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INDEPENDENT PATTERNS OF TRANSCRIPTION FOR THE PRODUCTS OF THE RAT CHOLINERGIC GENE LOCUS

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Abstract—The cholinergic phenotype requires the expression of the vesicular acetylcholine transporter and choline acetyltransferase proteins. Both genes are encoded at one chromosomal location called the cholinergic gene locus. We have identified by *in situ* hybridization histochemistry distinct patterns of transcription from the cholinergic gene locus in the subdivisions of the rat cholinergic nervous system.

The vesicular acetylcholine transporter and choline acetyltransferase are co-expressed in cholinergic neurons at all developmental stages in all major types of cholinergic neurons. The relative levels of vesicular acetylcholine transporter and choline acetyltransferase transcripts, however, change substantially during development in the CNS. They also differ dramatically in distinct subdivisions of the mature cholinergic nervous system, with vesicular acetylcholine transporter mRNA expressed at high levels relative to choline acetyltransferase mRNA in the peripheral nervous system, but at equivalent levels in the CNS.

Expression of the R-exon, the presumptive first non-coding exon common to both the vesicular acetylcholine transporter and choline acetyltransferase, was not detectable at any developmental stage in any of the cholinergic neuronal subtypes in the rat nervous system. Thus, in contrast to less complex metazoan organisms, production of the vesicular acetylcholine transporter and choline acetyltransferase via a common differentially spliced transcript does not seem to occur to a significant extent in the rat.

We suggest that separate transcriptional start sites within the cholinergic gene locus control vesicular acetylcholine transporter and choline acetyltransferase transcription, while additional elements are responsible for the specific transcriptional control of the entire locus in cholinergic versus non-cholinergic neurons. Independent transcription of the vesicular acetylcholine transporter and choline acetyltransferase genes provides a mechanism for regulating the relative expression of these two proteins to fine-tune acetylcholine quantal size in different types of cholinergic neurons, both centrally and peripherally. © 2001 IBRO. Published by Elsevier Science Ltd. All rights reserved.

Key words: acetylcholine, choline acetyltransferase, vesicular acetylcholine transporter, gene regulation.

The cholinergic gene locus (CGL) consists of the genes encoding the vesicular acetylcholine transporter (VAChT), responsible for the storage of acetylcholine in synaptic vesicles, and the biosynthetic enzyme for the synthesis of acetylcholine, choline acetyltransferase (ChAT) (Eiden, 1998). The coding region for VAChT lies within the first intron of the ChAT gene, an array conserved from nematode to man (Alfonso et al., 1994; Erickson et al., 1994; Kitamoto et al., 1998; Naciff et al., 1997). The conservation of VAChT and ChAT gene contiguity within the CGL is remarkable, given the considerable alteration in the locus, including loss of all introns from the VAChT gene, and addition of more than 2 kb of intervening sequence between the VAChT and ChAT coding exons during the approximately 600 million years of evolution separating nematode and mammal. Nevertheless, the transcriptional regulation of the CGL shows some important differences between worms and mammals, which may be relevant to the greater complexity of the mammalian cholinergic nervous system. The *Caenorhabditis elegans* CGL produces a primary transcript starting with a common non-coding exon for both VAChT and ChAT from which separate transcripts for VAChT and ChAT are generated by alternative splicing (Alfonso et al., 1994). The fly CGL appears to be similarly transcriptionally regulated, but with as yet unknown mechanisms for modulating VAChT/ChAT ratios throughout the organism (Kitamoto et al., 1998). Preliminary evidence suggests that the major transcriptional start sites for VAChT and ChAT are separate in

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Abbreviations: CGL, cholinergic gene locus; ChAT, choline acetyltransferase; c.p.m., counts per minute; d.p.m., disintegrations per minute; E, embryonic day; ISHH, *in situ* hybridization histochemistry; P, postnatal day; PBS, phosphate-buffered saline; PNS, peripheral nervous system; RT–PCR, reverse transcription–polymerase chain reaction; SDS, sodium dodecylsulfate; SSC, standard saline citrate; VAChT, vesicular acetylcholine transporter.

the mammalian CGL, unlike the fly and worm. Minor transcripts (defined by cDNA cloning and reverse transcription–polymerase chain reaction (RT–PCR) amplification) initiating at the R-exon, which, from its location, corresponds to the common exon in *C. elegans*, and the N-exon, an additional non-coding exon, for ChAT and at the R-exon for VAChT have been identified (Kengaku et al., 1993). However, recent *in situ* hybridization histochemical analysis (ISHH) (Hahm et al., 1997) and semi-quantitative analysis of whole rat brain tissue (Cervini et al., 1995; Kengaku et al., 1993; Misawa et al., 1992; Misawa et al., 1997) suggest that the major transcripts initiate at the M-exon for ChAT, and at the VAChT coding exon for VAChT.

The close relationship of the ChAT and VAChT genes and the existence of shared and unique transcriptional start sites for each gene have suggested that regulatory elements exist that either coordinate their concomitant expression or act independently. Copious evidence exists for a coordinate regulation of VAChT and ChAT transcription. Retinoic acid, leukemia inhibitory factor and nerve growth factor, stimuli known to enhance the cholinergic phenotype in cholinergic cells or to act as cholinergic differentiation factors, cause an up-regulation of both VAChT and ChAT mRNA in cultured sympathetic neurons (Berrard et al., 1995; Misawa et al., 1995) and in murine septal cell lines (Berse and Blusztajn, 1995; Oosawa et al., 1999). Recently, bone morphogenetic protein 9 has been identified as a potential differentiation factor for cholinergic neurons in the CNS because of its ability to directly induce the expression of VAChT and ChAT in septal cells in culture (Lopez-Coviella et al., 2000).

