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Developmental expression of the high affinity choline transporter in cholinergic sympathetic neurons

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Abstract

Choline uptake by the high affinity choline transporter (CHT) is the rate-limiting step in acetylcholine synthesis. Induction of CHT is therefore a critical step in cholinergic differentiation, and we examined the developmental expression of CHT in cholinergic sympathetic neurons that innervate rodent sweat glands. During postnatal development the earliest sympathetic axons in the rear footpads are noradrenergic, containing intense tyrosine hydroxylase immunoreactivity and lacking CHT-immunoreactivity (CHT-IR). By postnatal day 7 (P7) in mouse, and P10 in rat, weak CHT-IR appeared in axons associated with the sweat gland anlagen. CHT staining intensity increased during the following weeks in conjunction with plexus arborization and gland maturation. The pattern of CHT-immunoreactivity (CHT-IR) in the sweat gland innervation was similar to staining for the vesicular acetylcholine transporter and vasoactive intestinal peptide. Immunoblots of tissue from sympathectomized rats confirmed that most of the CHT in footpad was contained in sympathetic neurons. Although CHT expression has been reported in noradrenergic sympathetic neurons of the superior cervical ganglion, these data indicate that in the sympathetic neurons projecting to sweat glands CHT is present at detectable levels only after association with the glands.

Keywords

cholinergic differentiation; sympathetic development; sweat glands

Introduction

Post-ganglionic sympathetic neurons acquire a noradrenergic neurotransmitter phenotype early in embryonic development (Vitalis et al., 2002). These neurons are noradrenergic by the time ganglia coalesce, and long before axons are extended toward target tissues. Expression of noradrenergic receptors in effector organs is similarly autonomous; sympathetic innervation is not required for noradrenergic receptor expression.

Cholinergic sympathetic neurons are exceptional because their neurotransmitter expression is altered by their target tissue (Landis, 1990). Two sympathetic target tissues have been characterized that induce a switch in neurotransmitter phenotype from norepinephrine to acetylcholine/vasoactive intestinal peptide (ACh/VIP) in the neurons that innervate them. Eccrine sweat glands and bone periosteum both secrete a soluble factor that is taken up by

innervating axons to trigger a change in neurotransmitter properties (Guidry and Landis, 1998; Schotzinger and Landis, 1988; Schotzinger and Landis, 1990b; Asmus et al., 2000)

The mechanisms regulating this neurotransmitter switch have been partially delineated in studies focused on the neuropeptide VIP and the proteins choline acetyltransferase (ChAT) and the vesicular ACh transporter (VACHT) (Ernsberger and Rohrer, 1999; Francis and Landis, 1999; Guidry and Landis, 1998). ChAT and VACHT, which comprise the cholinergic gene locus (Eiden, 1998; Erickson et al., 1994), are responsible for the synthesis and vesicular packaging of ACh, respectively. These proteins are required but not sufficient for the acquisition of functional cholinergic transmission. Transport of choline into the nerve terminal through the Na⁺-dependent high affinity choline transporter (CHT) (Okuda et al., 2000) is the rate-limiting step in ACh synthesis (Birks and Macintosh, 1957), so CHT is also required for neurons to produce and release ACh.

The recent identification and cloning of the choline transporter (Apparsundaram et al., 2000; Apparsundaram et al., 2001; Okuda et al., 2000) revealed that CHT is a member of the sodium-dependent solute carrier (SLC5) family, which uses the cell electrochemical gradient to couple ion and substrate transport (Ferguson and Blakely, 2004; Wright and Turk, 2004). Identification of the transporter gene confirmed that it is not part of the cholinergic locus, and therefore is regulated independently of ChAT and VACHT. Finally, cloning of the CHT gene enabled the generation of reagents to study its expression and function. We have used these reagents and a newly generated CHT antiserum to investigate the expression of CHT in cholinergic sympathetic neurons.

Recent studies indicate the CHT is expressed in some noradrenergic sympathetic neurons that do not contain detectable ChAT or VACHT (Lecomte et al., 2005). This raises the possibility that CHT is present in cholinergic sympathetic neurons prior to target contact, when they are still noradrenergic and do not express ChAT, VACHT, and VIP. To determine if CHT expression preceded other aspects of cholinergic differentiation in these neurons, we examined CHT expression in the developing sweat gland innervation using immunohistochemistry.

