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Identification of a region from the human cholinergic gene locus that targets expression of the vesicular acetylcholine transporter to a subset of neurons in the medial habenular nucleus in transgenic mice

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Abstract

We use a transgenic mouse model system to elucidate the regulatory regions within the human cholinergic gene locus responsible for vesicular acetylcholine transporter gene expression *in vivo*. In this report we characterized two transgenes for their ability to confer cholinergic-specific expression of the encoded vesicular acetylcholine transporter. An 11.2 kb transgene (named hV11.2) that spanned from about 5 kb upstream of the start of vesicular acetylcholine transporter translation down to the first choline acetyltransferase coding exon gave expression in the somatomotor neurons and a subpopulation of cholinergic neurons in the medial habenular nucleus. The second transgene (named hV6.7), a 5-prime truncated version of hV11.2 that was devoid of 4.5 kb of gene-regulatory sequences completely lacked vesicular acetylcholine transporter expression *in vivo*. Our data indicate that vesicular acetylcholine transporter expression in somatomotor neurons and in the medial habenular nucleus is uniquely specified within the cholinergic gene locus, and separable from cholinergic expression elsewhere. The identification of these two subdivisions of the cholinergic nervous system suggests that other cholinergic neurons in the CNS and PNS are similarly regulated by additional discrete domains within the cholinergic gene locus.

Keywords: choline acetyltransferase, cholinergic gene locus, gene regulation, vesicular acetylcholine transporter

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Cholinergic neurons are characterized by the expression of choline acetyltransferase (ChAT), the biosynthetic enzyme for the synthesis of acetylcholine, and the vesicular acetylcholine transporter (VAChT), the protein needed for the uptake of acetylcholine into synaptic vesicles. In the genome, ChAT and VAChT are encoded within a single regulatory unit, called the cholinergic gene locus (CGL) (Eiden 1998). The coding region for VAChT resides upstream of the first coding exon of the ChAT gene, an arrangement that is conserved in the nematode (Alfonso *et al*. 1994), *Drosophila* (Kitamoto *et al*. 1998), and mammals (Erickson *et al*. 1994; Naciff *et al*. 1997). The striking anatomical co-localization of VAChT and ChAT in embryonic and adult neurons (Schäfer *et al*. 1997; Schäfer *et al*. 1998a,b; Schütz *et al*. 2001), the coordinated up-regulation of transcripts for both genes by various extracellular factors in cell culture experiments (Berrard *et al*. 1995; Berse and Blusztajn 1995; Misawa *et al*. 1995; Lopez-Coviella *et al*. 2000), and the co-suppression of ChAT and VAChT *in vitro* (Shimojo *et al*. 1998) and *in vivo* (Kaufer *et al*. 1998), suggests that the VAChT and ChAT genes may share regulatory sequences that

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Abbreviations used: CAT , chloramphenicol acetyltransferase; ChAT, choline acetyltransferase; CGL, cholinergic gene locus; Ct, threshold cycle; EGFP, enhanced green fluorescent protein; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; IHC, immunohistochemistry; IR, immunoreactivity; ISHH, in situ hybridization histochemistry; mRNA, messenger RNA; tRNA, transfer RNA; VAChT, vesicular acetylcholine transporter.

coordinate their expression in cholinergic neurons. In addition, VAChT and ChAT seem to be co-repressed in non-neuronal cells through a common neuron-restrictive silencer element (De Gois *et al*. 2000).

Little is known about regulatory regions within the CGL that are important for the expression of VAChT and ChAT *in vivo*. Two transgenic mouse studies have addressed the regulation of the ChAT gene. In the first study, reporter gene expression was observed in the ventral spinal cord and the brain of mice when 2 kb of the rat CGL, comprising the neuron-restrictive silencer element and sequences flanking it, were attached to a heterologous promoter driving the chloramphenicol acetyltransferase (CAT) gene (Lönnerberg *et al*. 1995). In another study, 6.1 kb from the mouse CGL that encompassed the VAChT exon were used to drive reporter gene expression from the first coding exon of ChAT (Naciff *et al*. 1999). Expression of β-galactosidase was detected in cholinergic neurons in the CNS, albeit at low levels. The removal of sequences from this construct that contained the VAChT open reading frame resulted in an increased expression of β-galactosidase within non-cholinergic neurons in the CNS. From these studies it was concluded that elements both upstream and downstream from the start of VAChT transcription control transcription of the ChAT gene.

We have focused on the regulation of VAChT expression from the human CGL. In our previous study we introduced 8.7 kb from the human CGL into mice, and monitored expression of transgenic VAChT using species-specific human VAChT PCR primer pairs, riboprobes for in situ hybridization histochemistry (ISHH) and antibodies for immunohistochemistry (IHC) (Schütz *et al*. 2000). Using VAChT as its own reporter, we were able to examine VAChT gene regulation without replacing potential *cis*-regulatory elements in the VAChT open reading frame with a reporter gene, thereby conserving the integrity of the locus. We found human VAChT expression restricted to the somatomotor subset of cholinergic neurons in the CNS of these mice, suggesting that regulation of the CGL in this subdivision of the cholinergic nervous system is unique and separable from cholinergic regulation elsewhere. Additional upstream or downstream sequences of the CGL therefore seemed to be needed for full cholinergic expression *in vivo*.

In the present study, we addressed this question by generating two additional human CGL transgenes and analysing expression of VAChT with the aforementioned tools. The addition of 2.5 kb of downstream sequence resulted in expression of human VAChT not only in somatomotor neurons, but also in a subset of cholinergic neurons of the medial habenular nucleus.

Our data provide additional and compelling evidence for the existence of multiple gene-regulatory elements within the CGL that control expression of VAChT in individual subdivisions of the cholinergic nervous system.