Discordant VAChT and ChAT transcript abundance has been observed during rat development and in adult peripheral autonomic ganglia. The semi-quantitative analysis of northern blots from total rat brain RNA preparations revealed that VAChT mRNA levels had already reached 60% of the maximal level at embryonic day (E) 21, with the most rapid increase between E19 and postnatal day (P) 3 (Holler et al., 1996). In contrast, ChAT mRNA levels were at only 20% of maximum at these stages, with the most pronounced changes seen during the second and third postnatal weeks. These data suggest that VAChT and ChAT are regulated differentially during development, although without indicating whether such regulation occurs in presumptive and mature cholinergic neurons, or is due to expression of VAChT transcripts in the developing brain in non-neuronal or neurons destined to be non-cholinergic. In the rat autonomic nervous system, an overexpression of VAChT mRNA compared to ChAT mRNA has been detected for the otic ganglion, using ISHH (Weihe et al., 1998). Here, we have addressed the question of VAChT and ChAT transcriptional regulation in the developing and mature cholinergic nervous system of the rat, using VAChT, ChAT and R-exon-specific probes and semi-quantitative ISHH.

EXPERIMENTAL PROCEDURES

Animals and tissue preparation

All animal procedures were performed according to National Institutes of Health (NIH; Bethesda, MD, USA) guidelines for the care and use of laboratory animals under an animal protocol approved by the NIMH-IRP Animal Care and Use Committee. All efforts were made to minimize animal suffering and to reduce the number of animals used.

Adult and timed pregnant Sprague–Dawley rats were obtained from Taconic Farms, (Germantown, NY, USA). Rat embryos were staged based on the presence of a copulation plug which was considered to be the first day of gestation (E0.5) and on measurements of crown rump lengths. The day of birth was considered to be P0.

For ISHH, pregnant females were killed by carbon dioxide inhalation on E11, 12, 14, 16, 18 and 20, the embryos carefully removed from the uterus, and immediately snap frozen in isopentane at −30°C. Whole embryos were stored at −70°C until further use. Three males and three females each of 2, 8, 14, 21 days and 4 months of age were killed by carbon dioxide inhalation, the brains, cervical spinal cords, submandibular glands, and duodenal parts of the small intestine quickly removed, and all tissues immediately frozen in isopentane for ISHH analysis.

Northern blot analysis

Sense and antisense RNAs from the rat VAChT and ChAT genes and the R-exon were generated by *in vitro* transcription from plasmids prVAChT-ORF, pChAT270 and pXSH987 (Hahm et al., 1997), using a T7/SP6 Riboprobe kit (Promega, Madison, WI, USA). The plasmid prVAChT-ORF was generated by subcloning a 275-bp *Nhe*I fragment from the rat VAChT open reading frame into pGEM-7. Serial 10-fold dilutions of sense RNA were electrophoresed on 1% agarose/formaldehyde gels, blotted onto Nytran membranes (Schleicher and Schuell, Keene, NH, USA), and immobilized by UV crosslinking. Antisense cRNA probes were labeled using [α-^{32}P]UTP. The membranes were prehybridized for 2 h at 60°C with a solution containing 50% formamide, 0.6 M NaCl, 10 mM Tris–HCl (pH 7.4), 1×Denhardt's, 100 μg/ml sheared salmon sperm DNA, 0.01% (w/v) *Escherichia coli* tRNA, and 0.1% sodium dodecylsulfate (SDS). Hybridization was carried out at 60°C overnight, with a probe concentration of 1×10^6 c.p.m./ml. Blots were washed twice for 20 min at 60°C in 2×standard saline citrate (SSC)/0.1% SDS, twice for 20 min at 60°C in 0.2×SSC/0.1% SDS, followed by a brief wash in 0.2×SSC, and exposed to autoradiography film for 20 h. Hybridization signals were documented with a digital camera and NIH Image computer software.

ISHH

Antisense riboprobes for ISHH analysis were generated from plasmids prVAChT-ORF, pChAT270 and pXSH987 (see above), labeled with [α-^{35}S]UTP to appropriate specific activity and reduced to uniform size by alkaline hydrolysis (Angerer et al., 1987).

In situ hybridization experiments were performed on 20-μm cryostat sections as previously described (Schäfer and Day, 1995; Schütz et al., 2000). In brief, sections were air-dried for 15 min, fixed in freshly prepared 4% (w/v) paraformaldehyde in phosphate-buffered saline (PBS) for 60 min at room temperature, and washed 3×10 min in 10 mM PBS. After a brief wash in distilled water, sections were acetylated with triethanolamine/acetic anhydride for 10 min at room temperature, followed by a 5-min wash in distilled water. The sections were then dehydrated through a graded series of ethanol (50%, 80%, 96%, 100%; each

