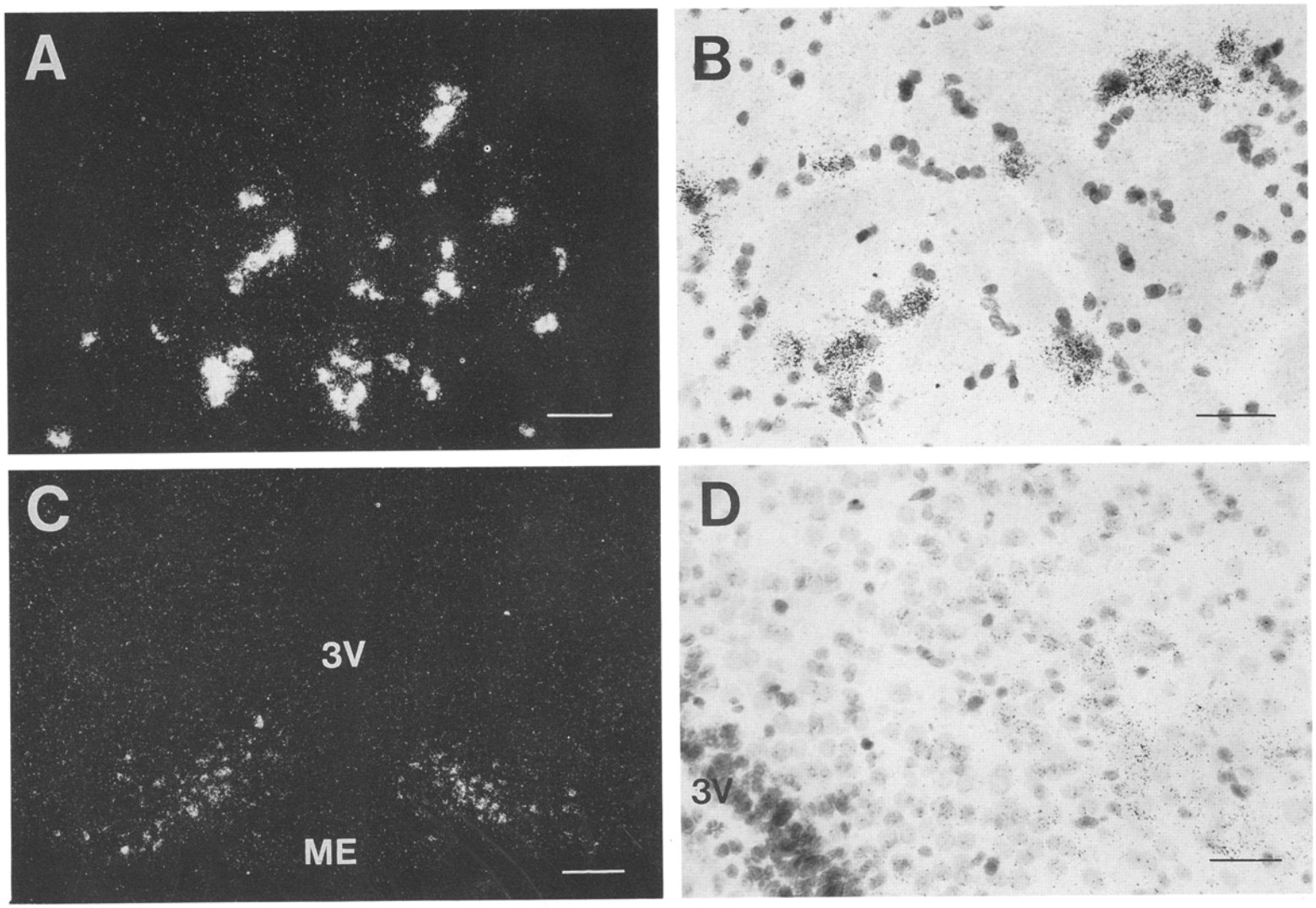


Fig. 4. Comparison of VAChT mRNA expression in the classical cholinergic region of the basal nucleus of Meynert (**A,B**) and in the presumed cholinergic region of the hypothalamic arcuate nucleus (**C,D**). Dark-field micrographs (A,C) and bright-field micrographs (B,D) demonstrate prominent differences in the labeling intensity between the basal nucleus (A,B), which shows high VAChT mRNA levels in relatively large neurons (B), and the arcute nucleus, which exhibits low VAChT mRNA levels in a subpopulation of concentrated small neurons. ME, median eminence; 3V, third ventricle. Exposure times 21 d. Scale bars: A, 100 μm; B,D 200 μm; C, 50 μm.