Experimental procedures

Generation of human cholinergic gene locus transgenic mice

A 2.5-kb genomic fragment from the human cholinergic gene locus that spanned from the *Xba*I site in exon 'M' to the ATG codon in the first ChAT encoding exon, was amplified from a human bacterial artificial chromosome (BAC) clone (Hahm *et al*. 1997) by PCR and cloned into pCRII (Invitrogen, Carlsbad, CA, USA). A modified lower PCR primer was used to introduce a *Hin*dIII site into the ATG codon. The DNA fragment was excised from the vector by *Xba*I digestion and cloned in proper orientation into the *Xba*I site of the previously described, VAChT encompassing 8.7-kb genomic fragment (Schütz *et al*. 2000). The resulting 11 137 bp clone (named hV11.2) was excised from the vector by *Hin*dIII digestion and separated from the plasmid DNA by a 10–40% sucrose gradient. Fractions containing only the insert DNA were pooled, the DNA concentrated and the sucrose removed. After ethanol precipitation, the fragment was resuspended in 10 mM Tris-HCl (pH 7.4), 0.1 M EDTA. From the hV11.2 clone a smaller, 6579-bp piece (named hV6.7) was excised by *Xho*I digestion and purified as described for the hV11.2 fragment. This truncated piece lacked 4558 bp of the 5′ end from hV11.2. Both constructs were injected into the male pronuclei of fertilized eggs of C57Bl/6J-129SJL chimeric mice and the eggs retransferred into foster mice. Transgene-harboring founder mice were identified by PCR (see below) and mated to C57Bl/6J mice. Three independent mouse lines were established with the hV6.7 transgene, and two lines with the hV11.2 transgene. From the F1 generation on, transgenic siblings were mated and occasionally backcrossed to C57Bl/6J mice to maintain heterozygosity. Heterozygous animals were used in all further expression analysis.

Standard PCR

For the identification of transgene-containing mice by PCR, genomic DNA was obtained from tail snips by a standard salt precipitation method. Three primer sets were used to test for the full integration of the transgenes. The first primer set, named 5′-dIII, generated a 479-bp amplicon that encompassed nucleotides 17–497 of the hV11.2 DNA and was used to detect the 5′-end of this construct. The second primer set, 5′-XhoI, amplified a 174-bp fragment located immediately downstream of the *Xho*I site (Fig. 1) and was used to detect the 5′ end of the hV6.7 construct. The third primer set, 3′-dIII, generated a 222-bp fragment immediately upstream of the 3′-end of both constructs. The PCR conditions were essentially as described earlier (Schütz *et al*. 2000). Briefly, in a DNA Thermal Cycler (Perkin-Elmer Cetus, Norwalk, CT, USA) 32 cycles of PCR were performed at 94°C for 1 min, 60°C for 45 s, and 72°C for 1 min.

Quantitative PCR

The mouse brainstem, ranging from the inferior colliculi to the border between medulla and spinal cord, was carefully dissected, divided into a left and right half, and stored in RNAlater (Ambion, Austin, TX, USA) at −20°C until further use. Total RNA was extracted from individual pieces using the RNeasy system (Qiagen, Valencia, CA, USA). One microgram of total RNA was then treated with Dnase I (Invitrogen) for 30 min at 37°C to eliminate contamination with genomic DNA. Reverse transcription was performed using the Omniscript RT system (Qiagen). A control

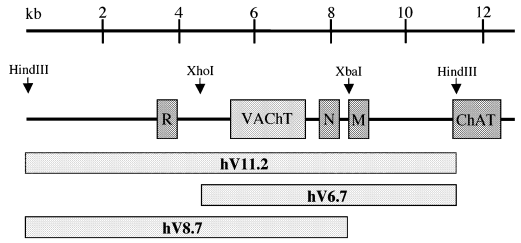


Fig. 1 The 5′-flanking region of the human CGL spans from about 4 kb upstream of the non-coding 'R' exon to the first ChAT encoding exon. Besides the 'R' exon this region contains two additional non-coding ChAT exons, named 'N' and 'M'. VAChT is encoded within a single exon that resides in the intron between 'R' and 'N'. The first construct to be used as a transgene in mice included about 11.2 kb upstream from the start of translation of ChAT and was named hV11.2. The second construct, named hV6.7, was a 5′-truncated version of hV11.2 that started 799 bp upstream of the start of VAChT translation. The location of restriction sites used for subcloning are indicated. For comparison, the location of our previously characterized transgene, hV8.7, is included.

reaction without reverse transcriptase enzyme (RT–) was included for each sample.

The quantitative PCR analysis was performed with the ABI Prism 7700 single reporter sequence detection system using QuantiTect Probe core reagents (Qiagen). Gene-specific PCR primers and probes were selected with the ABI Prism Primer Express™ 1.0 software (Applied Biosystems, Foster City, CA, USA). For the detection of the endogenous mouse VAChT (mVAChT) a forward primer TCCAAACACACTCGTATTCATTGAA, a reverse primer TGATCACCGGTACACCTCTGC, and a probe 6FAM-AGAGCCCAATGCCTCCCTGATCCA-TAMRA were used, in which 6FAM represents 6-carboxyfluorescein and TAMRA represents *N*,*N*,*N*′,*N*′-tetramethyl-6-carboxyrhodamine. This primer combination generated a 109-bp amplicon. For the detection of the transgenic human VAChT (hVAChT) a forward primer CCGTTCCATATCCCTTTCTCTCT, a reverse primer GTCCTCTTCACCTCCGCAAG, and a probe 6FAM-CGCTGGCCTCGGTGCTCCAA-TAMRA were used. This primer combination generated a 129-bp amplicon. Rodent glyceraldehyde 3-phosphate dehydrogenase (GAPDH) control reagents (Applied Biosystems) were used to normalize for input of total RNA. Single PCR reactions contained 1 × PCR Master Mix, each 0.3 μM forward and reverse primer, 0.15 μM probe, and 1 μL DNA in a volume of 50 μL. Amplification conditions were 2 min at 50°C, 10 min at 95°C, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. Fluorescent signals were normalized to a passive internal reference (ΔRn) and a threshold cycle (Ct) determined within the exponential phase of the reaction where ΔRn reached more then 10 times standard deviation of the baseline. Each PCR reaction was performed in triplicate and the mean Ct values (cycle number at which mean fluorescence exceeds threshold levels) calculated. Before the analysis of experimental samples, the hVAChT and mVAChT primer concentrations were calibrated to amplify equimolar amounts of plasmid-subcloned target sequences with equal efficiency. In addition, the linear range of detection of the primer sets was evaluated using multiple fourfold dilutions of target. The optimal forward/reverse primer concentrations resulting from these experiments were 300 nM/300 nM for both hVAChT and mVAChT.