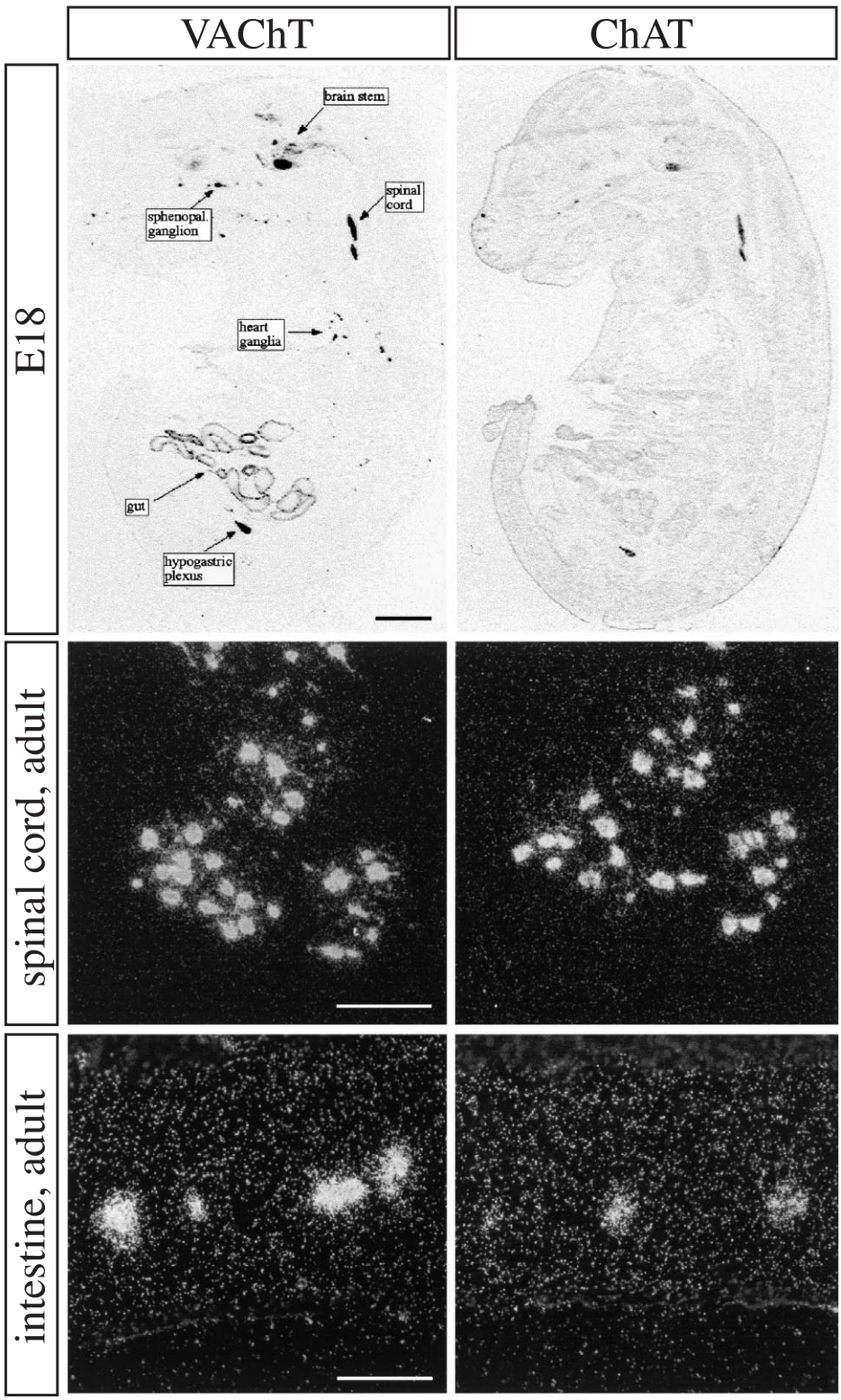

Fig. 1. Comparison of VAChT and ChAT mRNA expression in cholinergic cell groups of the embryonic and adult rat. ISHH signal intensities appear stronger for VAChT mRNA than for ChAT mRNA throughout the CNS and PNS on two adjacent parasagital sections through an E18 embryo (upper panel). In the CNS of the adult rat, shown here for the motor neurons in the ventral part of the spinal cord, VAChT and ChAT mRNA levels are similar (middle panel). In the PNS of the adult rat, levels for VAChT mRNA are still higher compared to ChAT mRNA levels, shown for the cholinergic neurons of the myenteric plexus of the intestine (lower panel). Scale bars = 2 mm (upper panel), 100 μm (middle and lower panel).

2 min) and incubated for 2×5 min in chloroform at room temperature. After a 2-min incubation in 100% and then 96% ethanol, sections were air-dried for 30 min and directly used for hybridization, or stored at −20°C until use. For hybridization, sections were covered with 30–40 µl of hybridization solution, containing 50% formamide, 0.6 M NaCl, 10 mM Tris (pH 7.4), 1 mM disodium ethylenediaminetetra-acetate, 1×Denhardt's, 10% dextran sulfate, 100 µg/ml sheared salmon sperm DNA, 0.05% (w/v) *E. coli* MRE600 tRNA, 20 mM dithiothreitol, 50 000 d.p.m./µl riboprobe, and coverslipped. Hybridization was carried out overnight at 60°C in a humid chamber. After hybridization, coverslips were removed in 2×SSC at room temperature and the sections washed in the following order: 20 min in 1×SSC, 45 min at 37°C in RNase solution containing 20 µg/ml RNase A and 1 U/ml RNase T1, 20 min in 1×SSC, 20 min in 0.5×SSC, 20 min in 0.2×SSC, 60 min in 0.2×SSC at 60°C, 10 min in 0.2×SSC at room temperature, and 10 min in distilled water. Finally, sections were dehydrated in 50% and 70% ethanol and air-dried at room temperature. For visualization of hybridization signals, all sections were first exposed to Amersham β-Max autoradiography film for 1–3 days to estimate further exposure times, then coated with Kodak NTB2 emulsion, exposed for exactly 14 days at 4°C and developed. Sections were counterstained with hematoxylin/eosin and hybridization signals documented on Kodak Ectachrome II color slide films using a Nikon Labophot 2 microscope and the slides digitized with a Polaroid slide scanner.

RESULTS

Comparative in situ hybridization for VAChT and ChAT mRNA during rat development and in adulthood

We initially determined the relative expression of VAChT and ChAT transcripts during rat development and in adulthood by ISHH on frozen sections. Three examples that summarize our major findings are shown in Fig. 1. Autoradiograms from two adjacent parasagital sections through an E18 rat embryo show an overexpression of VAChT mRNA relative to ChAT mRNA both centrally and peripherally (upper panel), when applying riboprobes with similar sensitivity (see also Fig. 2). Centrally, putative cholinergic neurons in the brainstem and spinal cord strongly expressed VAChT mRNA. In the periphery, the sphenopalatine ganglion, autonomic ganglia in the heart, the hypogastric plexus, and the gut intrinsic nervous system all expressed high levels of VAChT mRNA. All cholinergic cell groups expressed ChAT mRNA as well, albeit at much lower levels.

