BAC transgenic mice express enhanced green fluorescent protein in central and peripheral cholinergic neurons

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The “cholinergic locus” consists of the ChAT and the vesicular acetylcholine transporter (VACHT) genes, the latter gene entirely contained within the first intron of the former (11, 21). Because ChAT is essential for acetylcholine biosynthesis, several groups have used 5′-flanking DNA from this region to direct transgene expression in selective cholinergic neuron populations (16, 19, 20). While demonstrating expression confined to cholinergic neurons, these constructs do not provide robust peripheral expression. This lack of expression is probably due to the complex nature of ChAT and VACHT regulatory elements, which include enhancer and suppressor elements and significant tissue-specific splicing (14, 18). Thus, for example, Misawa and colleagues (19) recently reported the development of a mouse expressing Cre recombinase and enhanced green fluorescent protein (eGFP) under control of a 11.3-kb fragment of the cholinergic locus, placing the GFP-IRES-Cre within the VACHT coding region; no expression was observed in parasympathetic nerves in these mice. A bacterial artificial chromosome (BAC) transgenic approach utilizing the ChAT locus to direct expression of eGFP has been described (10). However, marker expression in peripheral cholinergic neurons was not reported in these mice, preventing determination of the effectiveness of the BAC strategy employed.

We reasoned that endogenous regulatory elements associated with the ChAT locus should effectively specify transgene expression in central and peripheral neurons, as well as other cells that synthesize acetylcholine, and employed a BAC transgenic approach to preserve these elements. Here we report that transgenic mice using a ChAT locus-spanning BAC, in which eGFP is knocked into exon 3 at the ChAT initiation codon, robustly and selectively express eGFP in all cholinergic nerves of the central and peripheral nervous systems (CNS and PNS), as well as in nonneuronal cells.

Materials and Methods

Generation of Tg(RP23-268L19-eGFP)Kot. The BAC clone RP23-268L19, containing ~78 kb and ~36 kb of DNA flanking the 5′ and 3′ ends of the ChAT locus, respectively, was obtained from the BAC resource at Children’s Hospital Oakland Research Institute. The eGFP cassette was inserted at the initiation codon of the ChAT coding sequence, which is located within exon 3, by homologous recombination using the targeting vector designed for BAC recombiner (13, 15, 31). ChAT homologous arms were amplified and inserted upstream of the ChAT locus and downstream of the eGFP sequence in plasmid pBS-eGFP-FRT-Neo/Kan-FRT (Kotlikoff laboratory); ChAT arm1 was a 596-bp fragment upstream of the ATG in exon 3 and was created using forward and reverse primers: 5′-GCG CTT AGA ATA CTG GTG...
GG-3′ and 5′-CCT AGC GAT TCT TAA TCC A-3′. An XhoI site was added to the 5′ end of the forward primer, and an SmaI site was added to the 5′ end of the reverse primer. ChAT arm2 was a 588-bp product downstream of the ATG codon of exon 3 using the forward primer 5′-CTC ATG GAA AAG GTC-3′, with a SpeI site added to the 5′ end, and the reverse primer 5′-GAG AAC ATAT CAT CCA GAC CA-3′ with a SacI site added to the 5′ end. Plasmid pBS-eGFP-FRT-Neo/Kan-FRT was cut with XhoI and SmaI and ChAT arm1 inserted followed by SpeI and SacI digestion and insertion of ChAT arm2. ChAT(arm1)-eGFP-FRT-Neo/Kan-FRT-ChAT(arm2) cassette was released from the plasmid by XhoI and SacI digestion. After electroporation of RP23-268L19 BAC into the recombinogenic arm2. ChAT(arm1)-eGFP-FRT-Neo/Kan-FRT-ChAT(arm2) cassette was released from the plasmid by XhoI and SacI digestion. EL250 cells carrying RP23-268L19 BAC were electroporated with ChAT(arm1)-eGFP-FRT-Neo/Kan-FRT-ChAT(arm2), and homologous recombinated positive clones were selected with kanamycin and chloramphenicol. The Neo/Kan cassette was removed by 0.5% arabinose induction of FLP recombinase, resulting in ChATBAC-eGFP. Homologous recombination was confirmed by PCR and sequencing the BAC using primers that flanked the upstream of ChAT arm1 to eGFP cassette and downstream of ChAT arm2 to eGFP cassette. The ChATBAC-eGFP DNA was prepared using a modified alkaline lysis protocol (Qiagen kit), and circular intact ChATBAC-eGFP DNA was microinjected into fertilized embryos by standard pronuclear injection techniques. Genomic DNA isolated from tissue was purified using Puragene (Gentra Systems), and founder mice carrying the BAC transgene were identified by PCR with primer sets RP23-268P1 5′-ATC CTT GTG GCC TCC CTG TTG-3′ and RP23-268P2 5′-CCG AAA TCG TCG TGG TAT-3′ in the BAC vector pBACe3.6, yielding a 505-bp product. Founders and offspring were subsequently genotyped with primers to a ChAT-eGFP fragment with a ChAT-upstream primer 5′-AGT AAG CCT ATG GGA TCC ATAT C-3′ and an eGFP-reverse primer 5′-AGT TCA CCT TGA TGC CTG CT-3′, yielding a 620-bp PCR product. Two founder lines were produced that transmitted the transgene in a normal Mendelian inheritance pattern with all offspring appearing grossly normal.

