Journal of Neuroscience Research 29:163–171 (1991)

Developmental and Regional Expression of Choline Acetyltransferase mRNA in the Rat Central Nervous System

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The developmental and regional expression of choline acetyltransferase (ChAT) mRNA was examined in the rat brain and spinal cord by northern blot analysis and in situ hybridization. ChAT mRNA expression in the brain showed a biphasic increase during development, with a first peak at two weeks postnatally, a marked decrease by the third week, and a second increase between the third and fifth week after birth, indicating that emergence of the cholinergic phenotype occurs at different times in different brain regions. In the spinal cord, ChAT mRNA was detected at similar levels from embryonic stage 13 (E13) until birth, increasing thereafter until adulthood. In the adult rat central nervous system, high levels of ChAT mRNA were detected in the spinal cord and brain stem structures. Lower levels were seen in midbrain, septum, striatum, thalamus, and olfactory bulb. ChAT mRNA containing cells were identified by in situ hybridization in the olfactory tubercule, piriform cortex, striatum, several basal forebrain nuclei, and spinal cord. A nearly two-fold increase in adult spinal cord ChAT mRNA levels were seen one week after a bilateral crush lesion of the sciatic nerve, indicating that ChAT mRNA expression is regulated during motoneuron regeneration.

Key words: brain, in situ hybridization, messenger RNA, northern blot, rat, spinal cord

INTRODUCTION

The anatomical organization of cholinergic systems has been extensively studied by mapping the distribution of acetylcholinesterase (AChE) using AChE histochemistry and by immunohistochemical staining for the acetylcholine-synthesizing enzyme, choline acetyltransferase (ChAT), currently the most specific marker for cholinergic neurons. The most prominent cholinergic system consists of cholinergic cells associated with cranial nerve nuclei and motoneurons of the spinal cord. The basal forebrain complex contains the major cholinergic cell bodies in the forebrain and provides the main source of cholinergic innervation for the entire neocortex, hippocampus, amygdala, and olfactory bulb (Mesulam et al., 1983; McGeer et al., 1984). Other cholinergic systems in the central nervous system (CNS) include the intrinsically organized cholinergic neurons of the striatum, nucleus accumbens, and olfactory tubercule (Kimura et al., 1981; Fibiger, 1982; Armstrong et al., 1983), and the cholinergic cells associated with the parabrachial and reticular complexes in the brain stem (Tago et al., 1989). Populations of small, intrinsic cholinergic neurons have also been described in cerebral cortex (Eckenstein and Thoenen, 1983; Parnavelas et al., 1986; Nishimura et al., 1988) and in hippocampus (Frotscher et al., 1986; Blaker et al., 1988).

ChAT activity is known to be developmentally regulated in rat basal forebrain, cerebral cortex, and hippocampus (Large et al., 1986). In cortex and hippocampus, a sharp increase in ChAT activity occurs ten days after birth, reaching close to adult levels at three weeks of age (Large et al., 1986). Recently, ChAT immunohistochemistry has also revealed a developmental regulation of intrinsic cholinergic innervation in cerebral cortex (Dori and Parnavelas, 1989) and striatum (Phelps et al., 1989). Only low levels of ChAT activity have been detected in rat striatum during the first two postnatal weeks, with a marked increase after postnatal day 15 (Mobley et al., 1989). A similar increase has also been seen in septum which, however, occurred six days earlier than in striatum, suggesting that different brain regions show a different developmental regulation of their cholinergic phenotype. However, it remains to be elucidated if these developmental changes are caused by an increased enzymatic activity or an increased level of the enzyme due to enhanced expression of the ChAT gene.

Received May 19, 1990; revised October 12, 1990; accepted October 15, 1990.

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The recent isolation of ChAT cDNA clones from the spinal cord of pig (Berrard et al., 1987), rat (Brice et al., 1989), and mouse (Ishii et al., 1990) has made it possible to study the developmental regulation of ChAT mRNA expression and, moreover, the distribution of ChAT-containing cells by the detection of ChAT-specific mRNA. In this study, the expression of ChAT mRNA in several regions of the rat brain was examined by northern blot analysis. ChAT mRNA-containing cells in the spinal cord and in various regions of the rat forebrain were identified by in situ hybridization. The developmental expression of ChAT mRNA in the rat brain and spinal cord is also described, and evidence is presented that ChAT mRNA expression is regulated during motoneuron regeneration.