Tissue harvesting for histological analysis

All animal procedures were performed according to the National Institutes of Health *Guidelines for Care and Use of Laboratory Animals* under an animal protocol approved by the NIMH-IRP Animal Care and Use Committee, which includes documentation of efforts to minimize the number, and potential distress, of experimental animals employed in the study.

From each of the five different mouse lines, six transgenic and four non-transgenic siblings were killed by carbon dioxide inhalation and the tissue to be examined was prepared in the following ways.

(i) For IHC, three transgenic and two non-transgenic mice were perfused transcardially with phosphate-buffered saline, followed by Bouin Hollande fixative, containing 4% (w/v) picric acid, 2.5% cupric acetate, 3.7% formaldehyde and 1% glacial acetic acid. Following perfusion, the brains, spinal cords and several peripheral organs were removed and post-fixed overnight. After extensive washing in 70% isopropanol, the tissues were dehydrated, cleared with xylene and embedded in paraffin. Sections were cut at 7 μm with a microtome and processed for antibody staining (see below).

(ii) For ISHH, the brains, spinal cords and peripheral organs were removed from three transgenic and two non-transgenic mice, immediately frozen in isopentane at −50°C and stored at −70°C. Sections were cut at 20 μm with a cryostat, mounted on silanized glass slides and stored at −70°C until use.

Human postmortem spinal cord specimens were obtained from a tissue bank of the Institute of Anatomy and Cell Biology, University of Marburg, Germany, with appropriate permission. Fixation was performed with buffered (pH 7.4) 10% (w/v) formalin. The tissue was cryopreserved by overnight incubation in 30% sucrose and frozen in isopentane at −50°C.

In situ hybridization histochemistry

The generation of a complementary RNA probe for the detection of mouse VAChT mRNA has been described earlier (Schütz *et al.* 2000). For the specific detection of human VAChT mRNA a 271-bp riboprobe was generated from a 341-bp *Sac*I/*Sac*II restriction fragment after linearization with *Bal*I. The riboprobe was homologous to the 3′-non-coding region of the human VAChT mRNA. The ISHH procedure was performed essentially as described in detail in our study on the human VAChT 8.7 transgene (Schütz *et al.* 2000). Briefly, as a pre-treatment, tissue sections were fixed in 4% (w/v) paraformaldehyde in phosphate-buffered saline, washed several times in phosphate-buffered saline, acetylated with triethanolamine/acetic anhydride, dehydrated through a graded series of ethanol, incubated in chloroform followed by a short wash in 100% and 96% isopropanol, and finally air dried. For the hybridization, gene specific riboprobes were generated by *in vitro* transcription using [^{35}S]UTP-labeled nucleotides. The 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 Na_2EDTA, 1 × Denhardts, 10% dextran sulfate, 100 μg/mL sheared salmon sperm DNA, 0.05% (w/v) *Escherichia coli* MRE600 tRNA, 20 mM dithiothreitol, and 50 000 dpm/μL riboprobe. After hybridization, the sections

were washed for 20 min in 1 × saline sodium citrate buffer, 45 min at 37°C in RNase A/T1 solution, 20 min in 0.5 × saline sodium citrate buffer, 20 min in 0.2 × saline sodium citrate buffer, 60 min in 0.2 × saline sodium citrate buffer at 60°C, and finally 10 min in twice-distilled water. The sections were dehydrated, air-dried and coated with Kodak NTB2 emulsion. After an exposure for 2 weeks at 4°C the slides were developed and analyzed.

Antibodies

Polyclonal antisera from rabbits were used to detect mouse and human VAChT on tissue sections by IHC. The antiserum #80259 was originally raised against the C-terminal undecapeptide of rat VAChT (Weihe *et al*. 1996). Since rat and mouse sequences in this region are identical, the antibody is denoted here as anti-rodentV-AChT (for rat/mouse). The antiserum #80153 was raised against the C-terminal dodecapeptide of human VAChT (Schäfer *et al*. 1995), and will be referred to in the text as anti-hVAChT. Loss of immunohistochemical staining after pre-incubation of the antibodies with the respective peptide immunogen against which the antisera were raised at a concentration of 10 μM was indicative of the specificity of the two antisera (data not shown). Moreover, the anti-human VAChT antiserum did not show any staining in non-transgenic mice (see Fig. 3).

Immunohistochemistry

The immunohistochemical detection of mouse and human VAChT on deparaffinized tissue sections was performed essentially as described in detail previously (Schütz *et al*. 2000). Briefly, the sections were deparaffinized, rehydrated, incubated in methanol/hydrogen peroxide to block endogenous peroxidase activity and then incubated in 10 mM sodium citrate buffer (pH 6.0) at 95°C for 10 min for antigen retrieval. After blocking in 5% (w/v) bovine serum albumin in phosphate-buffered saline, the primary antibodies (anti-rat VAChT 80259, raised in rabbit; anti-human VAChT 80153, raised in rabbit) were applied at a 1 : 1000 dilution in 1% bovine serum albumin/phosphate-buffered saline and incubated at 16°C overnight followed by 2 h at 37°C. After three washes in phosphate-buffered saline the sections were incubated with species specific biotinylated secondary antibodies for 45 min at 37°C, and the antigen–antibody complexes visualized with the Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA, USA), using the peroxidase/diaminobenzidine/nickel system.