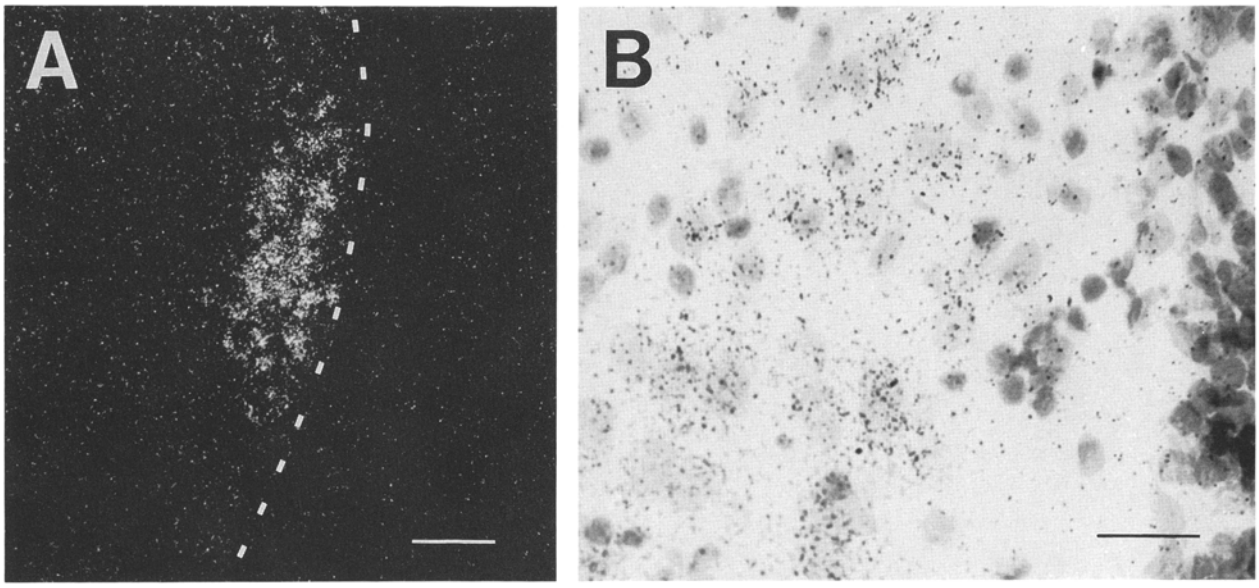


Fig. 5. Localization of VAChT mRNA in the parabigeminal nucleus. Dark-field illumination (**A**) demonstrates the selective accumulation of hybridization signals over the parabigeminal nucleus. The outer border of the brain is marked by a dotted line. The bright-field autoradiogram (**B**) shows the presence of moderate levels of VAChT mRNA over many neurons within the parabigeminal nucleus. Exposure times 21 d. Scale bars: A, 100 μm; B, 20 μm.

and Edinger Westphal nuclei, as reported for ChAT mRNA (Butcher et al., 1992, 1993; Oh et al., 1992; Lauterborn et al., 1993).

The functional significance of variable levels of VAChT mRNA throughout the cholinergic nervous system cannot be assessed at present, but may be a function of the extent of the terminal field and therefore the amount of protein required for a given cholinergic neuron, the rate of neurotransmission and therefore utilization of protein for different types of cholinergic neurons, or both.

VAChT Distribution in Regions of CNS Where Existence of Cholinergic Cells is Controversial

The presence of VAChT mRNA can provide experimental support for the cholinergic identity of cell groups within the CNS where this is in dispute because of technical difficulties inherent in the use of ChAT antibodies, or expression of cholinergic messenger RNA at relatively low levels. Thus, the existence of cholinergic cells in certain brain regions, including neocortex, hippocampal formation, ventrolateral septal nucleus, arcuate nucleus of the hypothalamus, and parabigeminal nucleus, is a matter of controversy. This is because of a lack of corroboration of histochemical evidence for ChAT expression by workers using other antibodies or of *in situ* hybridization histochemical evidence for the presence of ChAT mRNA (*see*, e.g., Butcher et al., 1992). Although some laboratories have reported the presence of cholinergic neurons in the neocortex by ChAT immunohistochemistry (Eckenstein and Sofroniew,