Animals were cared for in accordance with guidelines described in the Guide for the Care and Use of Laboratory Animals, using protocols approved by the Cornell Institutional Animal Care and Use Committee. Embryos were removed at specific days postcoitus (dpc) by dissection from the uterus. Pregnant dams and 7- to 18-wk-old adult mice were euthanized by carbon dioxide inhalation.

Immunohistochemistry. Tissues were removed, rinsed in saline, fixed in 10% formalin, dehydrated in graded ethanols, and embedded in paraffin. Paraffin-embedded tissues were cut into 5-μm sections then deparaffinized and rehydrated. Anti-GFP staining has previously been described with the following antibody concentration changes of rabbit anti-eGFP to 1:20 (Chemicon International, Temecula, CA) and biotinylated goat anti-rabbit IgG 1:200 (Vector Laboratories, Burlingame, CA) (30). Retrieval of antigen for anti-ChAT staining was performed by steaming in 0.01 M citrate buffer, pH 6.0, in a microwave. Sections were blocked with 10% normal rabbit serum with 2× casein for 20 min then incubated for 2 h at 37°C with polyclonal goat anti-ChAT antibody diluted 1:100 in PBS (Chemicon International). Biotinylated rabbit anti-goat IgG (Zymed, San Francisco, CA) was applied for 20 min at room temperature, followed by PBS washes and incubation for 20 min with streptavidin peroxidase (Zymed). AEC substrate kit was prepared as directed (Zymed) and applied for 10 min. Slides were counterstained with Gill’s #2 hematoxylin and mounted with Fluoromount G (both from Fisher Scientific Research, Pittsburgh, PA). Controls were included, substituting normal goat serum in place of primary antibody at the appropriate dilution. Immunohistochemistry images were captured either with an Aperio ScanScope (Aperio Technologies) or Olympus DP70 mounted to a Leica DMLB microscope.

In vivo and ex vivo imaging. In vivo and ex vivo images were obtained using a Leica MZFLIII stereomicroscope or OI100 macroimaging system (Olympus) with band-pass eGFP filter cubes, and fluorescent images were captured using a DP70 camera (Olympus). Reconstruction of some images was accomplished using ImageJ (National Institute of Health free software) extended depth-of-focus plug-in. The acquisition of multiphoton images has previously been described (32).