MATERIALS AND METHODS

Surgical Procedures and Collection of Tissue Samples

Adult rat tissues were dissected from 150–200 g male Sprague-Dawley rats. The spinal cord was collected from the vertebral column under pressure in phosphate buffer saline using a 50-cc syringe. The adult rat brain was dissected into 12 different regions. First the olfactory bulbs and the hypothalamus were removed from the ventral part of the brain. Dorsally, the corpus callosum was cut and the cerebral hemispheres were gently separated. The hippocampus was peeled off from the inner surface of the cortex. The septum and the striatum were then removed and the cortex was cut underneath and separated. The thalamus was then removed. The cerebellum was separated after cutting the middle cerebellar peduncle. Finally, the ventral midbrain was separated from the colliculi and the rest of the brain stem was divided into pons and medulla. For developmental studies, total brain and spinal cord were dissected from rat embryos collected from pregnant Sprague-Dawley rats at various stages of pregnancy which were determined by measuring the crown-rump length (Seiger and Olson, 1973). Bilateral compression of the sciatic nerve was performed on 150–200 g male Sprague-Dawley rats under chloral hydrate anaesthesia at the site where the nerve passes superficial to the tendon of the obturatus internus muscle. The method used produces a well-defined and complete transverse crush lesion of the nerve without breaking the mechanical continuity of its outer sheaths. All tissues were kept frozen at −70°C until use.

RNA Preparation

Frozen tissue samples were homogenized in 4 M guanidine isothiocyanate, 0.1 M β-mercaptoethanol, 0.025 M sodium citrate pH 7.0, three times for 15 sec with a Polytrone. Each homogenate was layered over a 4 ml cushion of 5.7 M CsCl in 0.025 M sodium citrate pH 5.5 and centrifuged at 15°C in a Beckman SW41 rotor at 35,000 rpm for 16 hr (Chirgwin et al., 1979). RNA pellets were dissolved in 10 mM Tris-HCl pH 7.5, 5 mM EDTA, 1% SDS, phenol-chloroform extracted, and ethanol precipitated. Polyadenylated RNA (Poly-$(A)^+$ RNA) was purified by oligo (dT) cellulose chromatography (Aviv and Leder, 1972) and the recovery of RNA was quantified spectrophotometrically.

Isolation of a Rat ChAT cDNA Probe

Adult rat spinal cord poly$(A)^+$ RNA was used as template for first strand cDNA synthesis. Five micrograms of poly$(A)^+$ RNA were incubated in 50 mM Tris-HCl pH 8.3, 75 mM KCl, 10 mM $MgCl_2$, 0.5 mM spermidine, 10 mM DTT, 4 mM Na pyrophosphate, 1 mM each of dATP, dCTP, dGTP, dTTP, and 1 unit/μl RNasin ribonuclease inhibitor (Promega, Madison, WI) with 370 ng of random primer (Pharmacia, Uppsala, Sweden) and 50 units of AMV reverse transcriptase (Life Sciences, St. Petersburg, FL), in a final volume of 60 μl for 1 hr at 42°C. The reaction was stopped on ice, purified through a QIAGEN-tip 20 (QIAGEN, Studio City, CA), and precipitated with isopropanol. One hundred nanograms of single stranded cDNA were used as template for the polymerase chain reaction (PCR) according to the manufacturer's protocol (Perkin Elmer Cetus, Norwalk, CT). The PCR reaction was primed with two synthetic oligonucleotides corresponding to the 5′ (positions 202–221) and 3′ (positions 2114–2133) ends of the protein coding part of the rat ChAT cDNA sequence (Brice et al., 1989). The oligonucleotides were synthesized on an Applied Biosystem 381A DNA Synthesizer according to the manufacturer's manual. A specific 1.93 kb band was detected in a 1% agarose gel after 40 amplification cycles. The 1.93 kb PCR-product was eluted from the gel and subcloned into pBS-KS+ (Stratagene, La Jolla, CA). Its identity with the ChAT cDNA sequence was confirmed by dideoxy chain termination sequence analysis (Sanger et al., 1977).

Northern Blot Analysis

Poly$(A)^+$ RNA (25 μg) or, where indicated, total cellular RNA (40 μg) from each sample was electrophoresed in a 1% agarose gel containing 0.7% formaldehyde and transferred to a nitrocellulose filter. The filter was then hybridized to the rat ChAT cDNA probe isolated by PCR. The probe was labeled with α-(^{32}P)-dCTP by nick-translation to a specific activity of approximately 5×10^8 cpm/μg. Hybridization was performed in 4 × SSC (1 × SSC is 150 mM NaCl, 15 mM sodium citrate pH 7.0), 40% formamide, 1 × Denharts' solution, and 10% dextrane sulfate at 42°C. Filters were

washed at high stringency (0.1 × SSC, 0.1% SDS, 55°C) and exposed to Kodak XAR-5 films.

