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Variable expression of GFP in different populations of peripheral cholinergic neurons of ChAT^{BAC}-eGFP transgenic mice

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ABSTRACT

Immunohistochemistry is used widely to identify cholinergic neurons, but this approach has some limitations. To address these problems, investigators developed transgenic mice that express enhanced green fluorescent protein (GFP) directed by the promoter for choline acetyltransferase (ChAT), the acetylcholine synthetic enzyme. Although, it was reported that these mice express GFP in all cholinergic neurons and non-neuronal cholinergic cells, we could not detect GFP in cardiac cholinergic nerves in preliminary experiments. Our goals for this study were to confirm our initial observation and perform a qualitative screen of other representative autonomic structures for the presences of GFP in cholinergic innervation of effector tissues. We evaluated GFP fluorescence of intact, unfixed tissues and the cellular localization of GFP and vesicular acetylcholine transporter (VAChT), a specific cholinergic marker, in tissue sections and intestinal whole mounts. Our experiments identified two major tissues where cholinergic neurons and/or nerve fibers lacked GFP: 1) most cholinergic neurons of the intrinsic cardiac ganglia and all cholinergic nerve fibers in the heart and 2) most cholinergic nerve fibers innervating airway smooth muscle. Most cholinergic neurons in airway ganglia stained for GFP. Cholinergic systems in the bladder and intestines were fully delineated by GFP staining. GFP labeling of input to ganglia with long preganglionic projections (vagal) was sparse or weak, while that to ganglia with short preganglionic projections (spinal) was strong. Total absence of GFP might be due to splicing out of the GFP gene. Lack of GFP in nerve projections from GFP-positive cell bodies might reflect a transport deficiency.

1. Introduction

Localization of cholinergic neurons in the central and peripheral nervous systems was accomplished initially using histochemistry for acetylcholinesterase, but this approach yielded some false positive results (Koelle, 1963; Hoover et al., 2004). Increased knowledge about the neurochemistry of cholinergic neurons and elucidation of the amino acid sequence of proteins that are required for the synthesis and storage of acetylcholine (ACh) in cholinergic neurons has led to the production of specific antibodies, which have been instrumental in the localization of cholinergic neurons and their processes in the central nervous system and periphery. Specific proteins targeted for immunohistochemistry are the high affinity choline transporter (CHT1) required for uptake of choline at cholinergic nerve endings (Misawa et al., 2001; Kus et al., 2003; Hoover et al., 2004), the ACh synthetic enzyme choline acetyltransferase (ChAT) (Kimura et al., 1980; Woolf, 1991), and the vesicular ACh transporter (VAChT) needed for storage of ACh in secretory vesicles (Weihe et al., 1996; Arvidsson et al., 1997; Schafer et al., 1998; Schäfer et al., 1998). Antibodies to cholinergic proteins can vary in their sensitivity for detection of the specific antigen and in the amount of background staining. The utility of polyclonal antibodies can also vary between lots. Variations in the structure of ChAT have been noted and probably contribute to the limited efficacy of ChAT antibodies for labeling all cholinergic perikarya and processes in the autonomic nervous system (Bellier and Kimura, 2011).

To overcome problems in detection of ChAT in peripheral neurons and to enable *in vivo* identification of central and peripheral cholinergic neurons and nerve fibers, some investigators have used a bacterial artificial chromosome strategy to insert an enhanced green fluorescent protein (GFP) gene into the ChAT locus (Tallini et al., 2006). It was

Abbreviations: ANS, autonomic nervous system; BSA, bovine serum albumin; ChAT, choline acetyltransferase; pChAT, peripheral choline acetyltransferase; GFP, enhanced green fluorescent protein; NDS, normal donkey serum; PBS, phosphate-buffered saline; VAChT, vesicular acetylcholine transporter