In the adult rat, VAChT and ChAT mRNA levels had

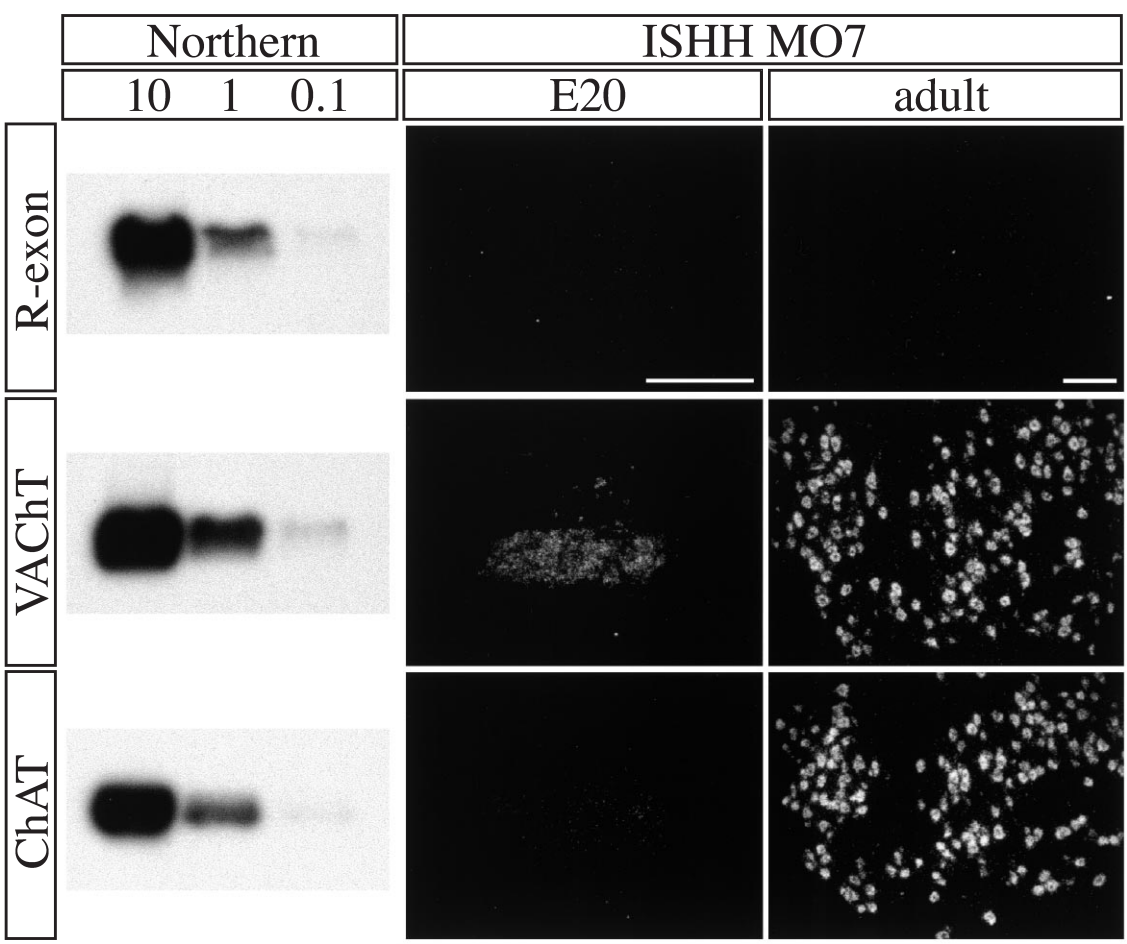

Fig. 2. The R-exon is not expressed in cholinergic neurons during rat development. The sensitivities of the R-exon, VAChT and ChAT riboprobes were determined by northern blot analysis. Equal amounts of 10-fold dilutions (10, 1, and 0.1 ng) of *in vitro* transcribed R-exon, VAChT, and ChAT sense RNA were electrophoresed, blotted onto nylon membranes and hybridized with the complementary antisense riboprobes under conditions similar to ISHH experiments. Hybridization signals appeared equally strong, indicating that the three riboprobes are similar sensitive. When analyzed by ISHH, R-exon expression was absent from embryonic (E20) and adult cholinergic neurons, exemplified for the motor nucleus of the facial nerve (MO7). Embryonic differences in mRNA abundance for VAChT and ChAT had equalized in the adult. Scale bars = 100 µm.