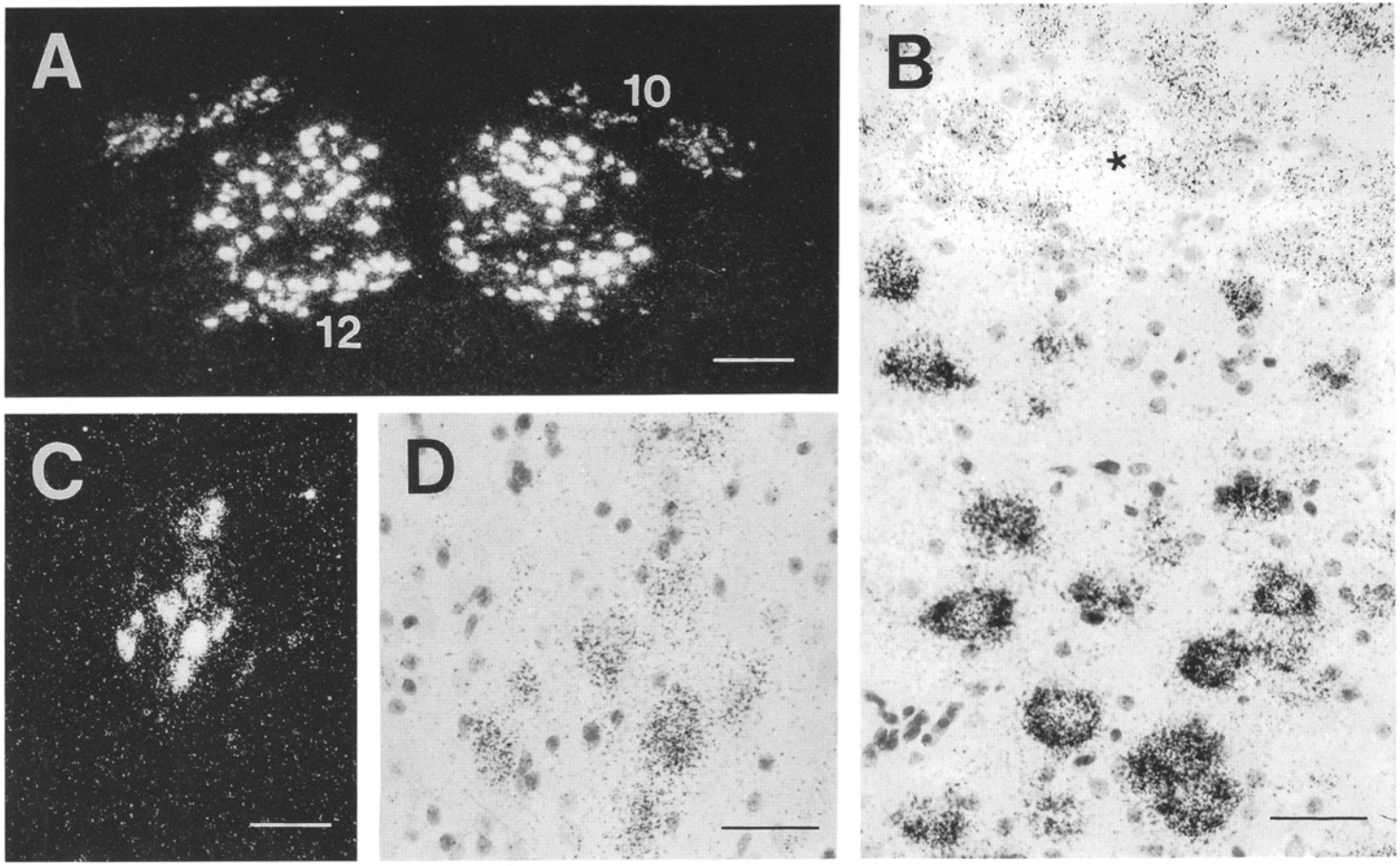


Fig. 6. Localization of VAChT mRNA in motor nuclei of cranial nerves. (**A**) Low-power dark-field autoradiogram demonstrates very high levels of VAChT mRNA in all neurons of the hypoglossal nucleus (12) and comparatively lower levels in all neurons of the dorsal motor nucleus of vagus (10). These differences become even more apparent under bright-field illumination (**B**), where the area of the dorsal motor nucleus of vagus is marked by an asterisk. (**C**) VAChT mRNA is also present in all neurons of the ambiguus nucleus, which is shown in dark-field and in bright-field illumination (**D**). Note that labeling intensities in the neurons of the dorsal motor nucleus of vagus (B) and in the ambiguus nucleus (D) are in a similar range (moderate to high). Exposure times were 21 d. Scale bars: A, 200 μm; B, 50 μm; C, 100 μm; D, 50 μm.

1983; Eckenstein and Thoenen, 1983; Levey et al., 1984; Eckenstein and Baughman, 1987; Chedotal et al., 1994) and by *in situ* hybridization with ChAT riboprobes (Lauterborn et al., 1993), for example, others have found no evidence for either immunoreactive ChAT (Butcher et al., 1992) or ChAT mRNA by either *in situ* (Butcher et al., 1992) or Northern blot (Ibanez, 1991a) analysis. Neither Oh et al. (1992) nor Lauterborn et al. (1993) reported ChAT mRNA-positive cells in the arcuate nucleus, in contrast to immunocytochemical studies showing ChAT immunoreactivity in this hypothalamic region, although at low levels (Tinner et al., 1989).

The present study demonstrates the existence of sparse VAChT mRNA-containing neurons in all regions of neocortex, predominantly in layers 2 and 3 and in arcuate nucleus, but not in hippocampus. This finding is consistent with our previous report of low levels of VAChT mRNA expression in rat neocortex by Northern blot hybridization (Erickson et al., 1994). A local cholinergic neuronal system is likely to exist in the neocortex, based on the present findings. The failure to detect these cells in previous studies may reflect their relative sparseness and low levels of VAChT, and presumably also ChAT mRNA. Although VAChT mRNA levels are low in cerebral cortex, VAChT mRNA abundance per neuron is qualitatively similar to that of the demonstrably functionally competent cholinergic neurons of the enteric nervous system (Kosterlitz and Lees, 1964; Furness et al., 1983) suggesting that expression of VAChT in cerebrocortical neurons is likely to impart functional cholinergic neurotransmission to these cells. Thus, the existence of an intracortical cholinergic system, if present also in primates, may be of significance in understanding the role of cholinergic neurotransmission in cognitive brain functions and of potential clinical significance in degenerative brain diseases, such as senile dementia of the Alzheimer's type (Perry et al., 1978).

VAChT mRNA-positive cells are distributed in the arcuate nucleus in a pattern like that of the dopaminergic tuberal cell group A12 (Björklund et al., 1973) consistent with the previous demonstration of ChAT immunoreactivity with tyrosine hydroxylase-positive cells in arcuate nucleus (Tinner et al., 1989, but *see* Rodriguez and Morley, 1985). It is therefore tempting to suggest that the VAChT mRNA-positive neurons described here have a mixed cholinergic/catecholaminergic phenotype, which may be relevant to the cholinergic physiology and pharmacology of hypothalamic neuroendocrine regulation.

There may well be additional examples of neurons in the central nervous system in which a mixed cholinergic/catecholaminergic phenotype persists into adulthood and has physiological relevance in CNS neurotransmission. This possibility is supported by in vitro experiments with clonal hybrid lines derived from embryonic septal nucleus neurons, in which mixed cholinergic/catecholaminergic expression at relatively low levels has been reported (Satoh et al., 1992) Determining the expression of biogenic amine biosynthetic enzymes, plasma membrane uptake systems and finally vesicular transporters for both catecholamines (Erickson et al., 1992; Liu et al., 1992) and acetylcholine (Erickson et al., 1994) will be required to assess the functional and neuropharmacological significance of the existence of such neurons.