In Situ Hybridization

A 43-mer oligonucleotide complementary to nucleotides 1818 to 1860 of the rat ChAT cDNA sequence (Brice et al., 1989) was 3′-end labeled with α-^{35}S-dATP using terminal deoxyribonucleotidyl transferase (International Biotech, Inc., New Haven, CT) to a specific activity of approximately 1×10^9 cpm/μg. The labeled probe was purified on a Nensorb column (DuPont, Wilmington, DE) prior to use. An unrelated oligonucleotide with a similar length and G + C content was used as control. Tissue sections (14 μm) of rat forebrain regions and lumbar spinal cord were cut on a cryostat (Dittes, Heidelberg, FRG) and thawed onto poly-L-lysine coated slides (50 μg/ml). The tissues were fixed in 10% formalin in phosphate buffered saline for 30 min, rinsed twice for 4 min in PBS, and delipidated in graded series of ethanol, including a 5 min-incubation with chloroform. The sections were then air-dried. Sections were hybridized in a cocktail containing 50% formamide, 4 × SSC, 1 × Denhardts solution, 1% Sarcosyl, 0.02M $NaPO_4$ (pH 7.0), 10% dextransulphate, 0.5 mg/ml yeast tRNA, 0.06M DTT, 0.1 mg/ml sheared salmon sperm DNA, and 1×10^7 cpm/ml of ^{35}S-labeled oligonucleotide probe. After hybridization (16 hr at 42°C), the sections were rinsed five times for 15 min each in 1 × SSC at 55° and in autoclaved water for 5 min at room temperature. They were then dehydrated through a series of graded alcohol and air-dried. Finally, the sections were dipped in Kodak NTB-2 photo emulsion (diluted 1:1 in water), exposed for six to eight days at −20°C, developed and fixed, followed by light counterstaining with cresyl violet.

RESULTS

Developmental Expression of ChAT mRNA in the Rat Brain

In the developing rat brain, a 4.0 kb ChAT mRNA was detected already at embryonic stage 17 (E17), the earliest stage tested (Fig. 1). Thereafter, ChAT mRNA levels increased and a peak was seen at the second postnatal week. At three weeks of age, the level of ChAT mRNA decreased, followed by a second increase between the third and fifth postnatal week. The levels of ChAT mRNA in the adult brain were slightly higher than the peak level seen at two weeks of age.

Regional Expression of ChAT mRNA in the Rat Brain

Polyadenylated RNA from 12 different regions of the adult rat brain was analyzed for ChAT mRNA expression. The highest levels were found in medulla and pons (Fig. 2). Lower levels were detected in the ventral part of the midbrain, septum, and striatum. Low levels were also found in thalamus and olfactory bulb. The levels of ChAT mRNA were below detection in cerebral cortex, hippocampus, colliculi, cerebellum, and hypothalamus.

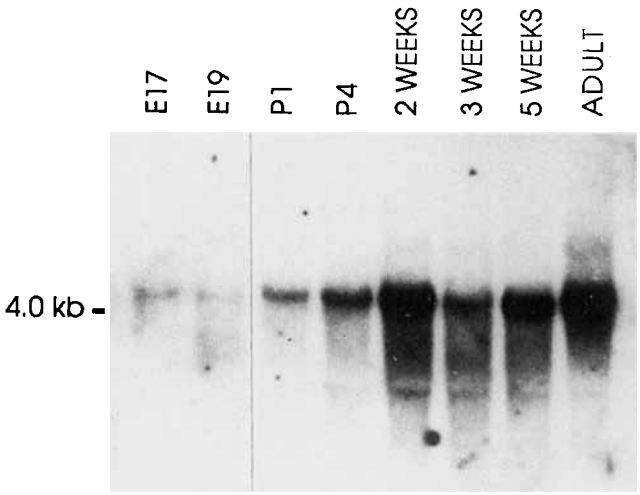

Fig. 1. Expression of ChAT mRNA during rat brain development. 25 μg of polyadenylated RNA from rat brain of the indicated ages was electrophoresed in a 1% denaturing agarose gel, transferred to a nitrocellulose filter, and hybridized to a nick-translated 1.93 kb rat ChAT cDNA fragment. The filter was washed at high stringency and exposed to Kodak XAR-5 film at −70°C.

Localization of Cells Expressing ChAT mRNA in the Rat Brain by In Situ Hybridization

In situ hybridization using a ChAT mRNA-specific oligonucleotide probe revealed ChAT mRNA-containing cells in several regions of the rat forebrain. Labeled cells with moderate intensity were seen in piriform cortex (Fig. 3A,B) and olfactory tubercule (Fig. 3C,D). The labeled cells had the size and morphology of neurons (Fig. 3B,D). Intensely labeled, scattered large neurons were seen throughout the striatum (Fig. 4A,B). In the basal forebrain, intensely labeled neurons were seen in the medial septum and in the diagonal band of Broca (Fig. 4C,D). Labeled neurons were also seen over more caudal sections in the magnocellular preoptic area and substantia innominata (not shown). No labeling was detected over any part of the sections after hybridization with an unrelated oligonucleotide probe of the same length and G + C content (not shown).

Expression of ChAT mRNA in Rat Spinal Cord

The developmental expression of ChAT mRNA in rat embryonic spinal cord was analyzed by northern blot