Methods

Animals. Pregnant Sprague-Dawley rats were obtained from Charles River (Cambridge, MA) and pregnant Swiss Webster mice were obtained from Taconic (Germantown, NY). Rat tissues from at least 3 animals were analyzed at several ages during postnatal development, and hind footpads were used for all experiments. Neonatal rats were sympathectomized with daily intraperitoneal injections of 6-hydroxydopamine hydrobromide (20 mg/kg in citrate buffer) from postnatal day 2 (P2) to P7 and on P10 (all reagents from Sigma, St. Louis IL). All procedures involving the use of animals conformed to guidelines set forth by the National Institutes of Health Animal Research Advisory Committee.

Immunohistochemistry. Animals were euthanized with carbon dioxide gas and perfused for 10 minutes with freshly depolymerized 4% paraformaldehyde. Dissected tissues were incubated in fixative for one hour then equilibrated in 30% sucrose in 0.1 M phosphate buffer. Optimal staining with antisera against the choline transporter was obtained with additional fixation. Dissected mouse tissue was incubated in paraformaldehyde at 37° for an additional 4 hours. Rat tissue sections mounted on microscope slides were incubated in paraformaldehyde for an additional 1.5 hours at room temperature (RT), and rinsed in phosphate buffered saline (PBS) prior to staining. The additional fixation required for optimal CHT staining disrupted immunostaining with other primary antibodies. Therefore, double labeling was not successful with either CHT antisera, and additional tissue from the same animals was used to identify other cholinergic and peptidergic markers.

Ten micron cryostat sections of mouse or rat tissue were mounted on gelatin coated or charged slides, rinsed in PBS, blocked with staining buffer (2% bovine serum albumin, 0.3% Triton X-100 and 0.1% sodium azide in PBS) for 1 hour, and then incubated with primary antiserum in staining buffer overnight at RT. Sections were then rinsed in PBS and incubated in species-specific secondary antiserum in buffer for 1 hour, rinsed with PBS and coverslipped with 50% glycerol in PBS. Some sections were labeled with avidin/biotin complex, which included serial application of biotin conjugated secondary antiserum then avidin-fluorochrome, both for 1 hour at RT with PBS washes.

Immunostaining for CHT was completed in rat tissue using a polyclonal antiserum generated by Ferguson and coworkers (Ferguson et al., 2003). The anti-CHT antiserum used for staining mouse tissue was generated in rabbits against a C-terminal peptide (DSSPEGSGTEDNLQ) corresponding to rodent CHT with an N-terminal cysteine for conjugation to keyhole limpet hemocyanin. The VIP antiserum was generated in guinea pig (Tyrrell and Landis, 1994). The VACHT antiserum was generated in rabbit (Roghani et al., 1998). Sheep anti-TH was from Chemicon (Temecula, CA). Secondary antibodies were conjugated to rhodamine, fluorescein, aminomethylcoumarin or biotin (from Jackson ImmunoResearch, West Grove, PA), or Oregon Green or Neutralite Avidin Texas Red (both from Molecular Probes, Eugene, OR).

Western Blots. Tissue extracts were diluted in SDS loading buffer (2% SDS, 60 mM Tris, pH 6.8, 0.01% bromophenol blue, 5% β -mercaptoethanol, and 10% glycerol), heated at 90°C for 5 min and centrifuged. Aliquots containing 175-200 μ g of total protein were separated on 10% SDS-polyacrylamide gels and then transferred to nitrocellulose membranes for identification of choline transporter or tubulin. Membranes were blocked in TBST containing 5% nonfat milk for one hour (TBST= 100 mM NaCl, 10 mM Tris, pH 7.5, and 0.1% Tween 20), and incubated at 4°C overnight with rabbit anti-CHT diluted 1:750 in TBST/5% BSA. Blots containing mouse tissue were incubated with the new CHT antiserum, while blots containing normal and sympathectomized rat tissue were incubated with the antiserum generated by Ferguson and coworkers (Ferguson et al., 2003). After washing, membranes were incubated at room temperature for 1 hour with horseradish peroxidase-conjugated IgG (diluted 1:5000 in TBST/5% nonfat milk), washed with TBST, and immunoreactive bands were visualized by chemiluminescence. Membranes were then stripped for 1 hour at room temperature in stripping solution (62.5mM Tris pH 6.8, 2%SDS, and .7% (v/v) β -mercaptoethanol), washed with TBST, blocked with TBST/5% nonfat milk, and incubated at 4°C overnight with mouse anti-tubulin diluted at 1:5000 in TBST/5% nonfat milk. Membranes were washed and incubated in secondary antibody (1:5000) in TBST/5% nonfat milk and visualized with chemiluminescence. Band intensity was recorded by a -40°C CCD camera and analyzed using LabWorks software (UVP, Upland, CA).