Table 1
Regional Distribution of VAChT mRNA in the Rat CNS

Region	Relative levels of labeling intensity	Relative density of labeled neurons
Olfactory areas		
Olfactory tubercle	++	Scattered, few
Piriform cortex	++	Scattered, few
Cerebral cortex	+	Very few
Hippocampal formation	0	0
Accumbens ncl.	+++	Scattered
Caudate-putamen	+++	Scattered, many
Globus pallidus	+++	Scattered, very few
Basal ncl. of Meynert	+++	All
Ventral pallidum	+++	Scattered, many
Substantia innominata	+++	Scattered
Substantia nigra, pars reticulata	+++	Scattered, few
Substantia nigra, pars compacta	0	0
Islands of Calleja	+++	Scattered, few
Amygdala		
Anterior amygdala region	+++	Scattered
Other regions	++/+++	Scattered, very few
Septum		
Medial septal ncl.	+++	Many
Lateral septal ncl., ventral part	+	Many
Ncl. of diagonal band		
Vertical limb	+++	Many
Horizontal limb	+++	Many
Medial preoptic area	+++	Scattered, many
Magnocellular preoptic ncl.	+++	All
Diencephalon		
Thalamus	0	0
Habenula		
Medial	++/+++	Many
Lateral	0	0
Hypothalamus		
Arcuate ncl.	+	Many
Lateral hypothalamic area	+++	Scattered
Mesencephalon, pons, medulla		
Cranial nerves		
Oculomotor ncl.	++++	All
Edinger Westphal ncl.	+	All
Trochlear ncl.	++++	All
Trigeminal ncl., motor	++++	All
Abducens ncl.	++++	All
Facial motor ncl.	++++	All

(continued)

Table 1 *(continued)*

Region	Relative levels of labeling intensity	Relative density of labeled neurons
Vestibular efferent ncl.	+/++	Many
Dorsal motor ncl. vagus	++/+++	All
Hypoglossal ncl.	++++	All
Ambiguus ncl.	++/+++	All
Pedunculopontine ncl.	+++	Scattered, many
Parabigeminal ncl.	++	All
Laterodorsal tegmental ncl.	+++	Scattered, many
Parabrachial ncl.	+++	Scattered
Spinal cord		
Ventral horn motoneurons (C, T, L, S)	++++	All
Dorsal horn (C, T, L, S)	+	Extremely few
Lamina 10 (C, T, L, S)	++/+++	Scattered
Intermediolateral cell column (IML) (T1-L2)	++	All
Intermediomedial cell column (IMM) (S2-S4)	+	All

Subjective rating of labeling intensity: (0) absent; (+) low; (++) moderate, (+++) high, (++++) very high.

A continuum of relative expression of cholinergic and catecholaminergic phenotypes may well be expected, both in development and in the adult nervous system, depending on the neuron-specific expression of combinations of receptors for various trophic factors, as well as the relative levels of such factors in the vicinity of neurons with potentially mixed phenotypes within the brain (Lindsay et al., 1994). Nerve growth factor (NGF), for example, may have both positive and negative effects on the survival and cholinergic phenotype of neurons expressing cholinergic markers in vivo and in vitro, depending in part on their relative commitment to the adrenergic vs cholinergic lineage (Patterson, 1990; Svendsen et al., 1994) It is of interest that NGF-responsive elements as well as tissue-specific silencer elements exist upstream of the several putative transcriptional start sites of the mammalian ChAT gene (Ibanez and Persson, 1991; Bejanin et al., 1992; Misawa et al., 1992; Hersh et al., 1993; Wu and Hersh, 1994) We have recently demonstrated that the mammalian VAChT gene, like that for the nematode *C. elegans* (Alfonso et al., 1994) is contained within the first ("R-type") intron of the ChAT gene locus (Erickson et al., 1994) Thus, various neurotrophic factors shown to affect regulation of ChAT expression are likely to regulate similary VAChT expression through the same *cis*-acting elements of this "cholinergic regulon," thus imparting a fully functional cholinergic phenotype to affected neurons.