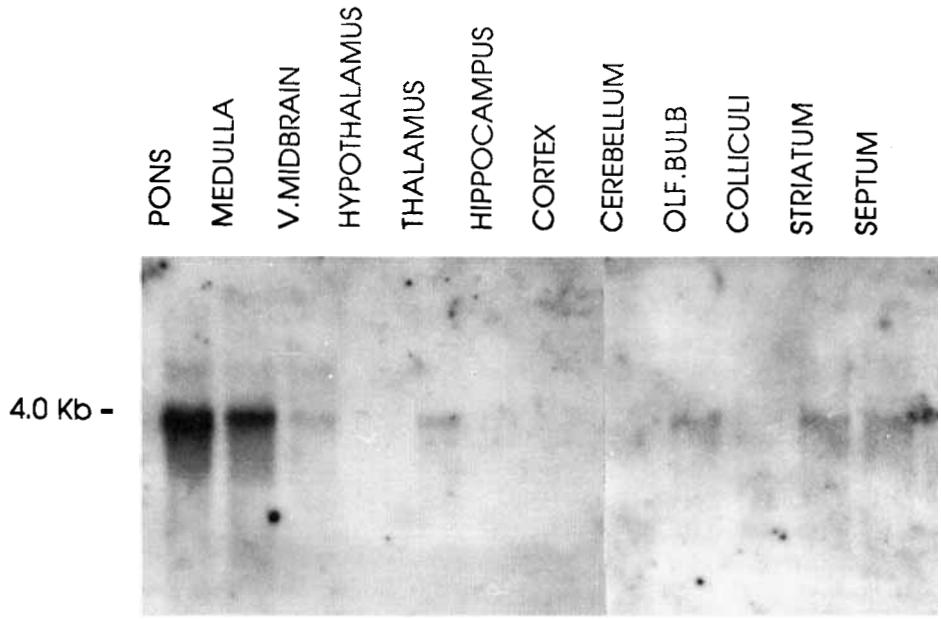

Fig. 2. Expression of ChAT mRNA in different regions of the adult rat brain. 25 μg of polyadenylated RNA from the indicated brain regions was electrophoresed in a 1% denaturing agarose gel, transferred to a nitrocellulose filter, and hybridized to a nick-translated 1.93 kb rat ChAT cDNA fragment. The filter was washed at high stringency and exposed to Kodak XAR-5 film at −70°C.

analysis. Low levels were already detected at E13–14, the earliest time point tested (Fig. 5). The levels remained relatively constant throughout the rest of the embryonic development but slightly increased shortly after birth. Densitometric scanning of the autoradiogram revealed that the levels of ChAT mRNA in the adult rat spinal cord were approximately seven-fold higher than at P1.

Transverse sections were prepared from the lower lumbar region of embryonic (E15) and adult rat spinal cord and analyzed for ChAT mRNA expression by in situ hybridization. Labeling was concentrated in the ventral horn of both embryonic and adult spinal cords (Fig. 6A,B). Both strongly and weakly labeled cells were observed in the adult spinal cord (Fig. 6B,C). Higher magnification revealed that the labeling was restricted to the cytoplasm of large motoneurons (Fig. 6C). Hybridization with a control oligonucleotide probe did not show any labeling (not shown).

Expression of ChAT mRNA in the Adult Rat Spinal Cord After a Crush Lesion of the Sciatic Nerve

To evaluate whether damage to the sciatic nerve affects the regulation of ChAT mRNA expression in motoneurons, a bilateral crush lesion of the sciatic nerve was performed. An approximately two-fold increase was seen seven days after the lesion as compared to the levels in control animals (Fig. 7).

DISCUSSION

In this study, a cDNA probe for choline acetyltransferase (ChAT), the key enzyme in the synthesis of the neurotransmitter acetylcholine (ACh), was used to study the developmental and regional distribution of ChAT mRNA in rat brain and spinal cord by northern blot analysis. Selected regions were further analyzed by in situ hybridization using an oligonucleotide probe complementary to rat ChAT mRNA.

In the adult rat brain, high levels of a 4.0 kb ChAT mRNA were detected in medulla and pons. Within these areas, high levels of ChAT immunoreactivity have been detected in nuclei associated with the parabrachial complex (mainly the pedunculopontine nucleus and the dorsal and ventral parabrachial nuclei), in neurons of the reticular formation (giganto and magnocellular ChAT-containing neurons which extend continuously from pons into the medulla), and in the cranial nerve motor nuclei (McGeer et al., 1984; Tago et al., 1989). Moderate levels of ChAT mRNA were also seen in the ventral midbrain, in which cholinergic cells have been detected in the red nucleus and in the cranial nerve nuclei 3 and 4 (Kimura et al., 1981; McGeer et al., 1984). In the basal forebrain, low levels of ChAT mRNA could be detected in septum by northern blot analysis. Within this area, ChAT mRNA-expressing neurons could also be seen by in situ hybridization. Strong labeling was seen in the medial septum. Clusters of intensely labeled neurons