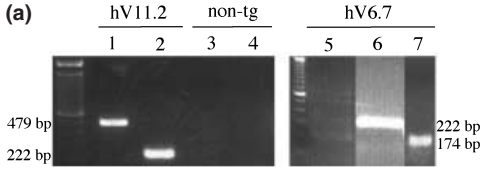
Results

Generation of human cholinergic gene locus transgenic mouse lines

In a previous study we identified a region from the human cholinergic gene locus that directed expression of hVAChT to somatomotor neurons when expressed as a transgene in mice (Schütz *et al*. 2000). This 8.7 kb fragment (named hV8.7) from the 5′ region of the ChAT gene spanned from about 4 kb upstream of the R exon to an *Xba*I site at the start of the M exon, thereby encompassing the VAChT coding exon (Fig. 1). The restricted expression in a subpopulation of central cholinergic neurons led us to generate and test two additional transgenes for their ability to confer cholinergic-specific expression of hVAChT. Linking an additional 2.5 kb of genomic sequence, ranging from the M exon to the first coding exon of ChAT, to the 3′-end of the hV8.7 transgene generated the first construct, hV11.2. The rationale for making this construct was based on observations by others, indicating the existence of gene-regulatory elements within this region (Quirin-Stricker *et al*. 1994; Li *et al*. 1995; Tanaka *et al*. 1998). Removing about 4.5 kb from the 5′-end of hV11.2 generated the second construct, named hV6.7. In the human CGL, this fragment started at an *Xho*I site, 799 bp upstream of the VAChT translation start site and could be regarded as the human equivalent to a mouse construct that was previously reported to confer cholinergic-specific expression of a reporter gene in the CNS of transgenic mice (Naciff *et al*. 1999).

Both constructs were microinjected into the male pronucleus of mouse zygotes and the resulting offspring analyzed by PCR for complete transgene integration with primer pairs that amplified fragments close to both ends of the constructs (Fig. 2a). From this analysis, three transgenic animals harboring the hV6.7 construct, and two with the hV11.2 construct, were identified. Individual mouse lines were

(a)



(b)

	hV6.7			hV11.2	
line	691	2180	6492	1520	6345
copy no.	1	2	31	2	16

Fig. 2 Analysis of transgene integration into the mouse genome. (a) Transgene-harboring mice were identified by PCR, using primer pairs that amplified fragments from both ends of the constructs. The PCR results shown are from one founder mouse of each construct and from a non-transgenic (non-tg) control mouse. The specific amplification of a 479-bp fragment from the 5′-end of hV11.2 (lane 1) and of a 222-bp fragment from the 3′-end (lane 2) is seen in hV11.2 transgenic mice, but not in non-tg control mice (lanes 3 and 4). In hV6.7 mice, lack of amplification of the 479 bp fragment from the 5′-end of hV11.2 (lane 5) is accompanied by the specific amplification of a 222-bp fragment from the 3′-end (lane 6) and of a 174-bp fragment from the 5′-end (lane 7) from hV6.7. (b) The transgene copy number for each of the five lines was determined using real time quantitative PCR on genomic DNA from tail snips of heterozygous mice. The copy number was calculated as $2^{\Delta Ct + 1}$, with ΔCt being $Ct_{hVAChT} - Ct_{mVAChT}$, assuming that the endogenous mouse VAChT has two copies per diploid genome.

established from each of the five founder mice. All mice mated normally and vertical transmission of the transgene occurred stably through multiple generations with the expected Mendelian frequency. All transgenic mice were indistinguishable from their non-transgenic siblings by gross morphological and behavioral examination. For all subsequent analysis, only heterozygous transgenic animals were used.

The transgene copy number was determined in all five lines using real time quantitative PCR. We favored this method over Southern blot analysis, because it allows copy number estimation from as low as 1 up to 10 or greater, with high accuracy in the same experiment. Genomic DNA from tail snips was used as a template and amplified with the hVAChT and mVAChT primer/probe sets. The threshold cycles (Ct) were recorded and the Ct differences (ΔCt) mVAChT–hVAChT calculated. The endogenous mouse VAChT is encoded at a single locus on chromosome 14. Therefore, a $\Delta Ct = 0$ equaled two copies of the transgene per diploid genome. From the hV6.7 lines, number 6492 showed the highest copy number (31 copies in heterozygous mice), followed by line 2180 with two copies and line 691 with one copy (Fig. 2b). From the hV11.2 construct, line 6345 had the highest copy number (16 copies in heterozygous mice), followed by line 1520 with two copies.

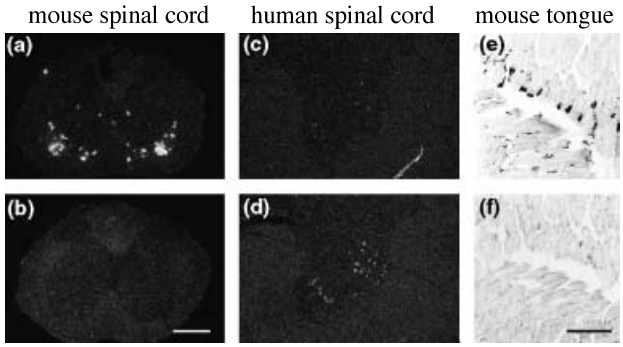


Fig. 3 Evaluation of the species-specificity of riboprobes and antibodies. The mouse VAChT riboprobe was derived from the coding region of the mRNA and strongly labeled motor neurons in mouse spinal cord (a) in ISHH experiments. It also showed some cross-reactivity with human VAChT mRNA, as seen by signals over motor neurons in the ventral horn of the human spinal cord (c). The human VAChT riboprobe, derived from the 3′-non-coding region of the mRNA, was specific for human (d) and did not cross-react with mouse VAChT mRNA (b). (e) Mouse VAChT protein was visualized in motor endplates in muscles of the tongue of a non-transgenic mouse with an anti-rodentVAChT polyclonal antiserum. (f) The human-specific anti-hVAChT polyclonal antiserum did not cross-react with mouse VAChT protein in an adjacent section. Scale bars: 250 μm (b, for a–d), 100 μm (f, for e and f).