VAChT mRNA-Positive Neurons in Spinal Cord

VAChT mRNA levels differ dramatically between motoneurons and preganglionic

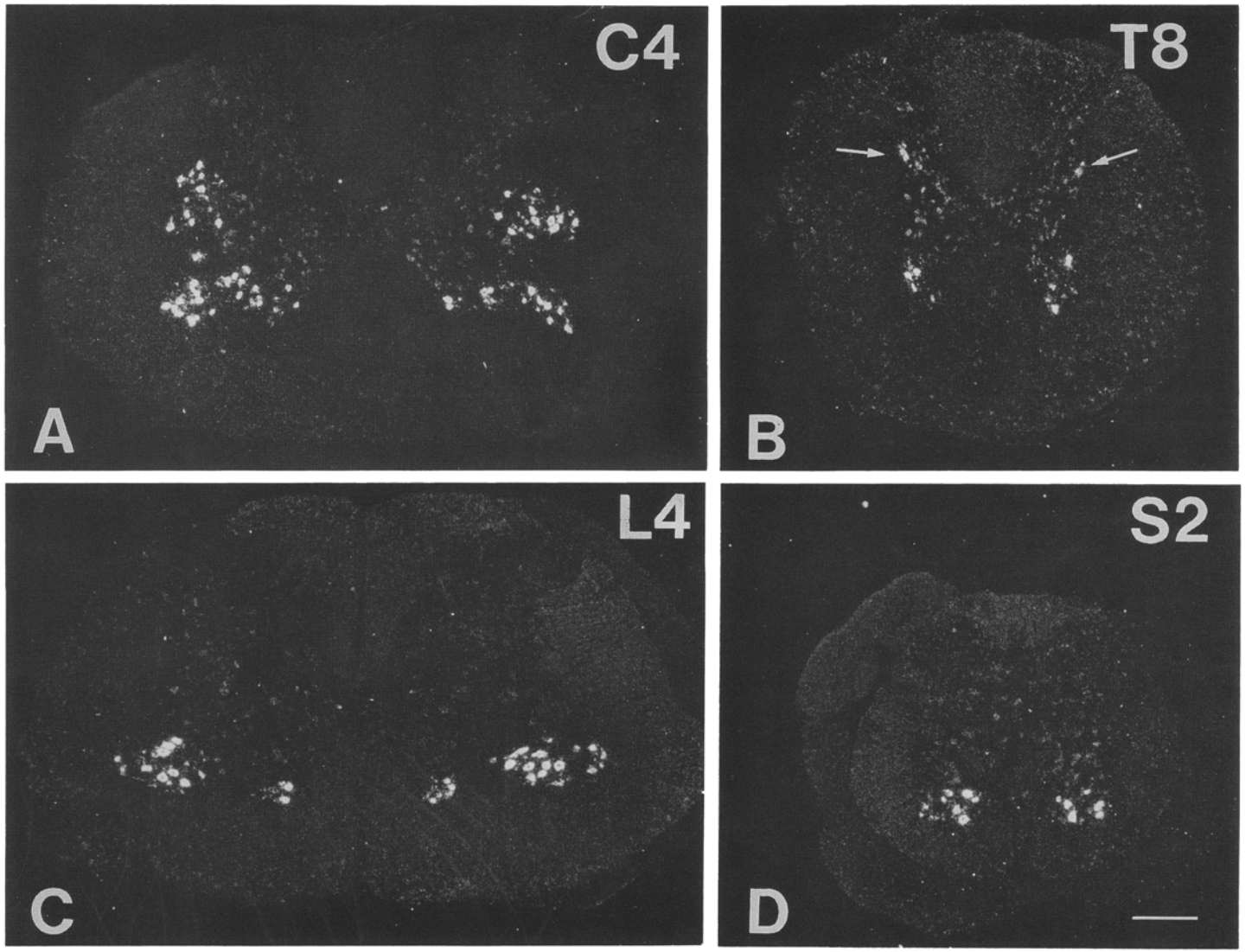


Fig. 7. Distribution of VAChT mRNA in the spinal cord. VAChT mRNA is present at very high levels in ventral horn motoneurons throughout all segments as shown in dark-field micrographs for (**A**) cervical (C4), (**B**) thoracic (T8), (**C**) lumbar (L4), and (**D**) sacral (S2) segmental levels. In addition, a moderate to high hybridization signal is present in the intermediolateral cell column (arrows in B). Exposure times were 21 d. Scale bar 400 μm.

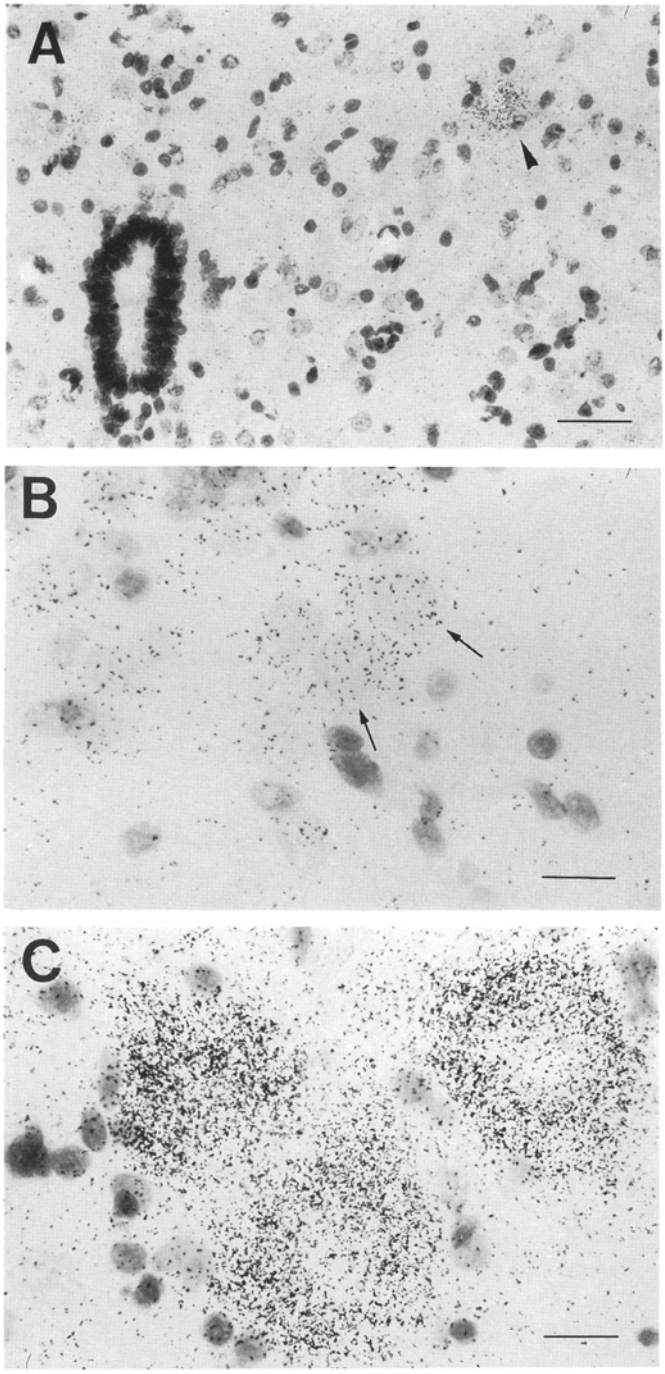
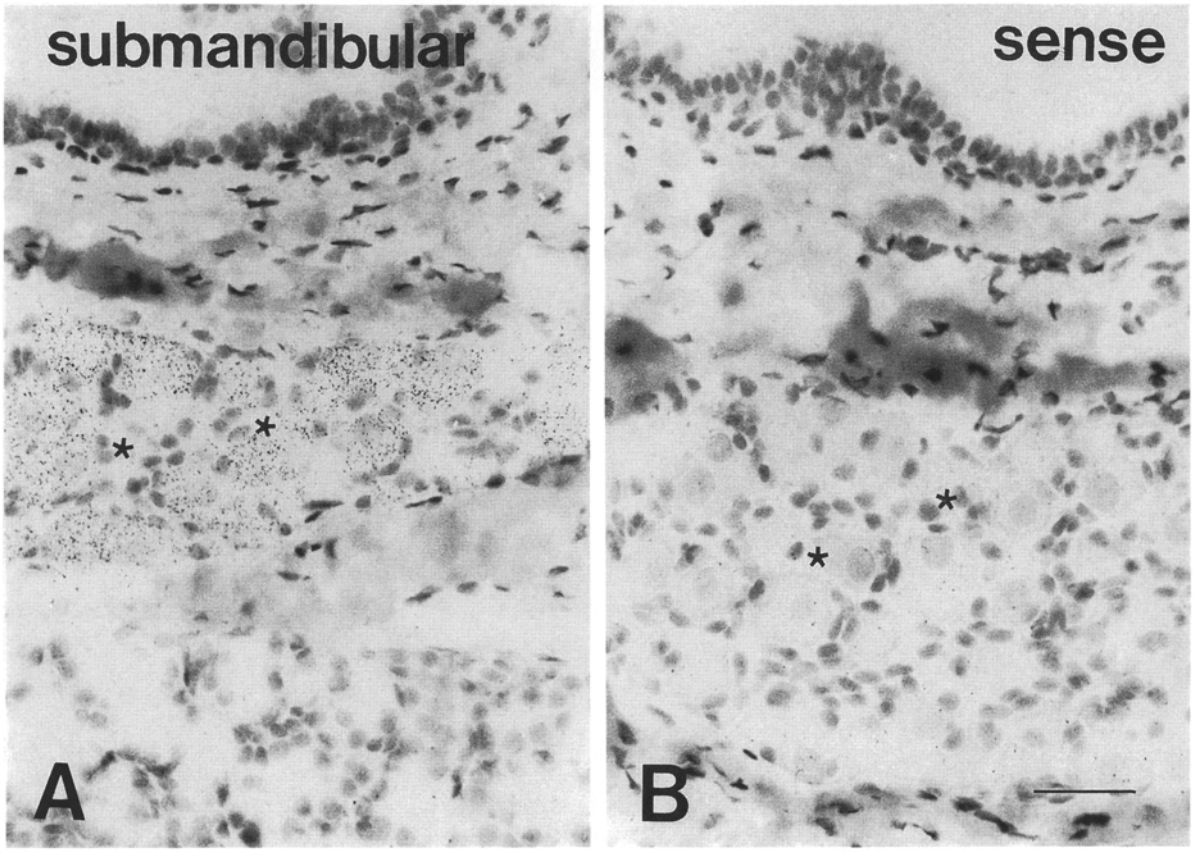