Results

The new anti-CHT antiserum was first characterized by western blot and immunohistochemistry using several tissues that contain cholinergic cells and/or axons. The anti-CHT antiserum stained a major band of approximately 50 kDa in extract from adult mouse rear footpad, stellate ganglia, striated muscle, spinal cord, and submandibular salivary gland (Figure 1a). Pre-absorption of the antiserum with the antigen peptide eliminated antibody binding (Figure 1b). In sections of adult mouse stellate ganglion, the antiserum labeled presumptive cholinergic principal neurons that sparsely populate the ganglion. Labeled neurons contained a faintly stained cytoplasm surrounding a dark, unstained nucleus (Fig. 2a). More intense staining was visible in the terminals of cholinergic neurons that project to the ganglion from the spinal cord. The terminals appeared as bright puncta, primarily clustered around the edges of ganglion principle neurons (Fig. 2a). The antiserum was very effective in staining motor neuron terminals. In sections of lumbricales, a plantar striated muscle, anti-

choline transporter staining revealed the branches characteristic of motoneuron end-plates (Fig. 2b). The antiserum also stained parasympathetic neurons in the submandibular salivary gland and the tongue. In sections of mouse tongue the intrinsic parasympathetic ganglia were labeled, as were parasympathetic fibers innervating smooth muscle that is a part of the larger vasculature (Fig. 2c).

Sympathetic axons reach the eccrine sweat glands in rear footpads of mice shortly after birth (Guidry and Landis, 1995; Guidry and Landis, 1998). To examine CHT expression in the developing gland innervation, immunohistochemistry was carried out on sections of hind footpad throughout early postnatal development. Antisera against the catecholamine synthesizing enzyme tyrosine hydroxylase (TH) was used to identify the sympathetic axons arriving at the footpad during the initial stages of target contact and plexus formation. At P5, TH-immunoreactive sympathetic axons are readily detected in the vicinity of developing sweat gland anlage (Fig. 3a). No CHT-immunoreactivity (CHT-IR) was visible in sweat gland innervation at P5. At P7 a plexus of TH-immunoreactive fibers surrounded the developing glands (Fig. 3b). Faint CHT immunoreactivity was first detected at this point as thin, weakly fluorescent axons near gland anlage (Fig. 3c). Choline transporter immunoreactivity increased steadily during the following week, and at P14 CHT-containing axons were present throughout the core of the footpad (Fig. 3d).

Choline transporter immunoreactivity was detected slightly later in the developing rat footpad, consistent with the later appearance of other cholinergic markers in the rat (Landis, 1990). At P4 and P7, tyrosine hydroxylase immunoreactivity was present in the axons innervating developing gland anlage (Fig. 4g and h, arrowheads), but CHT was not detectable (Fig. 4a and b). Faint CHT-IR appeared at P10 and persisted through P21 (Fig. 4 c-e), with more robust CHT staining present in adult footpad (f). In contrast, the abundant TH immunoreactivity which was evident in the early gland innervation (Fig. 4 g-i), was significantly reduced in the adult sweat gland innervation (l, arrowhead).

To confirm that the CHT-IR observed in the developing rear footpad was in sympathetic axons, and not sensory or motoneuron fibers, CHT expression was examined in tissue lacking sympathetic innervation. Rats were sympathectomized with 6-hydroxydopamine during the first postnatal week, and footpad samples taken at P30 for western blot analysis. Choline transporter band density was quantified and normalized to that for tubulin in the same samples. Sympathectomy resulted in a 90% decrease in footpad choline transporter immunoreactivity compared to control rats (Figure 5).

Neurotransmitter expression in the sweat gland innervation has been studied extensively, and the mature cholinergic innervation expresses TH, VIP, and VACht. We examined CHT together with these other markers in the adult gland innervation. In adult footpad, bright ribbons of TH-IR cholinergic fibers surrounded the secretory portion of coiled sweat glands (Fig. 6a). Additional sections from the same mouse were stained for VIP, VACht, and CHT. The morphology of CHT immunoreactivity in adult footpads was very similar to immunoreactivity for VACht and VIP (Fig. 6 b-d).