Expression of transgenic human VAChT can be unambiguously discriminated from that of endogenous mouse VAChT by the use of species-selective riboprobes and antibodies

Mouse VAChT mRNA expression was visualized in cholinergic neurons in the CNS and PNS by ISHH with an 808-bp antisense riboprobe that was derived from the end of the VAChT coding region. This probe strongly labeled cholinergic neurons in mouse (Fig. 3a), but also cross-reacted with human VAChT mRNA (Fig. 3c). A 271-bp antisense riboprobe from the 3′-non-coding region of human VAChT mRNA was used to detect expression from the transgene. This probe labeled cholinergic neurons in human spinal cord (Fig. 3d), but did not cross react with mouse VAChT mRNA (Fig. 3b). The specific detection of mVAChT on tissue sections by IHC with an anti-rodentVAChT polyclonal antibody is exemplified by staining of motor endplates on skeletal muscles in the tongue of a non-transgenic mouse (Fig. 3e). The anti-hVAChT polyclonal antibody did not cross-react with mouse VAChT, exemplified by the absence of immunoreactivity (IR) on an adjacent section (Fig. 3f).

The expression of human VAChT is extended from somatomotor neurons to a subset of neurons in the medial habenular nucleus in mice carrying the human VAChT 11.2 transgene

The expression pattern of human VAChT from both transgenes was monitored on a cellular level by ISHH and IHC. Previously characterized, species-specific riboprobes and polyclonal antibodies (Schütz *et al*. 2000) allowed the transgene-encoded human VAChT to serve as its own reporter and to compare its expression with the expression pattern of the endogenous mouse VAChT. Immunohistochemical analysis of the two hV11.2 lines and of two hV6.7 lines (6492 and 691) confirmed that differences in expression were due to the intrinsic properties of the constructs and were not caused by differences in transgene expression associated with the site of integration of the transgenic DNA in the mouse genome. Data is shown from the analysis of lines hV11.2-6345 and hV6.7-6492, because these two lines had the highest transgene copy number.

On the mRNA level, expression of hVAChT from the hV11.2 transgene (Fig. 4, middle panel) was found to be restricted to subpopulations of mouse cholinergic neurons that were clearly labeled with a mouse VAChT specific probe on adjacent sections (Fig. 4, left panel). In the central nervous system the somatomotor neurons, exemplified by the motor nucleus of the facial nerve and the motor neurons in the ventral horn of the spinal cord were labeled, although at weaker intensities compared to hybridization with the mouse VAChT specific probe. In addition, some neurons in the medial aspect of the medial habenular nucleus weakly expressed hVAChT mRNA. No expression was detected in the nucleus basalis of Meynert (Fig. 4), the laterodorsal tegmental nucleus (Fig. 4), or any other cholinergic nucleus or interneuron in the CNS (data not shown). Human VAChT mRNA was also absent from the cholinergically coded neurons in ganglia of the peripheral