Fig. 8. Comparison of the labeling intensities in different categories of spinal neurons. In (**A**) a small neuron in the dorsal lamina X exhibiting a moderate intensity is marked by an arrowhead. High power micrographs demonstrate that preganglionic sympathetic neurons (arrows) of the intermediolateral cell column (**B**) express substantially lower levels of VAChT mRNA as compared to ventral horn motoneurons (**C**). Exposure times 21 d. Scale bars: A, 50 μm; B,C, 20 μm.

Table 2
Distribution and Relative Levels of VAChT mRNA in Selected Regions of the Rat PNS

Region	Relative labeling intensity	Relative density of labeled neurons
Cranial parasympathetic ganglia		
Submandibular	++++	All
Pterygopalatine	++++	All
Ciliary	+++	All
Pelvic ganglia	+++	Majority
Thyroid ganglia	+++	All
Cardiac (atrial) ganglia	+++	All
Myenteric plexus (duodenum, colon)	++	Majority
Superior cervical ganglia	+	Single, extremely rare
Dorsal root ganglia (C, T, L)	0	Absent
Trigeminal ganglia	0	Absent

Subjective rating of labeling intensity: (0) absent; (+) low; (++) moderate, (+++) high, (++++) very high.



parasympathetic and sympathetic neurons of the spinal cord, and may reflect differences in functional activities between the two systems, the length of axonal processes through which VAChT proteins must be transported, the size of the terminal fields of each type of neuron, or all three factors, as discussed above. Although the functional identity of the VAChT mRNA-containing preganglionic autonomic and ventral horn motoneurons is well defined, the functional properties of VAChT mRNA-positive neurons in the dorsal lamina X and in the deep dorsal horn remain to be determined (Sherriff and Henderson, 1994). Cholinergic neurons in these locations may be involved in nociceptive processes (Weihe, 1992). The regulation of ChAT mRNA in motoneurons after peripheral nerve transection suggests that the regulation of VAChT might likewise be quite plastic in reflecting varying demands on secretory capacity of each class of cholinergic neuron, under various physiological states (Piehl et al., 1993).

VAChT Expression in the Peripheral Nervous System

The relatively high levels of VAChT mRNA in peripheral parasympathetic ganglia compared to low levels in enteric neurons may reflect differences in levels of neurotransmission or the extent to which each class of neuron is committed to a full cholinergic phenotype. Differences in projection fields, as discussed above, may also be reflected in variable biosynthetic demand for VAChT protein and, therefore, differences in levels of VAChT mRNA within cell bodies of each type of neuron. The demonstrated expression of VAChT mRNA in the majority, but not in all, enteric neurons is in agreement with immunocytochemical findings showing ChAT-like immunoreactivity in about 64% of myenteric neurons (Schemann et al., 1993).