Discussion

The functional development of the synapse between sympathetic neurons and eccrine sweat glands is dependent on reciprocal interactions between neuron and gland. When sympathetic axons first reach the developing glands, they contain catecholamines and immunoreactivity for catecholamine synthetic enzymes. During the three weeks after gland contact, catecholamine synthesis is suppressed and a cholinergic/peptidergic phenotype is induced. Final maturation of the sweat glands, characterized by secretory competence and sensitivity to cholinergic agonists, is correlated with the development of cholinergic transmission by

innervating axons. In mature animals cholinergic transmission is required to maintain the sweating response (Grant et al., 1995; Stevens and Landis, 1987; Stevens and Landis, 1988).

While many of the details of the noradrenergic to cholinergic switch in sympathetic neurons have been worked out, gaps remain. The suppression of noradrenergic properties involves decreasing the catecholamine synthesizing enzymes TH and dopamine- β -hydroxylase (DBH) (Landis et al., 1988), along with the TH cofactor BH4 (Habecker et al., 2002). The loss of catecholamines is accompanied by a coordinated increase in ChAT and VAcHT, and the expression of acetylcholinesterase, VIP and calcitonin-gene related peptide (CGRP) (Landis, 1990; Landis and Keefe, 1983; Schotzinger and Landis, 1990a). Despite our detailed knowledge of many aspects of this developmental switch in neurotransmitter phenotype, until recently the tools were not available to examine the expression of CHT, which controls the rate-limiting step in ACh production.

It is now possible to add characterization of CHT to our understanding of the noradrenergic to cholinergic switch in the sweat gland innervation. The choline transporter is critically important for development of the sympathetic/sweat gland synapse, since maturation of sweat glands depends on exposure to acetylcholine (Grant et al., 1995). It seemed logical to assume that the transporter would be expressed along with other cholinergic proteins in the sweat gland innervation, since we know these neurons acquire the capacity to synthesize and release ACh. However, recent reports indicate that noradrenergic sympathetic neurons of the superior cervical ganglia (SCG) express CHT in the absence of ChAT, VAcHT, or ACh production (Lecomte et al., 2005). This raised the possibility that all sympathetic neurons expressed the choline transporter, and that the onset of cholinergic transmission in the sweat gland innervation was due solely to the induction of ChAT and VAcHT by the sweat gland derived factor.

In contrast to the observation of CHT-IR in noradrenergic sympathetic neurons of the SCG (Lecomte et al., 2005), we detected CHT-IR in the sweat gland innervation only after axons contacted the developing glands. We cannot rule out the possibility that CHT was present at or before target contact, but at levels low enough to escape detection by immunohistochemistry. Nevertheless, our data clearly show that expression of CHT increases dramatically following initial target contact. These data are consistent with previous studies suggesting that the innervation lacks cholinergic markers before and during initial plexus development (Schotzinger and Landis, 1990a; Stevens and Landis, 1988).

The onset of CHT-IR in the sweat gland innervation was similar to the appearance of other cholinergic markers. In the mouse, CHT was first detected in rear footpads at P7 (Fig. 3) while VAcHT appears in these neurons at P6 (Guidry and Landis, 1998). In the rat rear footpad, CHT was initially detected at P10 (Fig. 4), which is consistent with the appearance of VIP and ChAT in these neurons (Landis, 1990). These data support a model whereby a gland-derived cholinergic differentiation factor stimulates coordinated expression of ChAT, VAcHT, and CHT.

The sweat gland-derived cholinergic differentiation factor has not been identified, but all evidence points to a cytokine using a receptor that incorporates the gp130 receptor subunit and intracellular message cascade that includes the phosphorylation of the STAT proteins (Duong et al., 2002; Geissen et al., 1998; Habecker et al., 1997). A number of structurally related gp130 binding cytokines have been tested and rejected as the cholinergic-inducing factor in sweat glands, including leukemia inhibitory factor (LIF), ciliary neurotrophic factor (CNTF), and oncostatin M (Francis et al., 1997; Rao et al., 1992; Rohrer, 1992). New evidence obtained through the application of chromatography and peptide identification via mass spectroscopy on large quantities of rat footpad extract suggests that the sweat gland factor may be cardiotrophin 1 (James Coulombe, personal communication). The observation that LIF triggers

the upregulation of ChAT, VAcHT, and CHT in cultured sympathetic neurons (Lecomte et al., 2005), while retinoic acid stimulates ChAT and VAcHT (Berrard et al., 1993; Berse and Blusztajn, 1995) but suppresses CHT (Lecomte et al., 2005), are consistent with our data suggesting a LIF-related cytokine stimulates expression of all three cholinergic genes in vivo.