RESULTS

Generation of ChATBAC-eGFP mouse. Two ChATBAC-eGFP founder lines, ChAT line 2 and ChAT line 44, were generated by replacing the initial codon of the ChAT gene contained in exon 3 with the entire eGFP sequence followed by a polyadenylation signal sequence, thus placing eGFP under control of the endogenous regulatory elements contained in the cholinergic gene locus (Fig. 1A) (5). Mice were identified by either a 620-bp or 505-bp PCR product, using primers specific for either the BAC vector or the sequence between ChAT and eGFP (Fig. 1B). The offspring appeared grossly normal, and the transgene was transmitted in a normal Mendelian inheritance pattern. We systemically evaluated both founder lines for eGFP specificity and expression using fluorescent imaging and
immunohistochemistry, and no apparent differences were observed between the two founder lines. Following the Mouse and Rat Strain Nomenclature Guidelines, we registered the ChAT<sup>βAC</sup>-eGFP mouse with Mouse Genome Informatics as Tg(RP23–268L19-eGFP)Kot.

Expression of eGFP in cholinergic nerves of the CNS. We analyzed the cholinergic CNS regions expressing eGFP and cell specificity of the fluorescence in our mice. Whole brain mounts from adult ChAT<sup>βAC</sup>-eGFP mice express light fluorescence throughout the cerebrum, with intense staining running on the surface of the diencephalon caudally to the mesencephalon (Fig. 2A). Highly fluorescent fibers in the pons and medulla were identified as cranial nerves III and V-XII and cervical spinal nerves (Fig. 2, A and B). No fluorescence was observed in the cerebellum, consistent with minimal cholinergic neurotransmission in that region. Immunodetection of eGFP revealed specific neuronal populations expressing the transgene (Fig. 2C); fibers and/or cell bodies were specifically labeled in the cortex, caudate putamen, accumbens, medial habenular, basolateral amygdaloid, anteriorolfactory, facial, spinal V, medial vestibular, prepositus hypoglossal, solitary tract, and ambiguous nuclei, as well as in the spinal trigeminal tract, consistent with previous findings based on ChAT immunodetection (20, 22).

The entire spinal cord was intensely fluorescent, and cross sections at the thoracic and lumbar spinal cord segments showed eGFP-positive immunostaining in large cell bodies, as well as in varicose nerve fibers and ventral nerve roots of the ventral horn, consistent with expression in cell bodies and axons of α-motor neurons (Fig. 2, D and E). Prominent eGFP expression was also detected in the intermediolateral cell column (lamina VII) located in the lateral horn of the thoracic and upper lumbar spinal cord, which gives rise to autonomic preganglionic sympathetic neurons. Cell bodies and processes in the dorsal horns (lamina I-VI) of the spinal cord and dorsal root ganglia, representing nerves involved in carrying sensory information from peripheral organs, were also stained (Fig. 2E).

To document the cholinergic-specific expression of eGFP at the cellular level, we performed immunohistochemistry using antibodies against ChAT and eGFP in sequential brain slices. Anti-eGFP immunostaining was more prominent in cell bodies and neuronal processes compared with anti-ChAT immunostaining (Fig. 2F). Comparison of the same regions in serial sections indicated that eGFP and ChAT were expressed in the same neuronal cell populations throughout the brain except in two regions: olfactory cortex and amygdaloid. To our surprise anti-ChAT did not stain these two areas even though reports by other groups and our ChAT<sup>βAC</sup>-eGFP mice expressed eGFP in these regions; this is most likely attributable to differences in immunohistochemistry techniques (20, 22). In double-labeling experiments, eGFP-expressing neurons were colabeled by antibodies directed against neuronal nitric oxide synthetase (nNOS), a neuron-specific synthetase, or synaptophysin, an integral membrane protein expressed in the presynaptic vesicle in neurons (not shown). Moreover, an astrocyte-specific antibody directed against glial fibrillary acidic protein did not label eGFP-positive cells in brain slices (not shown). Taken together these data demonstrate that ChAT<sup>βAC</sup>-eGFP mice express eGFP in cholinergic cell bodies and processes of the CNS, including elements specific to the autonomic nervous system.