The occasional occurrence of VAChT mRNA-positive cells in the superior cervical ganglion (SCG) is most likely attributed to sudomotor neurons. The presence of single neurons of cholinergic phenotype has also been demonstrated in the superior mesenteric ganglion (Schemann et al., 1993).

The absence of VAChT expression in sensory ganglia is in contrast to recent reports demonstrating ChAT immunoreactivity at low levels in all sensory neurons of developing and mature chick dorsal root ganglia (Tata et al., 1994) Although species differences or uncoupling of ChAT and VAChT expression in these neurons cannot be ruled out, there is no other convincing evidence that ACh acts as a neurotransmitter in sensory neurons.

VAChT as a Functionally Relevant Cholinergic Marker

The apparently invariable coexpression of VAChT mRNA in reported ChAT-positive cholinergic neurons is consistent with the proposal that the contiguity of the VAChT and ChAT genes and their proxim-

Fig. 9. *(opposite page)* Distribution of VAChT mRNA in the submandibular ganglion. Hybridization with a VAChT antisense cRNA probe reveals a selective signal in all neurons of the depicted part of the submandibluar ganglion (asterisk). No hybridization signal is seen over duct epithelium and periductal tissue (upper part of **A**) or over juxtaganglionic exocrine glands (lower part of A). No specific hybridization signal is detected in an adjacent section hybridized with the sense probe (**B**). Exposure times 21 d. Scale bar 50 μm.

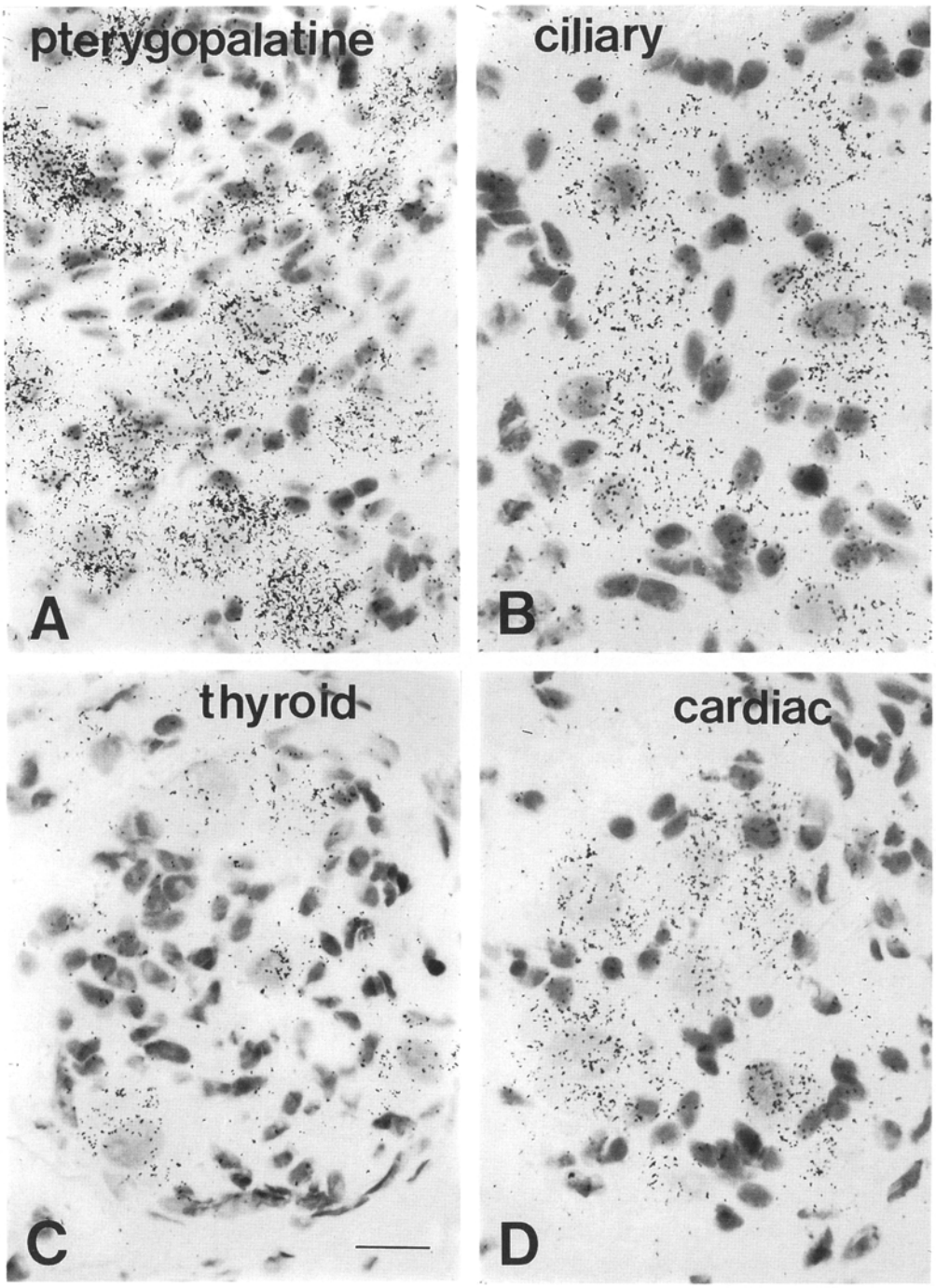


Fig. 10. Distribution of VAChT mRNA in cranial parasympathetic ganglia (**A,B**) and in peripheral parasympathetic ganglia (**C,D**). Highest levels of VAChT mRNA were seen in the pterygopalatine ganglion (A) followed by the ciliary ganglion (B) and an intramural thyroid (C), and an atrial cardiac ganglion (D), which both exhibited somewhat lower levels. Note that in all of the examined