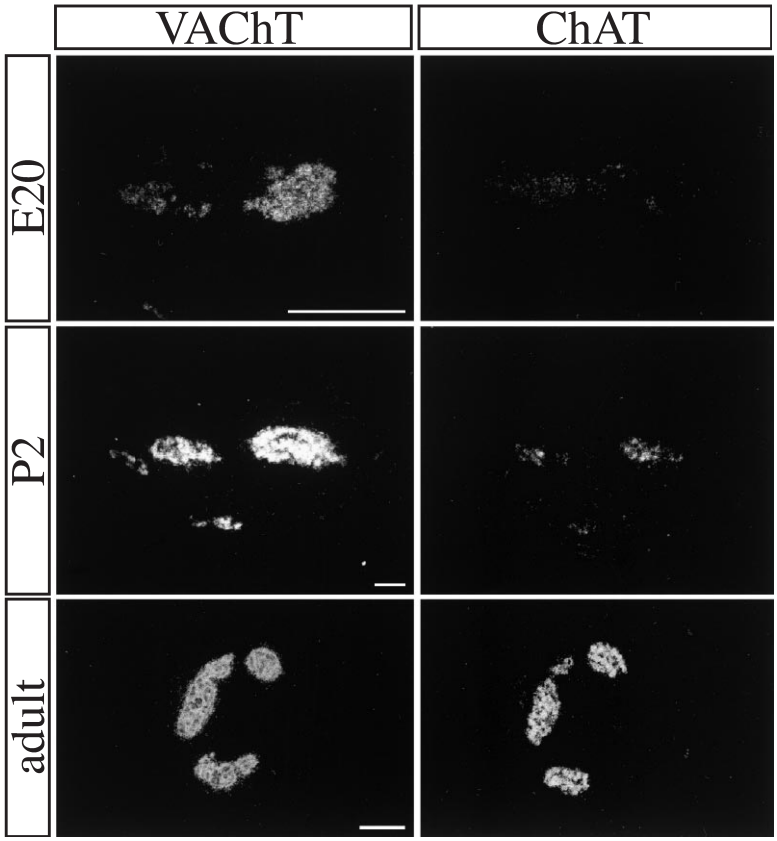

Fig. 3. ISHH of VAChT and ChAT mRNA expression in the submandibular ganglion throughout development. At E20, mRNA levels for VAChT are high compared to those for ChAT mRNA. This overexpression of VAChT relative to ChAT is maintained throughout postnatal development (P2) into the adult. Near-saturation of signals for VAChT and ChAT mRNA obscures this to some degree in adult tissues. Scale bars = 100 μm.

equalized in central cholinergic cell groups, exemplified on transverse sections through the ventral part of the spinal cord (middle panel). The motor neurons expressed similar amounts of VAChT and ChAT message. Expression levels in peripheral cholinergic ganglia, shown here for the myenteric plexus of the intestine (lower panel), resembled those seen during development, with higher abundance of VAChT messages relative to those for ChAT.

Expression of the R-exon is negligible during development and in adulthood

In our next analysis we determined if changes in transcript abundancies of the VAChT and ChAT genes resulted from differential expression that initiated at the R-exon, the presumptive first non-coding exon common to both genes. We first established that differences in ISHH signal intensities of the R-exon, VAChT and ChAT riboprobes used in our studies were not an artifact of differential hybridization. Labeled antisense riboprobes were hybridized directly to known amounts of synthetic sense RNA transcripts of the target sequences for each probe. All three riboprobes showed similar sensitivities of hybridization (Fig. 2, left panel), under conditions equivalent to those of the ISHH. A further comparison of R-exon transcript abundancies with those for VAChT and ChAT by ISHH revealed that expression of the R-exon is negligible during development and in adulthood: R-exon transcripts were absent from the facial motor nucleus both at E20 and in adulthood, whereas the messages for VAChT and ChAT were clearly detectable (Fig. 2). A lack of R-exon transcript detection was also observed in all other cholinergic cell groups at all developmental stages analyzed. These results also indicated that the differences in signal intensities observed between the VAChT and ChAT riboprobes during embryogenesis and in the adult peripheral nervous system (PNS) represent true differences in mRNA abundance.

Individual cholinergic nuclei display different ratios of VAChT to ChAT mRNA abundance

In order to screen for cell-specific differences in VAChT to ChAT mRNA ratios we studied the developmental time-course of VAChT and ChAT expression throughout the subdivisions of the cholinergic nervous system in detail on a cellular level.

In the PNS, cholinergic neurons participate in the formation of sympathetic, parasympathetic and gut intrinsic ganglia. The cranial parasympathetic ganglia are entirely cholinergically coded. As shown for the submandibular ganglion at E20, VAChT mRNA levels were already