Fig. 2. eGFP fluorescence is restricted to the central nervous system in ChAT<sup>βAC</sup>-eGFP mice. A: light (left) and fluorescence (right) dissecting scope ex vivo whole brain images from adult ChAT<sup>βAC</sup>-eGFP mice. B: close-up in vivo view of the ventral surface of the pons, medulla, and rostral spinal cord showing fluorescence of the cranial and spinal nerves. C: anti-eGFP antibody immunostaining from rostral to caudal cross sections of the brain. D: ventral view of spinal cord from postnatal day 2 mouse. E: anti-eGFP immunostaining in the thoracic and lumbar segments of the spinal cord. Note the labeling of the ventral nerve root exiting the spinal cord and dorsal root ganglia. F: anti-ChAT (left) and anti-eGFP (right) serial sections of the medial habenular nucleus of the brain shows the same cell bodies and nerve processes possess eGFP and ChAT. III, oculomotor; V, trigeminal; VI, abducens; VII, facial; IX, glossopharyngeal; X, vagus; XI, accessory; XII, hypoglossal; AO, anterior olfactory nucleus; CPu, caudate putamen; ACh, accumbens nucleus core; AChSh, accumbens nucleus shell; OC, olfactory cortex; LGp, lateral globus pallidus; Mnb, medial habenular nucleus; BL, basolateral amygdaloid nucleus; 7, facial nucleus; Sp5, spinal 5 nucleus; MVeMC, medial vestibular nucleus; Pr, prepositus hypoglossal nucleus; 7PA, facial nucleus proximal axons; 12, hypoglossal nucleus; Sol, solitary tract nucleus; Amb, ambiguous nucleus; VH, ventral horn; L9, lamina 9; DH, dorsal horn; VNR, ventral nerve root; DRG, dorsal root ganglia. Scale bars: A, 1 mm; B, cranial left 1 mm; cranial right 500 μm; cervical nerves 1 mm; C, 500 μm; D, 1 mm; E, 250 μm; and F, 50 μm.
Expression of eGFP in cholinergic nerves of the PNS. We next evaluated eGFP fluorescence in elements of the PNS in both in vivo and ex vivo preparations, to determine the extent to which eGFP is expressed in ChATBAC-eGFP mice cell bodies and extensive processes of cholinergic nerves, including motor neuron axons and pre- and postsynaptic autonomic neurons. As shown in Fig. 3A, adult mice display robust eGFP fluorescence on the lateral surface of the jaw in all branches of the facial nerve, which carries motor axons from cell bodies in cranial nerve VII to facial muscles. Fluorescence extended through nerve branches to the neuromuscular junction (Fig. 3B), indicating efficient axonal transport of transgene protein and/or mRNA. Superficial motor neurons running along the lateral and medial surfaces of the thoracic and pelvic limbs also expressed eGFP (data not shown). Similarly, phrenic motor neurons projecting to the diaphragm from cervical nerves 3–5 displayed intense in vivo fluorescence (Fig. 3C). These data indicate effective marking of postganglionic motor neurons of the somatic PNS.

Of perhaps more significance, eGFP was observed in the parasympathetic nervous system, which has not been effectively labeled by transgenic methods (16, 20). As shown in Fig. 3C, the vagus nerve trunk displayed clear fluorescence within the thoracic cavity, although the fluorescence intensity of the vagus both in the neck and thoracic cavity was distinctly lower than observed in somatic motor nerves. This likely reflects the smaller diameter preganglionic autonomic axons and lower axon density of the nerves, although lower promoter strength in vagal preganglionic neurons could not be excluded.