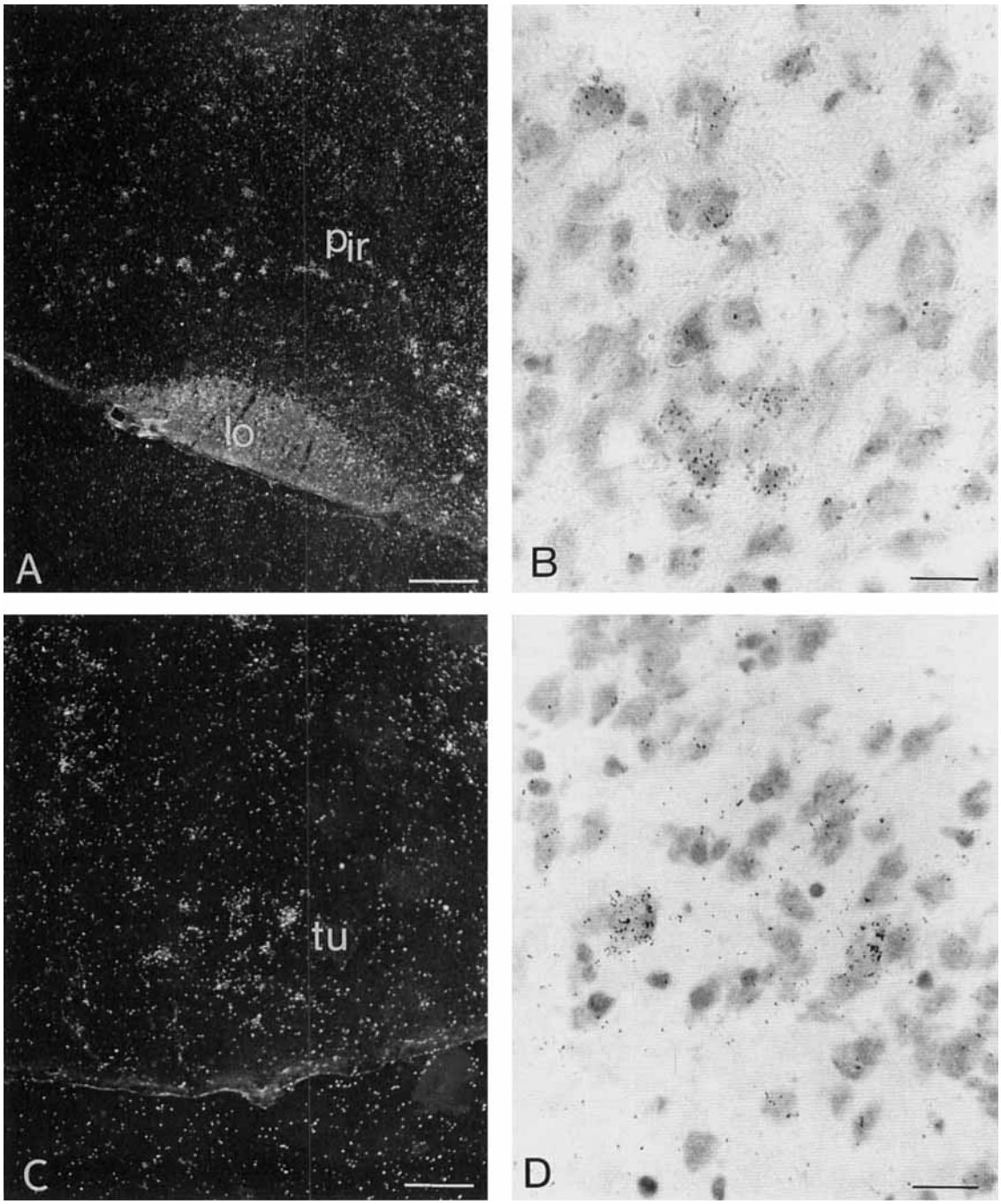

Fig. 3. Detection of ChAT mRNA expressing neurons in rat piriform cortex and olfactory tubercule by in situ hybridization. A rat ChAT antisense oligonucleotide was labeled with ^{35}S and hybridized to 14 μm coronal sections of rat brain piriform cortex **(A,B)** and olfactory tubercule **(C,D).** B and D are high magnification bright-field micrographs of individual cells from the sections showed in panels A and C, respectively. lo, lateral olfactory tract; pir, piriform cortex; tu, olfactory tubercule. Scale bars: A = 200 μm; B and D = 20 μm; C = 60 μm.

could also be seen in the vertical limb of the diagonal band of Broca and, caudally, in the magnocellular preoptic area, the horizontal limb of the diagonal band, and the substantia innominata. In all these regions, cholinergic neurons have been previously demonstrated by immunohistochemical staining with ChAT antibodies (Fibiger, 1982; Mesulam et al., 1983). RNA blot analysis also showed ChAT mRNA in the striatum and, in