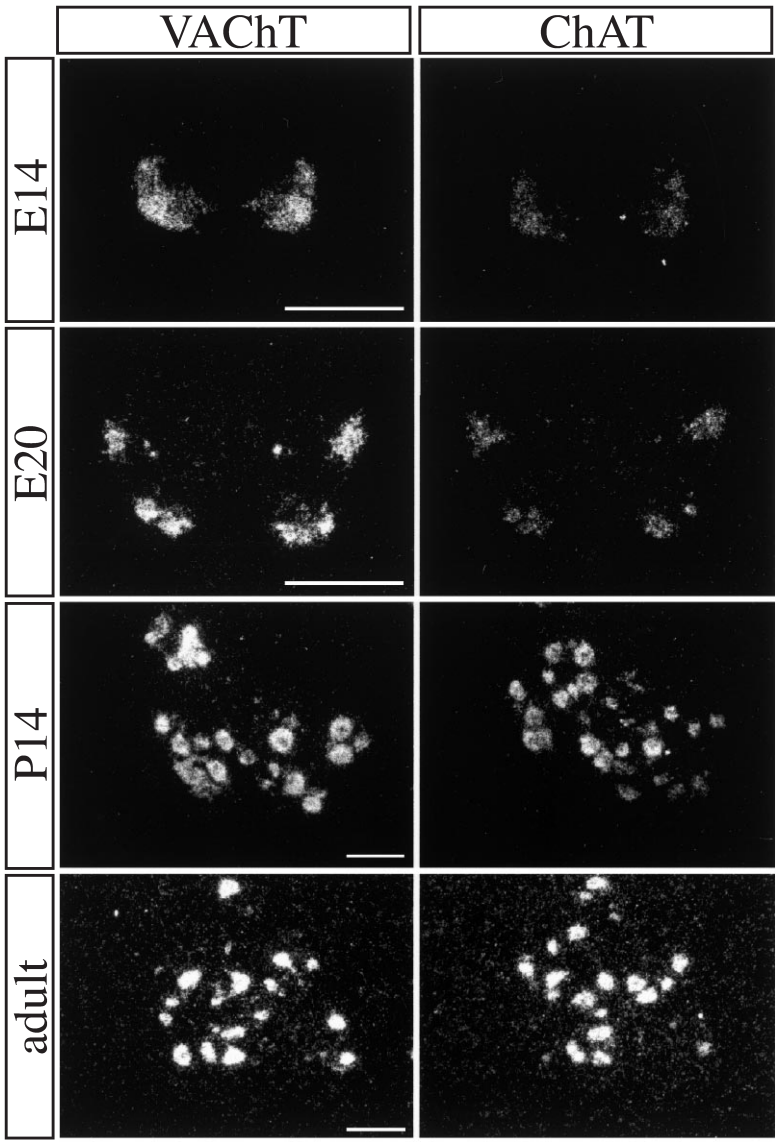

Fig. 4. ISHH for VAChT and ChAT mRNA expression in the embryonic and adult spinal cord. Transcript levels for VAChT were high compared to ChAT mRNA throughout prenatal development (E14 and E20). ChAT mRNA levels increased significantly during the first 2 postnatal weeks. From P14 on both messages could be detected with similar intensities. Scale bars = 100 μm.

high compared to those for ChAT at an early stage of ganglion formation (Fig. 3, upper panel). This pattern of VAChT mRNA overexpression relative to that for ChAT mRNA was maintained through early postnatal stages (P2, middle panel) and into adulthood (lower panel; the near-saturation of signals for VAChT and ChAT mRNA obscures this to some degree in adult tissues).

In the cholinergically coded motor neurons of the ventral spinal cord, the onset of VAChT and ChAT mRNA expression was accompanied by an overexpression of VAChT relative to ChAT (Fig. 4): transcript levels for VAChT started off relatively high (shown for E14) with the most pronounced increase prenatally between E14 and E20. ChAT mRNA levels instead remained low throughout all embryonic stages but increased significantly during the first 2 postnatal weeks. From P14 on both messages could be detected with similar intensities. The same temporal patterns of expression for the VAChT and ChAT mRNAs were detected in the cholinergic motor nuclei and projection neurons in the brainstem and in the cholinergic projection neurons of the ventral forebrain (data not shown).

Comparing and rating relative levels of gene expression from a single ISHH exposure provides qualitative but not quantitative information. For a quantitative assessment of the ratios of VAChT to ChAT mRNA expression, hybridized sections from ISHH experiments were exposed on X-ray films across a broad range of exposure times. The shortest times needed to detect a hybridization signal for VAChT and ChAT for a given structure and developmental stage were compared. We selected the motor neurons in the spinal cord, the motor nucleus of the trigeminal nerve, and the medial

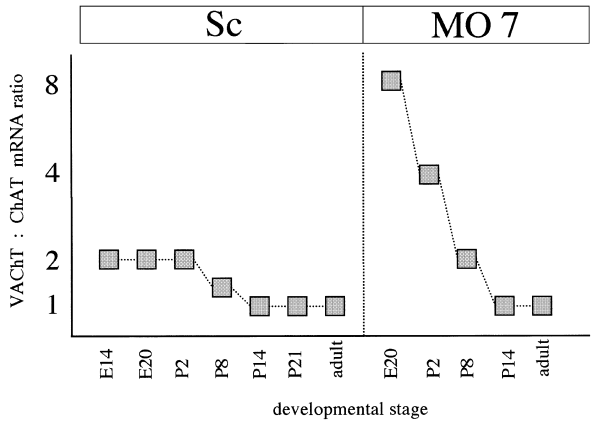

Fig. 5. Semi-quantitative determination of VAChT to ChAT mRNA ratios during pre- and postnatal development in cholinergic nuclei of the CNS. VAChT to ChAT mRNA ratios were assessed by exposing ISHH slides to X-ray films across a broad range of times. The shortest exposure time needed to detect a VAChT or ChAT mRNA signal for a given structure and developmental stage was used for comparison. A ratio of 1 means equal expression levels. Two-fold higher VAChT mRNA levels in the spinal cord (Sc) motor neurons during embryonic life gradually diminished to become equal at P14. An eight-fold VAChT overexpression relative to ChAT in the motor nucleus of the facial nerve (MO7) at E20 gradually equalized throughout the first 2 postnatal weeks.

habenular nucleus (see below) for this analysis because the density of cholinergic neurons in these structures is similarly high and therefore well suited for comparison. For the spinal cord, a ratio of 2:1 in favor of VAChT mRNA levels was assessed during embryonic and early postnatal stages (Fig. 5). VAChT to ChAT mRNA differences gradually diminished thereafter to become equal at around P14. The initial overexpression of VAChT compared to ChAT mRNA was far more pronounced in the facial motor nucleus. A VAChT to ChAT ratio of 8:1, seen at E20, gradually equalized during the first 2 postnatal weeks.

In the medial habenular nucleus of the epithalamus, VAChT and ChAT expression was first detectable at E18 (data not shown). At P2, similar VAChT and ChAT mRNA levels could be discerned (Fig. 6). Signal intensities increased minimal for VAChT but more pronounced for ChAT thereafter up to a peak at P21 and then dropped again slightly into adulthood. ChAT message abundancies were always higher than those for VAChT, starting from P8 onwards. On the semi-quantitative level, the VAChT to ChAT mRNA ratio changed from 1:1 (P2 through P8), to 1:2 (P14 through P21), to end up with 1:4 in favor of ChAT mRNA in the adult (Fig. 7).