eGFP expression was also observed in ganglionic neurons; three different ganglia, representing predominately parasympathetic, sympathetic, or mixed neurons (6, 28), were evaluated. The parasympathetic ciliary ganglion located on the dorsal surface of the eye receives preganglionic fibers from the oculomotor nerve (III) and synapses on postganglionic fibers projecting to smooth muscle of the eye (6); eGFP was expressed in the preganglionic oculomotor nerve running parallel with the optic nerve, the preganglionic oculomotor nerve at the ciliary ganglion, and postganglionic cell bodies and ciliary nerve fibers (Fig. 3E). The second ganglia we assessed was the paravertebral chain ganglia containing sympathetic preganglionic nerves that exit the ventral root of the thoracic and lumbar spine to form synapses on postganglionic cell bodies. As shown in Fig. 3F, paravertebral ganglia display eGFP fluorescence in processes running to, and synapsing within, the ganglia. Finally, we evaluated mixed sympathetic and parasympathetic neurons within the pelvic ganglia located on the dorsal surface of the prostate gland. These ganglia receive inputs from lumbar and sacral regions of the spinal cord and are the major autonomic ganglia supplying postganglionic nerves to reproductive organs, the lower urinary tract, and the lower bowel (28). As shown in Fig. 3G, the major nerves of the pelvic ganglia were easily distinguished by eGFP fluorescence in ChATBAC-eGFP mice (28).

Postganglionic, parasympathetic process innervating target organs also display strong eGFP fluorescence, an important advantage of ChATBAC-eGFP mice for physiological studies. As shown in Fig. 3H, postganglionic fibers were labeled in a variety of target organs, including within the extensive submucosal plexus of the gastrointestinal tract. Single nonneuronal epithelial cells were also labeled within the gastrointestinal mucosa (also see below). The sweat gland is unusual in that sympathetic innervation switches during development from catecholaminergic to cholinergic; Fig. 3I demonstrates that eGFP fluorescence is observed in hind footpad sweat glands in adult ChATBAC-eGFP mice, demonstrating that this transition
has occurred (26). Taken together, these data demonstrate the ability to visualize postganglionic projections of the somatic motor, sympathetic and parasympathetic PNS in vivo and ex vivo.

**Cellular expression of eGFP in the PNS.** To more carefully examine the cellular pattern and neuron-specific nature of ChAT driven eGFP expression in target tissues we performed immunostaining using anti-GFP antibodies. As shown in Fig. 4A, eGFP was detected in the cytoplasm of cell bodies present within ganglia and processes of nerves similar to earlier findings (12). To further document the neuronal specificity of ChAT\textsuperscript{BAC}, eGFP mice in the PNS we performed immunohistochemistry using antibodies against eGFP and nNOS in the intestine. Approximately 50% of the cells within the ganglia of Auerbach’s and Meissner’s plexus colabeled with eGFP and nNOS, a ratio similar to previous reports in the mouse (data not shown) (24). ChAT has also been reported to be present in nonneuronal cells within the lung and intestine (29). Prominent eGFP immunostaining was observed in nonneuronal tissues in ChAT\textsuperscript{BAC}, eGFP mice (Fig. 4B), consistent with the observation of intense focal fluorescence in these tissues (Fig. 3H). To identify the cell populations in the lung and intestine expressing eGFP, we used specific markers for epithelial cells (keratin), neuroendocrine cells (synaptophysin), lymphocytes (CD38), macrophages (MAC387), and goblet cells (Ascan blue and periodic acid-Schiff). eGFP colocalized with keratin and a subpopulation of lymphocytes but not with neuroendocrine or goblet cell markers, consistent with the expression of the transgene in epithelial cells and T lymphocytes (data not shown) (29).