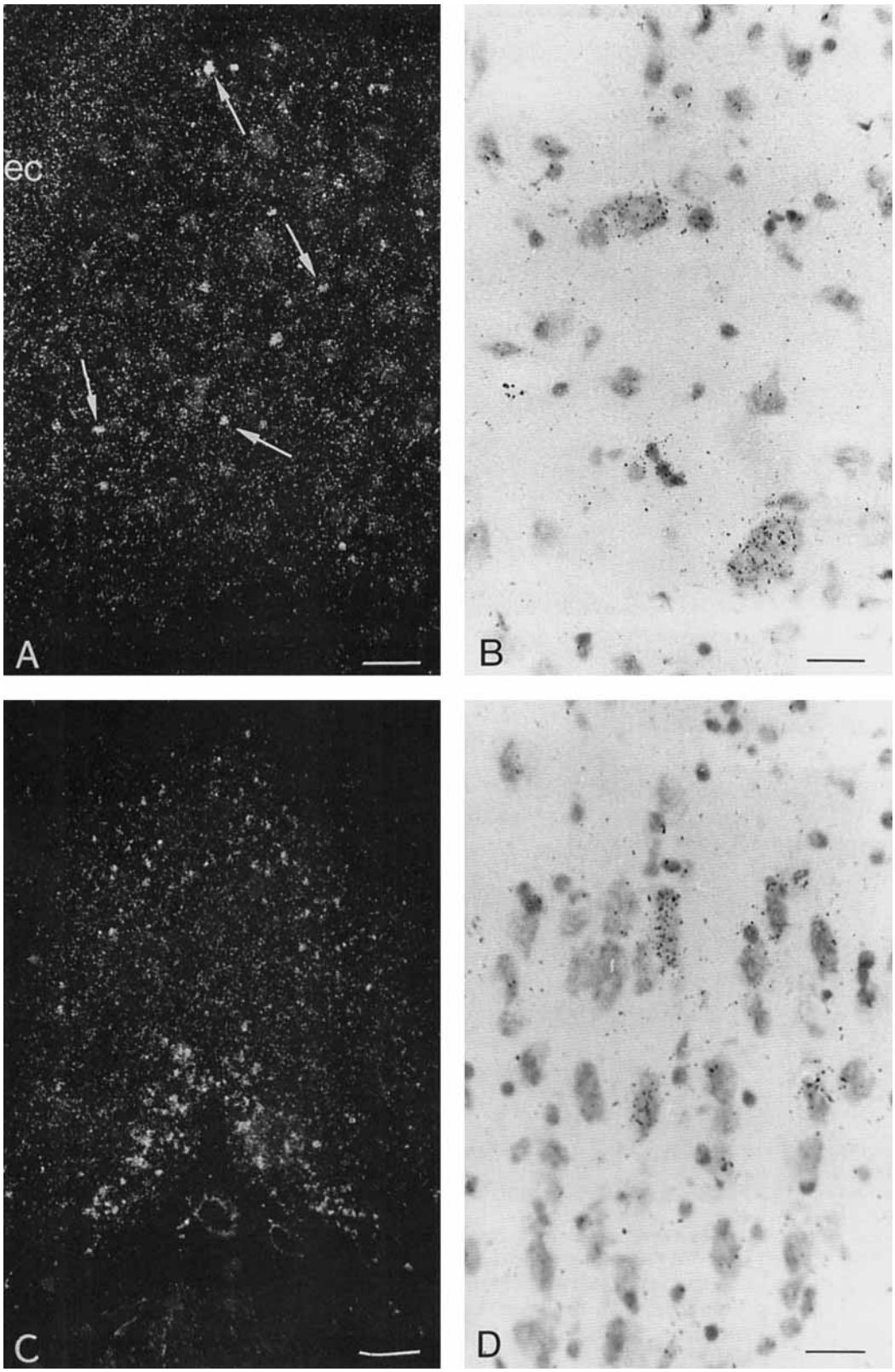

Fig. 4. Detection of ChAT mRNA-expressing neurons in rat striatum, septum, and diagonal band of Broca by in situ hybridization. A rat ChAT antisense oligonucleotide was labeled with ^{35}S and hybridized to 14 μm coronal sections of rat striatum **(A,B)** and medial septum and diagonal band **(C,D).** B and D are high magnification bright-field micrographs of individual cells from the sections showed in panels A and C, respectively. Arrows in A indicate some of the labeled cells. ec, external capsule. Scale bars: A and C = 200 μm; B and D = 20 μm.

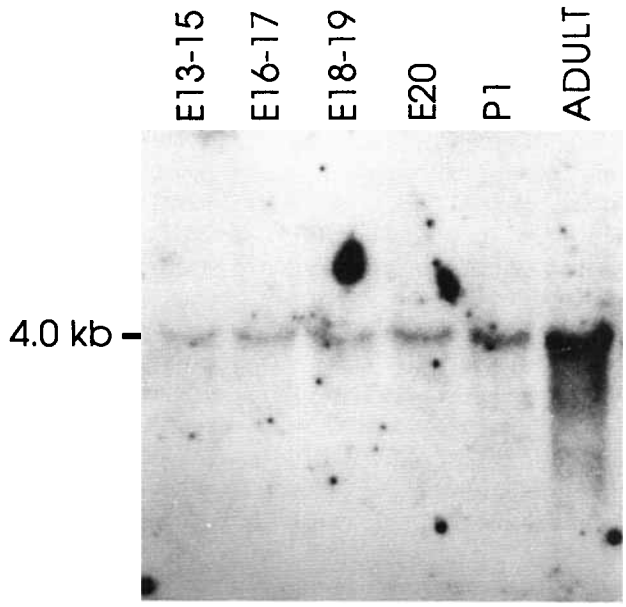

Fig. 5. Developmental expression of ChAT mRNA in rat spinal cord. 40 μg of total RNA from rat spinal cord of the indicated ages was electrophoresed in a 1% denaturing agarose gel, transferred to a nitrocellulose filter, and hybridized to a nick-translated 1.93 kb rat ChAT cDNA fragment. The filter was washed at high stringency and exposed to Kodak XAR-5 films at −70°C.