Although gp130 cytokines can stimulate the simultaneous expression of ChAT, VAcHT, CHT, and VIP mRNAs in sympathetic neurons, the transcription factors involved have not all been identified. The VIP gene has been the most extensively studied, and a number of cis-regulatory elements have been identified (Hahm et al., 2003; Hamelink et al., 2004; Waschek et al., 1988), including a 180 base pair cytokine response element that confers responsiveness to LIF and related cytokines (Pitts et al., 2001; Symes et al., 1994). Our data indicate that expression of CHT is induced together with ChAT, VAcHT, and VIP in the developing sweat gland innervation. Further studies are required in order to ascertain if these genes are turned on by common regulatory mechanisms.

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Figure 1.

Characterization of a new CHT antiserum by immunoblot. A. Extracts from adult mouse footpad (1), stellate ganglia (2), striated muscle (3), spinal cord, (4) and submandibular salivary gland (5) were transferred onto nitrocellulose membrane and blotted using a newly generated anti-CHT antiserum. The antiserum recognized a single band of the expected size. B. Pre-absorption of the antiserum with the antigen peptide (corresponding to a C-terminal fragment of the CHT) prevented binding to the 50 kDa protein CHT in footpad extracts.

Figure 2.

Characterization of a new CHT antiserum by immunohistochemistry in adult mouse. (a) In sections of mouse stellate ganglion, the new anti-CHT antiserum stained a small number of neuron cell bodies (arrow) and diffuse terminals of cholinergic projections from spinal cord neurons, which appear as puncta at this magnification. (b) Motoneuron endplates (arrow) in a hind foot striated muscle, a slip of the lumbricales, were brightly stained with the antiserum. (c) In sections of mouse tongue, the antiserum recognized cholinergic parasympathetic fibers (arrow) innervating smooth muscle cells lining the wall of a blood vessel. Scale bar = 50 μ m.

Figure 3.

Developmental expression of the choline transporter in mouse hind footpad innervation. (a) Mouse hind footpad stained with anti-TH antisera at postnatal day 5 (P5) contain numerous TH positive sympathetic axons (arrow) surrounding developing sweat gland anlage (asterisk). At this age, the innervation lacked reactivity for choline transporter. (b, c) TH and CHT staining in separate sections from the rear footpad of a single P7 mouse. TH containing innervation (b, arrow) surrounds developing glands and immunoreactivity for choline transporter first appears in the gland innervation (c arrow, asterisk marks gland). (d) At P13, choline transporter immunoreactivity (arrows) is widespread in axons innervating mature sweat glands. Scale bar = 50 μ m

Figure 4.

Developmental expression of the choline transporter in rat hind footpad innervation. Sections of rat footpad from P4 (A, G), P 7 (B, H), P10 (C, I), P14 (D, J), P21 (E, K), and adult (F, L) were stained with an antibody directed against the high affinity choline transporter (A-F; diluted 1:500) or tyrosine hydroxylase (G-L; diluted 1:300). TH immunoreactivity was present in the neurons innervating developing gland anlage at P4 and P7 (G and H, arrowheads), but CHT immunoreactivity was undetectable in additional sections from the same animals (A and B). Faint CHT immunoreactivity was detected at P10, P14, and P21 (C-E), and robust CHT staining was present in adult footpad (F). In contrast, abundant TH immunoreactivity is present early in development (G-I), with lower levels in the adult sweat gland innervation (L, arrowhead). Scale bar = 10 μ m.

Figure 5.

Choline transporter expression in sympathectomized sweat glands. (A) Protein from the rear footpads of sympathectomized (6-OHDA) and control rats were blotted for the choline transporter. Membranes were stripped and re-blotted for tubulin. (B) Band density was quantified as described in the methods, with CHT normalized to tubulin in the same samples. The mean $\pm$ sd for 3 sympathectomized and 2 control rats are graphed ($p=0.001$, t-test).

Figure 6.

Expression of CHT and other cholinergic markers in the adult mouse sweat gland innervation. (a) In adult mouse hind footpad, the TH-IR sympathetic innervation is concentrated around sweat glands (asterisk in all micrographs). Additional sections from the same animal show that the mature gland innervation also contains immunoreactivity for VIP (b), VAcHT (c), and CHT (d). Scale bar = 50 μ m