**Embryonic development.** The high degree of fidelity of eGFP expression in cholinergic cells conferred by the BAC transgene provides an effective way to study the development and adaptation of cholinergic neurons, including the targeting of motor neurons to specific muscles and the complex ramifications of the parasympathetic nervous system. To confirm the early embryonic expression of eGFP we examined ChAT\textsuperscript{BAC}, eGFP embryos at several stages of development. At all stages of fetal development evaluated ChAT\textsuperscript{BAC}, eGFP positive embryos could easily be distinguished from negative littermates (Fig. 5). At 10.5 dpc, cranial ganglia displayed robust eGFP fluorescence; the eGFP fluorescence between the somites was identified in a cross section taken below the level of the heart as ventral motor neurons. In addition, sympathetic ganglion and enteric neurons were identified in this cross section (Fig. 5, A–C). By 13.5 dpc eGFP fluorescence was prominent between the digits of both fore- and hindlimbs (Fig. 5, D and E). Figure 5F shows a cross section taken at the brachial level with cholinergic-dependent eGFP fluorescence in the bilateral ventral roots, bilateral sympathetic ganglia, left vagal sympathetic trunk, and fine projections to the limbs. At this stage fluorescent nerve projections to the gastrointestinal organs were apparent including significant ramifications within the smooth muscle and mucosal layers, which became progressively more extensive by 16.5 dpc and 18.5 dpc, consistent with previous literature (Fig. 5G) (7).

**DISCUSSION**

The PNS is an extensive, diverse, and highly adaptive system that plays essential roles both as the conduit for central control of higher order function and as a distributed nervous system capable of complex pattern generation and processing in local circuits. The extensive ramifications, complex wiring, and diverse expression of neurotransmitters, particularly in the autonomic nervous system, have complicated neurophysiological evaluation of the PNS. Recently, substantial progress has been made in the understanding of complex central processing through the use of genetic approaches that enable the targeting of specific neuronal populations and also provide a mechanism to regulate gene expression in these cells. Here we report specific and effective labeling of the cholinergic nervous system using BAC transgenic methods that exploit the ChAT gene locus. The ChAT gene resides in a complex “cholinergic locus” with numerous poorly understood tissue-specific regulatory elements and a second gene, VACH\textsuperscript{T}, contained within the first intron (11, 20). Transgenic mice expressing eGFP under control of minimal ChAT promoter constructs lack cholinergic specificity and parasympathetic neuronal labeling (16, 20), emphasizing the difficulty of reconstituting endogenous gene expression patterns with this approach. More recently, a BAC strategy utilizing the ChAT locus was employed as part of a large scale CNS gene expression atlas (10). This strategy resulted in robust expression of GFP in central neurons; however, no evaluation of peripheral expression was reported, and therefore neither the degree of expression in the CNS or other cholinergic cells nor the extent to which neuronal processes are labeled is available. We employed a similar strategy in which eGFP cDNA is knocked into the first ChAT coding exon within a large (173 kb) genomic DNA fragment by homologous recombination; however, the BAC targeting approach differed in two significant ways. First, we used homology sequences upstream and downstream of the initiation codon, which flanked the insertion eGFP cassette, and confirmed the insertion site within the BAC by PCR. Second, we removed the recombination selection (NEO) cassette before
Fig. 5. Embryonic expression of eGFP in ChATBAC-eGFP mice. A: light (left) fluorescence (right) of a 10.5-day postcoitus (dpc) embryo. B: enlarged view of boxed area in A showing cranial and spinal nerves (indicated by A). C: cross section showing ventral motor neuron (indicated by B), single arrow pointing to sympathetic ganglion and double arrow to enteric neurons. D: light (left) fluorescence (right) of a 13.5-dpc embryo. E: enlarged view of boxed area in C showing fluorescence in the forelimb. F: cross section taken at the brachial level of a 13.5-dpc embryo. Ventral motor neuron (*), single arrow pointing to sympathetic ganglion and double arrow to left vagal sympathetic trunk. G: organs from a 13.5-dpc embryo showing cholinergic projections in the stomach and intestine. X, ganglia vagus. Scale bar: A, D, and F, 1 mm; B and E, 250 μm; C, 100 μm; and G, 50 μm.

injection of the BAC (13). The high throughput strategy employed by Gong et al. (10) results in the retention of the selection cassette, as well as vector sequences such as the R6Kγ origin of replication within the ChAT locus. The extent to which these differences affected peripheral expression is not clear, however, and in both strategies the retention of extensive 5′ and 3′ flanking sequence and the insertion of the eGFP reporter gene at the ChAT start codon should preserve 5′ untranslated nucleotides associated with the normal transcript. A further advantage of this approach is that the use of this construct as a randomly inserted genetic element (pronuclear injection) enables preservation of the endogenous ChAT alleles, whereas knock-in strategies usually require inactivation of the targeted allele.