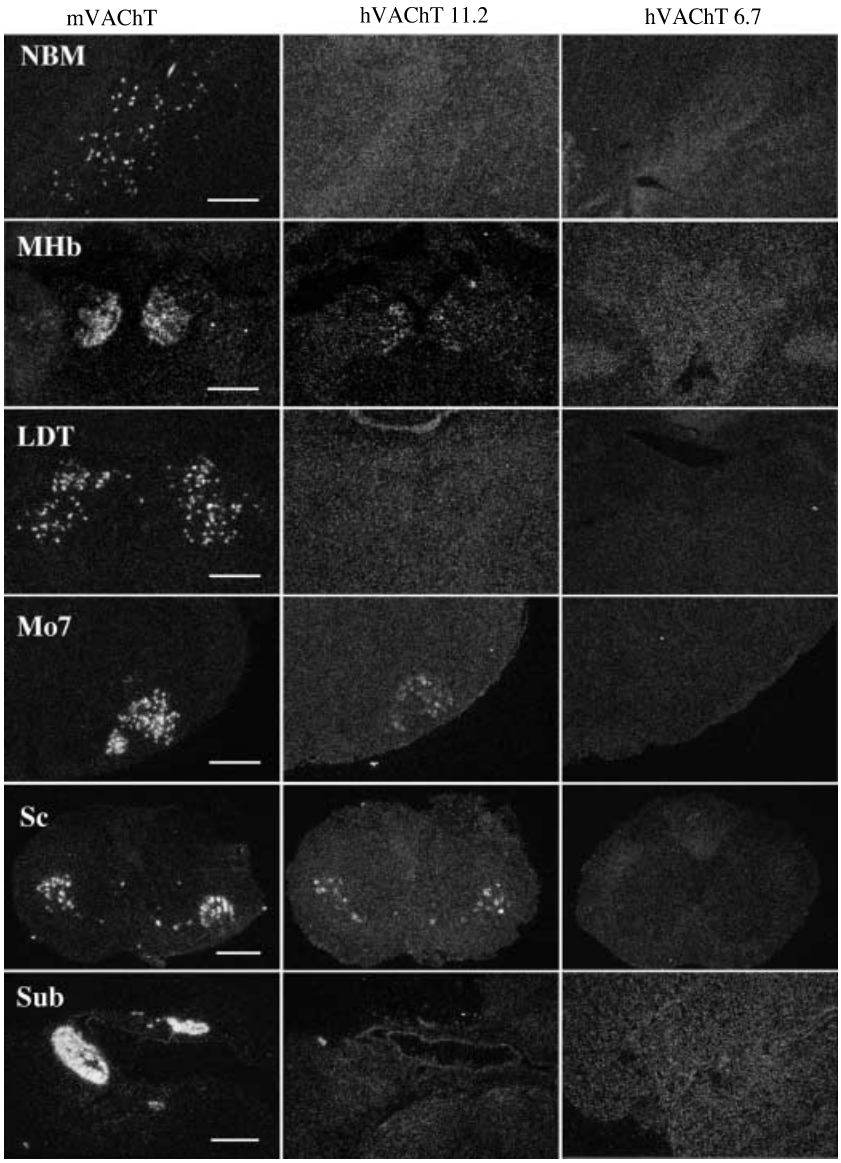


Fig. 4 Expression pattern of mouse VAChT mRNA compared to human VAChT mRNA in selected structures in hV11.2 and hV6.7 transgenic mice. Dark-field photomicrographs of ISHH with mVAChT (left panel) and hVAChT (middle and right panel) specific riboprobes on coronal brain sections showed that hVAChT was expressed in a subset of neurons of the medial habenular nucleus (MHb), in numerous neurons in the somatomotor nuclei of the cranial nerves (shown here for the facial motor nucleus, Mo7), and in motor neurons in the ventral horn of the spinal cord (Sc) in hV11.2 mice (middle panel). hVAChT labeling intensities were always weaker compared to mVAChT signals. Human VAChT mRNA was absent from the nucleus basalis of Meynert (NBM), the laterodorsal tegmental nucleus (LDT), and all other cholinergic and non-cholinergic nuclei in the CNS (data not shown). In the PNS, mVAChT mRNA, but not hVAChT mRNA was detected in neurons of a parasympathetic ganglion in the submandibular gland (Sub). HV6.7 transgenic mice completely lacked hVAChT mRNA in all structures analysed (right panel). Scale bars: 250 μm (MHb, Mo7, SC, and Sub), 500 μm (NBM, LDT).

nervous system, shown here for the submandibular ganglion. All corresponding structures analysed throughout the central and peripheral nervous systems in hV6.7 mice were negative for hVAChT mRNA signals (Fig. 4, right panel). While not definitive proof that the 6.7 hVAChT gene is transcriptionally silent in mouse cholinergic neurons, these data clearly demonstrate that promoter strength is much less than that of the endogenous gene, and less than that of the 8.7 kb and 11.2 human CGL gene fragments examined previously, and in this report, respectively.

With the usage of species-specific antisera the observed mRNA expression pattern could be confirmed on the protein level (Fig. 5). The medial habenular nucleus is densely packed with cholinergic neurons that could be visualized for rodent VAChT IR (Fig. 5a). The interpeduncular nucleus is the main target of fibers from the medial habenular nucleus and appears as a strongly labeled structure (Fig. 5b). hVAChT IR was detected in a subset of neuronal cell bodies of the medial habenular nucleus (Fig. 5c), and in a subset of fibers and terminals of the interpeduncular nucleus (Fig. 5d). In the medial habenular nucleus, mainly neurons in the medial part of the nucleus were labeled, whereas the lateral part was almost devoid of immunoreactivity. A comparable restriction of labeling could be seen in the interpeduncular nucleus, where the most dense fiber staining appeared in the ventral part of the nucleus. All cholinergic somatomotor neurons of the cranial nerves exhibited strong rodent VAChT IR, exemplified here for the motor nucleus of the trigeminus (Fig. 5e). Numerous neuronal cell bodies were found stained for hVAChT IR (Fig. 5f). Compared to the uniform labeling

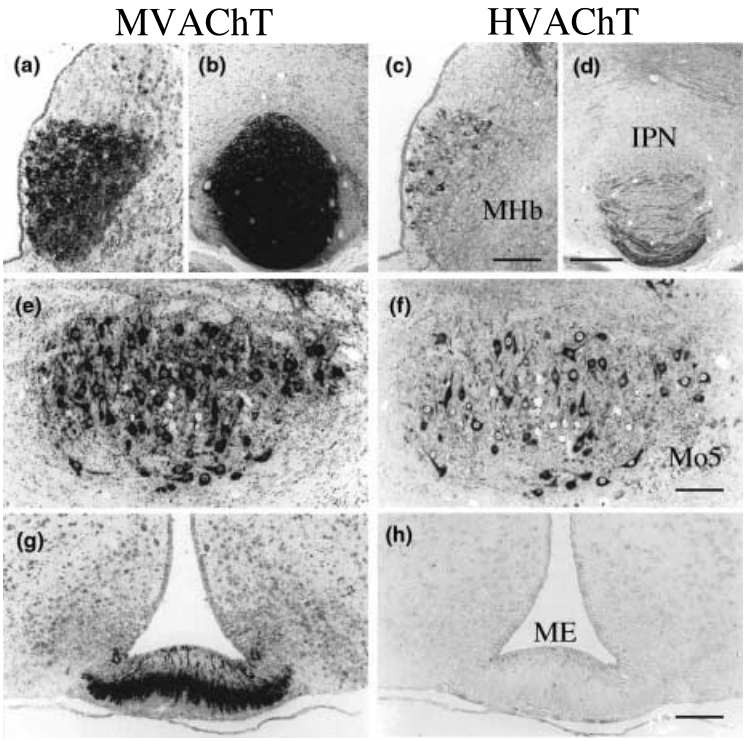


Fig. 5 Human VAChT IR in neuronal cell bodies and fibers in hV11.2 transgenic mice. In the medial habenular nucleus, rodent VAChT IR detected all cholinergic neurons (a), whereas hVAChT IR was restricted to a subpopulation of neurons in the medial part of the nucleus (c). In the interpeduncular nucleus, the main target of fibers from the MHb, rodent VAChT IR marked an intense cholinergic fiber staining (b). hVAChT IR was most prominent in fibers in the ventral part of the nucleus, but sparse in the medial and dorsal part (d). In the cholinergically coded somatomotor nuclei of the cranial nerves, exemplified by rodent VAChT IR labeling of the motor nucleus of the trigeminus (e), hVAChT IR was detectable in numerous neurons (f). The absence of hVAChT IR from other cholinergic projections is illustrated by the lack of fiber staining in the median eminence (h), a structure strongly labeled for rodent VAChT IR (g). No hVAChT IR was detectable in the IPN in hV6.7 and non-transgenic mice (data not shown). IPN, interpeduncular nucleus; ME, median eminence; MHb, medial habenular nucleus; Mo5, motor nucleus of the trigeminus. Scale bars: 100 μm (c, for a and c), 250 μm (d, for b and d), 100 μm (f, for e and f), 50 μm (h, for g and h).

of all neurons for rodent VAChT IR, differences in the staining intensity could be observed for hVAChT. In addition, a dense network of cholinergic synapses surrounding the cell bodies could be observed with rodent VAChT IR, but not with hVAChT IR. Absence of additional cell body or fiber staining for the transgenic human VAChT in every other region of the CNS or PNS analysed was most obvious in structures with a high endogenous VAChT load. This is exemplified by an intense rodent VAChT IR fiber staining in the median eminence (Fig. 5g), which completely lacked hVAChT IR (Fig. 5h). A complete absence of hVAChT IR from hV6.7 transgenic animals confirmed the results from our ISHH analysis on the protein level (data not shown).