In summary, by performing semi-quantitative ISHH, we discerned three categories of VAChT and ChAT mRNA expression patterns in rat cholinergic neurons. Throughout the PNS, VAChT mRNA is expressed at high levels relative to ChAT mRNA. This pattern is established early in development and persists into adulthood. In spinal cord, basal forebrain, and brainstem cholinergic nuclei, VAChT mRNA is initially expressed at high levels relative to ChAT mRNA, as in the PNS, but this pattern changes developmentally, so that in the adult rat these cholinergic cell groups express similar relative levels of VAChT and ChAT mRNA. Finally, the medial habenular nucleus is exceptional compared to other cholinergic nuclei of the brain in that the relative abundance of VAChT and ChAT mRNAs is similar in early development, and ChAT mRNA levels become relatively higher during development and into adulthood.

DISCUSSION

The CGL encodes the two proteins that functionally define the cholinergic neuronal phenotype, within a single regulatory domain. In mammals, four transcriptional start sites (R1, R2, N, and M) requiring at least three separate promotors exist for ChAT, and at least six transcriptional start sites (R1, R2, V1a, V1b, V2a, and V2b) probably requiring three separate promotors exist for VAChT (Bejanin et al., 1994; Cervini et al., 1995; Kengaku et al., 1993; Misawa et al., 1992; Misawa et al., 1997; Oda et al., 1992). The use of the N and M start sites for ChAT, and the V1 and V2 sites for VAChT is predicted to generate separate primary transcripts for each of the two gene products. The use of the R-type start sites, however, is predicted to generate a primary transcript spanning the entire locus, which would then be differentially processed to R-exon containing VAChT and ChAT messages by alternative splicing, reflecting the situation seen in simpler organisms. These alternative scenarios raise the question of how frequently various VAChT and ChAT transcriptional start sites are used, and how tightly VAChT and ChAT transcription are coupled *in vivo*.

In our present ISHH study we showed on a cellular level that the R-exon is not expressed significantly in rat cholinergic neurons during development and in the adult. This argues against the possibility of the R-exon being the major/common transcriptional start site for the expression of both VAChT and ChAT from the mammalian CGL. The previously reported cloning of a cDNA from rat spinal cord where ChAT coding sequences were fused directly to the R-exon (Misawa et al., 1992), the detection of R-exon containing VAChT transcripts by RT–PCR (Bejanin et al., 1994), and the detection of weak staining in brainstem cholinergic neurons with an R-exon riboprobe (Hahm et al., 1997) have not been assessed quantitatively. Comparing our current results with these data it can be assumed that initiation of transcription from a weak R-exon promotor exists in the mammalian CGL, but that these transcripts contribute only marginally to the expression of both genes.

If not transcribed as a unit from a common first exon, how could expression of the VAChT and ChAT genes be regulated? Co-expression of VAChT and ChAT messages in all cholinergic neurons at all developmental stages suggests that expression from the CGL is tightly coupled, at least to the extent that neither gene is ever expressed in neuronal cells without concomitant expression of the other. This is supported by findings that various stimuli simultaneously increase the expression