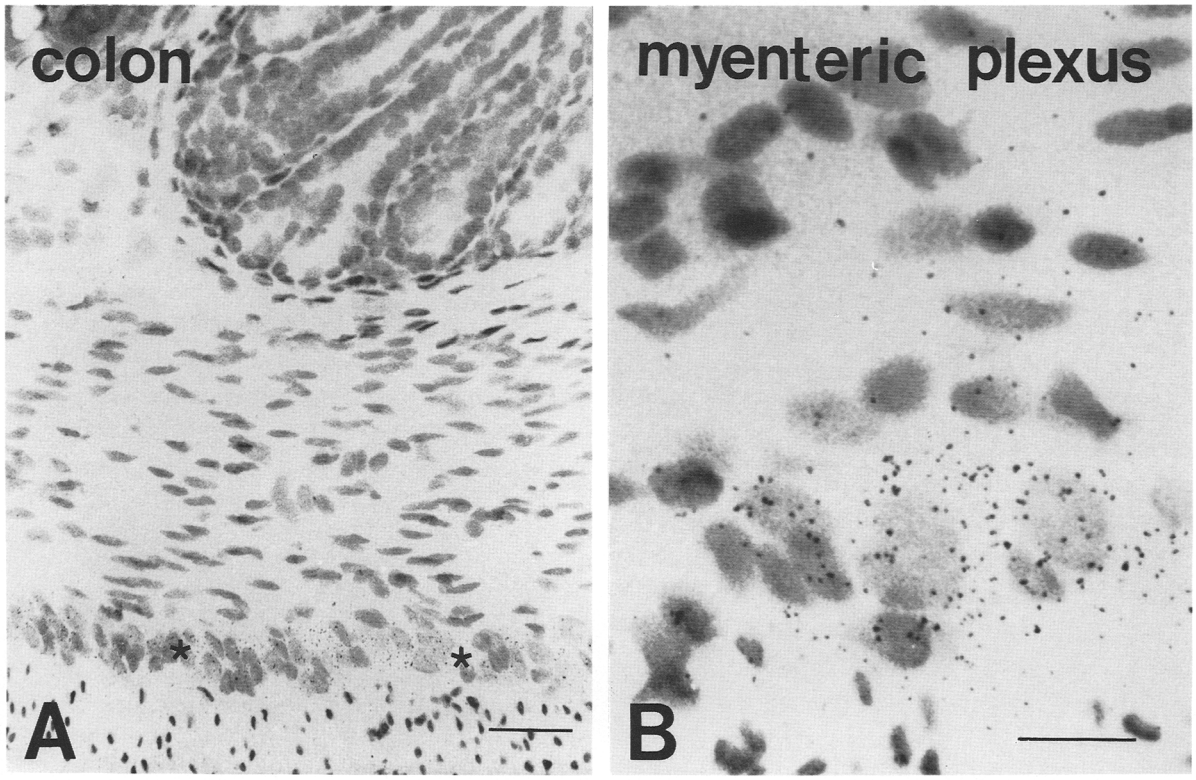


Fig. 11. Distribution of VAChT mRNA in colon. The hybridization signal for VAChT mRNA is concentrated over the myenteric plexus (asterisks) and absent from the mucosa (top), from the circular muscle and from the longitudinal muscle as shown in low-power bright-field illumination (**A**). It appears that the majority of the myenteric plexus neurons are labeled at moderate labeling intensities. The concentration of hybridization signal over neurons of the myenteric plexus and absence of VAChT mRNA from nonneuronal cells is shown in high-power bright-field illumination (**B**). Exposure times 21 d. Scale bars: A, 100 μm; B, 10 μm.

ity to demonstrated tissue- and neurotrophin-specific *cis*-regulatory sequences constitute a structural basis for coregulation of the expression of the two proteins within a cholinergic "regulon" (Erickson et al., 1994). A full understanding of VAChT/ChAT coregulation will need to include an analysis of the expression of the messenger RNAs encoding each of these proteins during the ontogeny of the nervous system and in extra-neuronal tissues (Bruce et al., 1985; Ibanez et al., 1991b; Grando et al., 1993; Rinner and Schauestein, 1993).

The complete concordance of VAChT mRNA expression with both the known and previously suspected distribution of

ganglia, virtually all neurons are labeled and that there are no apparent differences in labeling intensities of individual neurons in a respective ganglion. Schwann cells and connective tissue elements are negative. Exposure times 21 d. Scale bar 20 μm.

neurons expressing the cholinergic phenotype throughout the central and peripheral nervous systems, and the identification of a single genetic locus for the mammalian VAChT gene (Erickson et al., 1994) also strongly suggest that a single species of VAChT subserves vesicular accumulation of ACh in all cholinergic neurons, in contrast to biogenic amine uptake, which is mediated by separate species VMAT1 and VMAT2, for nomenclature *see* Erickson and Eiden [1993] note added in proof, and Schuldiner [1994]) in the central and peripheral nervous systems, and in the adrenal medulla (Mahata et al., 1993). Finally, VAChT expression may well be a dynamic indicator of the overall levels of synaptic activity for a given type or anatomical locus of cholinergic neurons, given the wide range of expression of its mRNA within a variety of indisputably functional cholinergic neurons of the central and peripheral nervous systems. For these reasons, VAChT would appear to be an ideal marker for the cholinergic nervous system. By raising antisera against the VChAT protein, it may be possible to create better immunocytochemical tools to map the perikarya and terminal fields of cholinergic neurons throughout the CNS and PNS, and during ontogeny of the cholinergic nervous system.

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