Comparison of human and mouse VAChT mRNA levels in hV11.2 mice

ISHH and IHC provide the critical qualitative information about the requirement for specific domains of the CGL to drive hVAChT expression in the mouse, including potential *cis*-active elements present within the open reading frame and 3′ untranslated region of the VAChT gene itself. Our transgenic model system also offers the unique opportunity to directly measure and compare hVAChT and mVAChT mRNA expression levels *in vivo*. We therefore analyzed cDNA from brainstem samples of hV11.2-6345 mice by quantitative RT-PCR to obtain information about the strength with which hVAChT is expressed compared to its endogenous counterpart. The brainstem, rich in cholinergic somatomotor nuclei that express both mVAChT and hVAChT, was most suitable for this analysis. The graph in Fig. 6 shows representative PCR amplification curves for GAPDH, mVAChT and hVAChT mRNAs from duplicate experiments. Target-specific amplification was detected starting after cycle 20 with the emergence of GAPDH, followed by mVAChT and hVAChT signals. Ct values were 25.99 ± 0.47 for GAPDH, 29.59 ± 0.21 for mVAChT and 32.76 ± 0.27 for hVAChT. A ΔCt_{hV-mV} of 3.05 indicated hVAChT transcriptional activity (uncorrected for percentage

of cells expressing the transgene) that was at least within an order of magnitude of the transcriptional activity of the endogenous mouse VAChT gene.

Discussion

We have begun to identify regions within the CGL that are important for the expression of VAChT *in vivo*. In our transgenic analysis we employ the human CGL, because we are able to monitor expression of human VAChT in mice with species-specific tools for PCR, IHC and ISHH. This approach preserves the integrity of the locus because it omits the utilization of reporter genes for the detection of promoter activity. The high sequence homology between the mouse and human CGL even on the genomic level makes it very likely that a human transgene is treated very similarly compared to its endogenous counterpart in the mouse.

In our first analysis, 8.7 kb of human CGL sequence gave cholinergic-specific, but somatomotor neuron-restricted expression of VAChT (Schütz *et al*. 2000). These results implied that regulation of VAChT in this subdivision of the cholinergic nervous system is unique and separable from cholinergic regulation elsewhere, and that additional upstream or downstream sequences of the CGL are required for full cholinergic expression *in vivo*. The addition of another 2.5 kb to hV8.7, giving hV11.2, resulted in an extended expression of VAChT that now included a subset of neurons in the medial habenular nucleus, whereas the removal of the upstream regulatory region including the R exon (hV6.7) completely abolished VAChT expression.

The hV6.7 transgene is not capable of promoting VAChT expression

Lack of VAChT expression in hV6.7 transgenic mice underlines the importance of the far best characterized gene-regulatory region of the mammalian CGL, i.e. the sequence spanning approximately 4 kb upstream of the R exon which contains multiple *cis*-acting elements, needed to activate the CGL in cholinergic cells and to repress its activity in non-neuronal cells. In comparison to the fruit fly CGL, the mammalian R exon has been thought to be the common first exon for both VAChT and ChAT. Alternative splicing of a primary message would then lead to VAChT and ChAT specific messenger RNAs. When the upstream fragment from the rat CGL was attached to a heterologous promoter that initiated transcription at the R exon, CAT reporter gene expression could be demonstrated in the ventral spinal cord and brain of transgenic mice, although at very low levels (Lönnerberg *et al*. 1995). Recently, promoters specific for VAChT have been described in rat (Cervini *et al*. 1995), and the lack of detectable levels of R exon-containing transcripts in developing and adult rat cholinergic neurons by ISHH suggests that this exon is used as most as a minor transcriptional start site, with the majority of VAChT and ChAT transcripts arising individually from more proximally located promoters (Schütz *et al*. 2001). We therefore believe that absence of VAChT transcription from hV6.7 is likely due to the absence of the 4 kb of gene-regulatory sequence, rather than of the R exon.

From its length and location in the CGL, hV6.7 is almost identical to a fragment from the mouse CGL that, when expressed as a transgene in mice, conferred β-galactosidase expression to cholinergic neurons in the CNS (Naciff *et al*. 1999). Although β-galactosidase expression was used to display ChAT transcription initiating at the M exon, the authors also indicated an increase of VAChT message by Northern blot analysis and PCR. Our data argue against this possibility, despite the fact that hV6.7 has 799 bp of sequence upstream of the start of VAChT translation that contains one of the VAChT proximal promoters. The detection of ChAT-like expression from the mouse transgene and the absence of VAChT expression from our human transgene most likely indicates that the VAChT and ChAT genes are regulated at least in part by separate *cis*-acting elements *in vivo*.