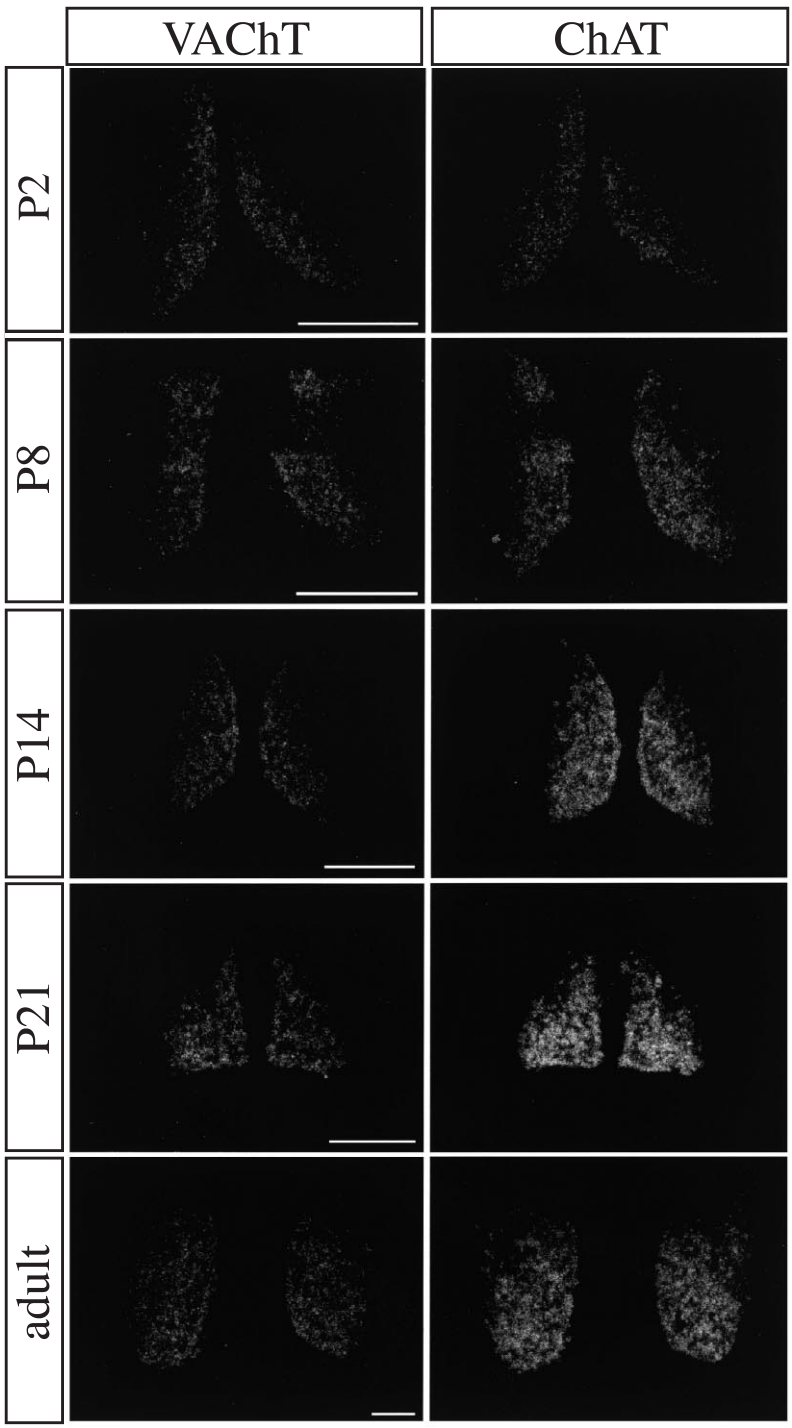

Fig. 6. ISHH for VAChT and ChAT mRNA expression in the medial habenular nucleus throughout development. At P2, VAChT and ChAT mRNA were expressed equally strong. Signal intensities increased minimal for VAChT but more pronounced for ChAT thereafter to a peak at P21 and then dropped again slightly into adulthood. ChAT message abundancies were always higher than those for VAChT, starting from P8 on. Scale bars = 100 μm.

of both VAChT and ChAT in model systems for cholinergic differentiation and reaction to axonal injury (Berrard et al., 1995; Lopez-Coviella et al., 2000; Misawa et al., 1995).

In our study, discordant VAChT and ChAT transcript abundance during development and in the PNS of the adult rat occurred within cholinergic neurons. This was not due to ectopic expression of VAChT in non-neuronal cells, or neurons not destined to become cholinergic, but to differential expression of VAChT and ChAT mRNAs in the same, presumptive cholinergic neurons. Our data specify on a cellular level previously reported differences in mRNA levels in the brain assessed by northern blot analysis (Holler et al., 1996), and extend these observa-

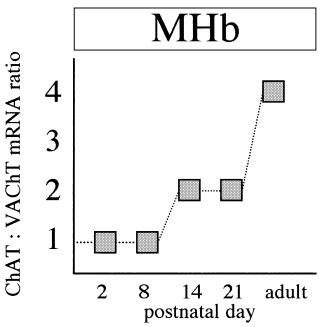

Fig. 7. Semi-quantitative determination of ChAT to VAChT mRNA ratios during postnatal development in the medial habenular nucleus (MHb). Initially equal expression levels changed to a four-fold overexpression of ChAT mRNA relative to VAChT mRNA in the adult.

tions to the developing and adult peripheral cholinergic nervous system. A unique pattern of regulation of VAChT and ChAT mRNA expression in the medial habenular nucleus has also been uncovered in the present analysis.

Discordancies in mRNA levels are probably due to unequally strong proximal promotors, and/or unequal responsivity of the individual promotors to induction by extrinsic signaling pathways during development. For example, in the murine septal cell line SN56, cAMP increased VAChT mRNA levels more efficiently than ChAT mRNA levels (Berse and Blusztajn, 1995). Whether the resulting differences in mRNA levels for VAChT and ChAT have an effect on quantal sizes of acetylcholine per synapse remains to be determined. Evidence for an additional regulation on the post-transcriptional level comes from studies by Holler et al. (1996). Although VAChT mRNA levels had already reached 60% of the maximal level at E21 in the rat, ChAT mRNA levels were at only 20% of maximum at these stages. The developmental timetable for VAChT protein abundance paralleled that of ChAT activity, with protein levels in the brain reaching only one-half of adult levels at around 10 days postnatally. It has previously been noted that the concentration of acetylcholine estimated to be present in the cytoplasm of cholinergic motor neurons is similar ($\sim$1 mM) to the K_m for acetylcholine transport by VAChT (Varoqui and Erickson, 1996). In this situation, any change in the ratio of expressed VAChT to ChAT protein could be expected to change cholinergic quantal size (Song et al., 1997) and modulate the efficacy of cholinergic neurotransmission, in a given neuron. Thus, while expression of both VAChT and ChAT from the CGL is required for the cholinergic phenotype, a loose coupling of VAChT and ChAT expression in individual neurons *in vivo* could function as a mechanism to fine-tune cholinergic function.

The power of semi-quantitative ISHH in comparing levels of gene expression is limited by the potential for differential RNA sensitivity in the *in situ* hybridization protocol, and the intrinsic limits to comparisons based on differential exposure time, or signal intensity at the level of individual cells or nuclei. Comparison of hybridization efficiency for each probe by northern blot hybridization of similarly formalin-fixed mRNA, and the use of similarly sized riboprobes for the *in situ* analysis were employed to mitigate this concern. Nevertheless, fully quantitative measurement of VAChT and ChAT transcript abundance and the relative usage of non-coding exons in individual cholinergic nuclei would help to understand how transcription from the CGL is initiated under normal and pathophysiological situations in the brain and autonomic nervous system.

Furthermore, identification of *cis*-acting elements in the CGL and of the transactivating factors that bind to these elements should unravel the regulatory mechanisms that are responsible for the separate versus common activation of the VAChT and ChAT genes *in vivo*. Recent transgenic analyses have addressed this question on an individual gene level. Transcription of the lacZ gene from the first ChAT coding exon in central cholinergic neurons was achieved in transgenic mice from a 6.4-kb fragment of the mouse CGL that spanned from 633 bases upstream of the start of translation of VAChT through the start of translation of ChAT (Naciff and Dedman, 1999). A somatomotor neuron-specific expression of VAChT was achieved by introducing 8.7 kb of the human CGL into mice, pointing towards a mechanism of cell-specific regulation of the CGL (Schütz et al., 2000). It will be of special interest to determine if these regions are also involved in the regulation of cholinergic-specific expression of the other member of the CGL, or if they contain elements that are exclusive for VAChT or ChAT.

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