agreement with this, in situ hybridization revealed intensely labeled, scattered neurons in striatum with a distribution very similar to the pattern of ChAT immunoreactivity in this region (Armstrong et al., 1983). Thus, overall a strong correlation exists between the distribution of ChAT mRNA-expressing cells in the brain and previously described distribution of ChAT immunoreactivity, verifying the specificity of the results obtained using immunohistochemical detection of ChAT. However, RNA blot analysis also revealed low levels of ChAT mRNA in thalamus, although no cholinergic cell bodies have been described in this region. The strong labeling detected by in situ hybridization in the most caudal extensions of the substantia innominata, at the level of the thalamic nuclei, suggests that the thalamus sample contained a portion of the most caudal parts of the basal nuclei, which would explain the detection of ChAT mRNA in this region. The low levels of ChAT mRNA detected in the olfactory bulb were probably due to ChAT mRNA expression in the olfactory tubercule (Fibiger, 1982; Armstrong et al., 1983), which was included in the olfactory bulb sample used to prepare RNA for northern blot analysis. This possibility was confirmed by in situ hybridization, which revealed specifically labeled neurons in the olfactory tubercule region. Levels of

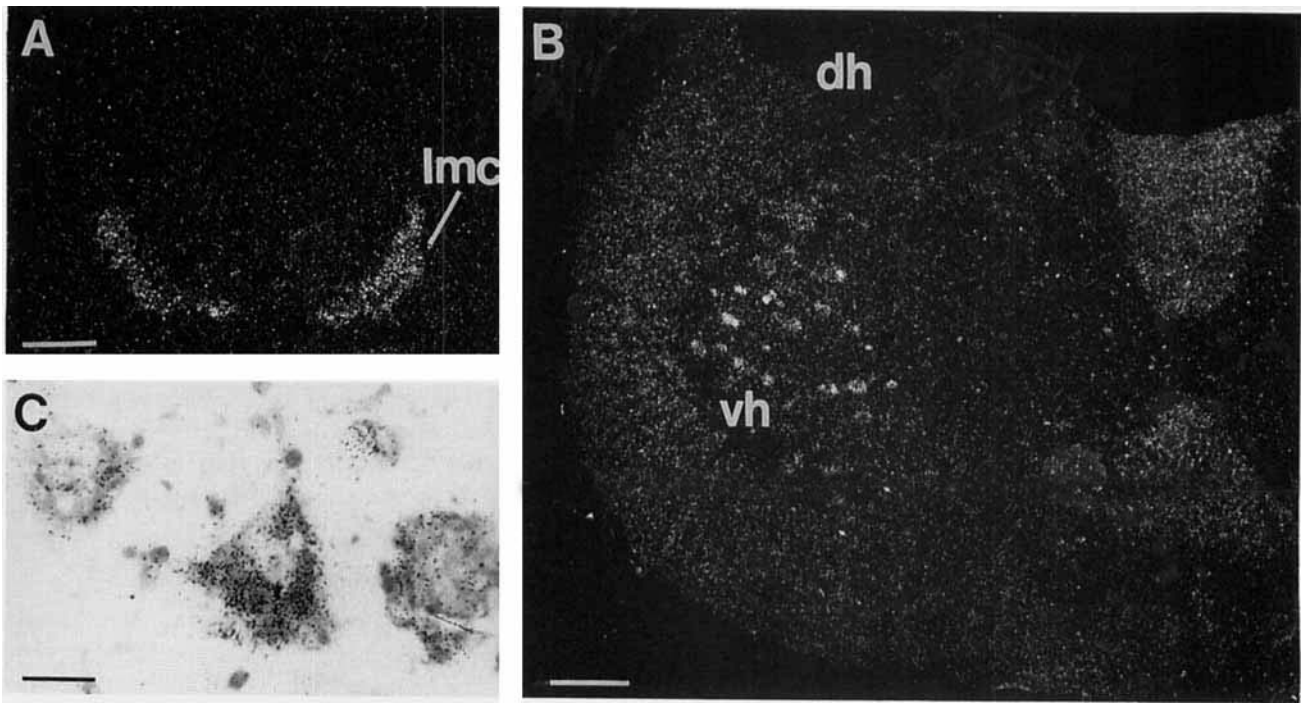

Fig. 6. Detection of ChAT mRNA expressing neurons in embryonic and adult rat spinal cord by in situ hybridization. A rat ChAT antisense oligonucleotide probe was labeled with ^{35}S and hybridized to 14 μm transverse sections of rat E15 **(A)** or adult **(B)** spinal cord at the level of the lumbar enlargement. C is a high magnification bright-field micrograph of individual motoneurons from the section showed in panel B. Note the presence of both intensely and weakly labeled motoneurons. dh, dorsal horn; vh, ventral horn; lmc, lateral motor column. Scale bars: A and B = 200 μm; C = 20 μm.