The hV11.2 transgene extends expression of VAChT from the somatomotor neurons to a subset of neurons in the medial habenular nucleus, suggesting a mosaic model of VAChT expression *in vivo*

Our results from the analysis of the hV11.2 transgene indicate that the genomic region between the M exon and the

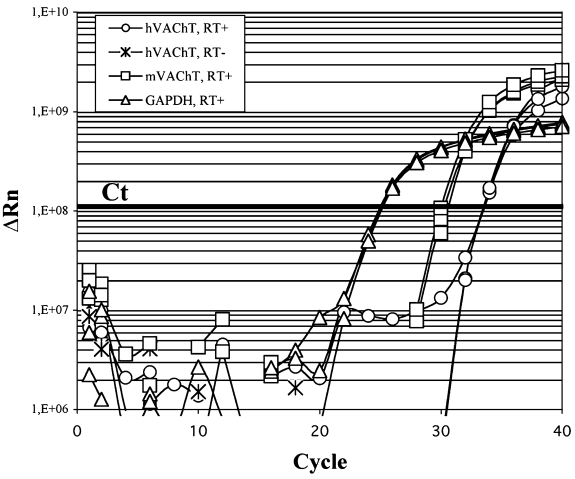


Fig. 6 Quantitative RT-PCR analysis of human and mouse VAChT mRNA levels in hV11.2 mice. cDNA from brainstem tissue was analyzed by quantitative PCR for GAPDH, mVAChT and hVAChT expression levels. The graph shows representative amplification curves from triplicate PCR reactions. Note that during the first 20 PCR cycles, only background fluorescence was detectable. The emergence of target-specific amplification curves was seen first for GAPDH, followed by mVAChT and hVAChT. Ct, threshold cycle, ΔRn, normalized reporter signal; RT+, PCR after reverse transcription; RT–, PCR without reverse transcription.

first ChAT coding exon is responsible for the extension of VAChT expression to a specific population of non-somatomotor neurons. By itself this region is not sufficient to promote hVAChT expression in the medial habenular nucleus but rather seems to depend on the upstream 4 kb genomic sequence absent in the hV6.7 construct. This is an important finding, because it suggests a mosaic model of hVAChT expression *in vivo*. In other words, while hVAChT expression in the somatomotor neurons seems to require a core promoter, additional sequences have to be added to extend this expression to other cholinergic neuronal populations, one of them being the medial habenular nucleus. Our observations make it promising that also other cholinergic systems will be identified to be regulated in this manner *in vivo*. The identification and characterization of the underlying cis-active domains and the gene-regulatory factors that bind to them would be especially valuable for those neurons that are damaged in neuropathological situations, i.e. the basal forebrain neurons in Alzheimers disease or sympathetic and parasympathetic neurons in peripheral neuropathies.

Where within the CGL might these elements lie, and what are the minimal requirements for full cholinergic expression *in vivo*? Recently, transgenic mice were generated where green fluorescent protein (EGFP) was inserted at the translational start of ChAT in a 150-kb mouse bacterial artificial chromosome clone (von Engelhardt *et al*. 2002). EGFP-labeled cells were found to be co-labeled with ChAT-immunopositive cells in the basal ganglia, septum, tegmental and brainstem nuclei. In addition, putative cholinergic cells were also found in hippocampus and neocortex. It will be of particular interest to know if this construct gives full cholinergic expression, because data concerning the expression profile in the PNS is missing. Experimental support for our mosaic model of CGL regulation comes from studies in *Drosophila*, where a cholinergic subdivision-specific transcriptional activator has been identified. Mutant flies that lack expression of the transcription factor *abnormal chemosensory jump 6* (*acj6*) showed decreased ChAT mRNA levels and enzyme activity in primary olfactory neurons, whereas expression in mechanosensory neurons was unaffected (Lee and Salvaterra 2002).

Our ISHH and IHC analysis pointed out that there are differences in hVAChT compared to mVAChT mRNA expression levels, and in hVAChT IR between the somatomotor nuclei and the medial habenular nucleus. The weaker hVAChT mRNA signals not only result from a lower transcription rate compared to the endogenous one (see below) but are also due to the better performance of the mouse-specific riboprobe. Therefore, differences in ISHH signal intensities are not per se indicative of differences in expression levels. In the motor nucleus of the trigeminus almost all neurons stained positive for hVAChT IR, albeit with different intensities. In contrast, only a subset of neurons in the medial habenular nucleus seemed to express hVAChT. These cells were mainly located in the medial part of the nucleus. This indicates that the addition of 2.5 kb of sequence to the hV8.7 transgene seems to be not sufficient to give full penetrance to all cholinergic neurons in the medial habenular nucleus.

One major limitation with the use of reporter genes in transgenic analysis is that a quantitative evaluation of the promoter strength is not possible, due to lack of comparability. A reporter molecule like β-galactosidase can only be used qualitatively to determine an expression pattern that reflects the cell-specificity of the fused promoter. Attempts to circumvent this limitation and to investigate the strength of CGL promoter fragments in transgenic analysis ranged from standard PCR analysis (Naciff *et al*. 1997) to measurements of relative CAT activities (Lönnerberg *et al*. 1995). The high structural and sequence similarity between the mouse and human CGL and the use of VAChT as its own reporter now allows for the first time a direct quantitative comparison of the levels of mVAChT and hVAChT transcription by qRT-PCR, albeit uncorrected for the percentage of cholinergic cells (which we estimate to be 10–15% of the total) in which the transgene is expressed in the brain stem. The less than 10-fold lower expression of hVAChT compared to mVAChT message in the brainstem indicates that hVAChT is expressed relatively robustly compared to endogenous mVAChT. The fact that the human CGL is capable of driving high level expression in a mouse background, generating near-physiological levels of protein expression, suggests that the human CGL is recognized in mice similarly to the endogenous CGL.

In summary, the transcriptional regulation of VAChT and ChAT expression from the mammalian CGL *in vivo* is highly complex. The minimal genomic sequence required for full cholinergic-specific expression of VAChT and ChAT remains to be determined, and the entire functional CGL could include as much as 150 kb. Likewise, the nature of the individual *cis*-regulatory elements and *trans*-acting factors that control VAChT expression in the individual cholinergic circuits of the CNS and PNS *in vivo* remain open to further investigation. Our transgenic mouse model has so far provided significant new information about the nature of this regulation including evidence for subdivision-specific activation of VAChT expression. A more complete explication of the mechanisms of CGL transcriptional control should have a major impact on our understanding of cholinergic functioning in health and disease.

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