Progenies from both ChATBAC-eGFP lines displayed eGFP expression in a pattern that was qualitatively the same, suggesting BAC transgenic mice may have less integration site positional insertion effects and are insulated from regulatory elements of neighboring genes because they harbor the majority, if not all, of the chromatin domain insulator elements (25). While the random insertion of large DNA fragments may occasion fragmentation or recombination, lines can be effectively screened by PCR strategies that take advantage of the BAC vector DNA. By this tactic, random primers to the BAC vector were designed, and one such example is shown in Fig. 1A. While we did not attempt to quantify the gene copy number in our ChATBAC-eGFP lines, pronuclear injection of BAC transgenes usually results in the integration of only a few copies/genome of the BAC DNA (1, 23). Despite the likely lower copy number of large BAC inserts, eGFP expression was found to be remarkably high, and excellent axonal transport was achieved without fusion of the tau microtubule binding protein (23). We have observed this phenomenon in several BAC transgenics and attribute the strong expression to the fact that the large genomic provides the complete regulatory context required for robust gene expression. Finally, as previously noted, the BAC approach requires little or no knowledge of the underlying gene regulatory elements and is amenable to both relatively high throughput approaches (10). The specific strategy used here should be an effective way to obtain lineage-specific transgene expression that extends to cholinergic nerves within the autonomic nervous system, such as the expression of Cre recombinase or specific neural factors.

Immunostaining using antibodies directed against eGFP and ChAT indicated extensive colabeling of central and peripheral cholinergic neurons, indicating that the genetic indicator serves to accurately reflect ChAT expression. The single exception to this overlap was the failure of the anti-ChAT antibody to recognize a small subset of neurons with previously reported ChAT indicated extensive colabeling of central and peripheral cholinergic nerves, indicating that the genetic indicator serves to accurately reflect ChAT expression. The single exception to this overlap was the failure of the anti-ChAT antibody to recognize a small subset of neurons with previously reported ChAT expression. However, in our ChATBAC-eGFP lines, the eGFP signal in the autonomic nervous system, such as in the Meissner’s plexus, demonstrated eGFP-specific expression in cholinergic neurons of peripheral organs. The expression of eGFP in central and peripheral cells was observed at the earliest time point evaluated, 10.5 dpc, with progressive rostral to caudal innervation of the intestinal tract during embryonic developments. Interestingly, at 13.5 dpc intense fluorescence was observed between the fore- and hindlimb digits; these axons may be responsible for maintaining the appropriate synapse to skeletal muscle cell ratio as homozygous knockout ChAT mouse embryos develop wrist drop (3).
In contrast to the common role of acetylcholine in neuronal cells of the CNS and PNS, its cellular function(s) in nonneuronal cells is limited. Our observation of intense eGFP fluorescence and staining in epithelial cells of airways and alimentary tract and in a subset of lymphocytes is consistent with reports of ChAT mRNA and protein within these cell types (29) and demonstrates the potential use of ChAT\textsuperscript{BAC}-eGFP mice as an experimental model to study the nonclassical role of acetylcholine under normal and pathological conditions.

The availability of a genetic marker of cholinergic activity should be useful for the study of the development of the cholinergic nervous system, as well as genetic and acquired autonomic neuropathies. Moreover, we demonstrate the ability to effectively target this system by genetic means, enabling tissue-specific gene expression and gene inactivation. ChAT\textsuperscript{BAC}-eGFP mice should advance our understanding of the in vivo development of the cholinergic nervous system, adaptive changes that occur under pathophysiological conditions, and the in vivo regenerative capabilities of the CNS and PNS after injury.

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