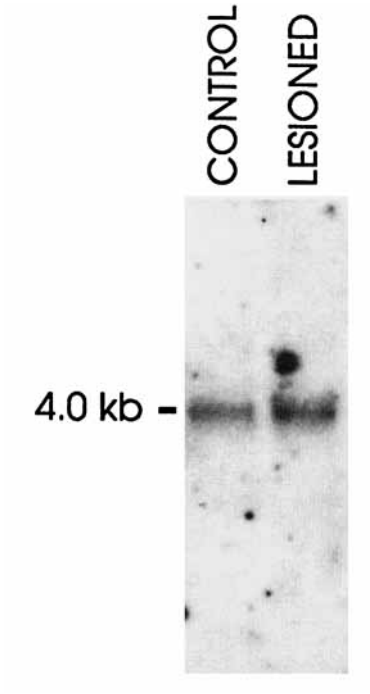

Fig. 7. Expression of ChAT mRNA in adult rat spinal cord after a bilateral crush lesion of the sciatic nerve. RNA was prepared from the spinal cord of either nonlesioned control adult rats (control) or adult rats seven days after a bilateral compression of the sciatic nerve (lesioned). 40 μg of total RNA was electrophoresed in a 1% denaturing agarose gel, transferred to a nitrocellulose filter, and hybridized to a nick-translated 1.93 kb rat ChAT cDNA fragment. The filter was washed at high stringency and exposed to Kodak XAR-5 films at −70°C.

ChAT mRNA in hippocampus and cerebral cortex were below detection of the northern blot technique. However, ChAT mRNA expressing cells were detected along the piriform cortex by in situ hybridization, in agreement with previous immunohistochemical studies performed on the adult (Hellendall et al., 1986) and embryonic (Schambra et al., 1989) rodent forebrain. The failure to detect ChAT mRNA in cerebral cortex by northern blot analysis could be due to low levels of ChAT mRNA or low density of cholinergic cell bodies within this region.

In the developing brain, ChAT mRNA expression showed a biphasic increase with a first peak around the second postnatal week, a decrease by the third week and a second increase between the third and fifth postnatal week. This discontinuous pattern indicates that the emergence of the cholinergic phenotype occurs at different times in different brain regions, as has recently been reported for septum and striatum (Mobley et al., 1989; Phelps et al., 1989).

ChAT mRNA expressing cells were detected by in situ hybridization in transverse sections from the lumbar enlargement of embryonic and adult rat spinal cord. It could be shown that ChAT mRNA labeling was restricted to the ventral horn of the spinal cord as early as E15. Both strongly and weakly labeled motoneurons were seen in the adult spinal cord, indicating that a subpopulation of adult spinal cord motoneurons express varying levels of ChAT mRNA. In the embryonic spinal cord, ChAT mRNA was already detected at E13, when the motoneurons undergo naturally occurring cell death (Flanagan, 1969), and the levels remained the same throughout embryonic development. A significant increase in ChAT mRNA was seen at postnatal day 1, at which time most of the synapses are already formed. Expression of ChAT mRNA in the spinal cord increased from P1 to adulthood, and the adult spinal cord contained significantly higher levels of ChAT mRNA than the embryonic spinal cord. The profile of ChAT mRNA expression during spinal cord development is inversely correlated to the expression of nerve growth factor (NGF) receptor mRNA, which is highly expressed at early embryonic stages, decreases slowly until E18, and drops again, almost below detectable levels, between P1 and adulthood (Ernfors et al., 1989b). However, the levels of NGF receptor mRNA in the adult spinal cord are increased approximately ten-fold seven days after a crush lesion of the sciatic nerve (Ernfors et al., 1989b). A similar, but less pronounced, increase was seen for ChAT mRNA after the same lesion. This suggests that ChAT mRNA expression is upregulated during motoneuron regeneration although by what mechanism is obscure. NGF is known to increase ChAT activity in basal forebrain (Honegger and Lenoir, 1982; Hefti et al., 1984; Martínez et al., 1987) and in striatum (Mobley et al., 1985). By extension, it is possible that NGF, or the newly described NGF-related neurotrophic factor, hippocampus-derived neurotrophic factor/neurotrophin-3 (HDNF/NT-3), which is known to interact with the NGF receptor (Ernfors et al., 1990), may upregulate ChAT mRNA expression during regeneration of motoneurons. Validation of this hypothesis is possible using biologically active recombinant NGF and HDNF/NT-3 (Ernfors et al., 1989a, 1990; Ibáñez et al., 1990) together with the ChAT-specific probe described in this study.

ACKNOWLEDGMENTS

Technical assistance was given by Mrs. Mona Gullmert. Support was obtained from the Swedish Natural Science Research Council, The Swedish Board for Technical Development, Petrus och Augusta Hedlunds Stiftelse, Magnus Bergvalls Stiftelse, and funds from the Karolinska Institute. C.F.I. was supported by a fellowship from the Karolinska Institute and P.E. by the Swedish Medical Research Council.

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