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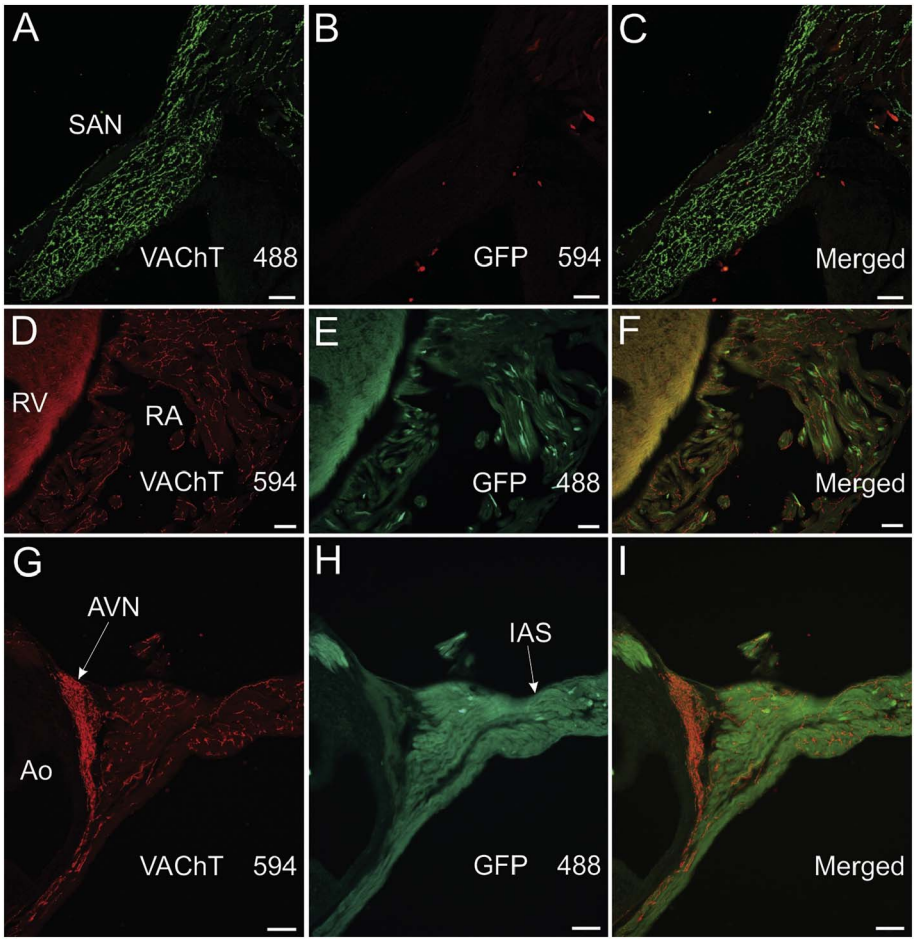

Fig. 1. VAChT-positive nerves fibers in nodal and contractile regions of atrium lack GFP staining in ChAT^{BAC}-eGFP mice. (A–C) Confocal images of section through the sinoatrial node that was double labeled for VAChT (A) and GFP (B). The rich cholinergic innervation revealed by VAChT staining (A) lacked GFP (B and merged image in C). Alexa Fluor 594 was used to detect GFP in this and some other experiments to assure that lack of staining for GFP could not be attributed to the fluorophore used for detection. (D–I) Standard fluorescence photomicrographs of right atrial and ventricular myocardium (D–F) and the atrioventricular node (AVN) and interatrial septum (IAS) (G–I). Fluorophores used for detection are included with images. Cholinergic innervation of all these regions was stained for VAChT and lacked GFP staining. Ao, aorta; RA, right atrium; and RV, right ventricle. Note that cholinergic nerves are more abundant in atrial regions (A, D, and G) than in RV (D). Scale bars are 50 μm (A–C) and 100 μm (D–I).

reported that this approach results in the expression of GFP in central and peripheral cholinergic neurons and in non-neuronal cholinergic cells (Tallini et al., 2006).

ChAT^{BAC}-eGFP mice have been used extensively in recent years to identify and facilitate the study of cholinergic immune cells, specifically T and B cells (Rosas-Ballina et al., 2011; Reardon et al., 2013). We purchased these transgenic mice primarily for this purpose and have confirmed their value for detecting abundant cholinergic leukocytes in the spleen and mesenteric lymph node (Hoover, 2017). Additionally, we have a long-term interest in cardiac cholinergic innervation and regulatory mechanisms, so we decided to evaluate the utility of ChAT^{BAC}-eGFP mice for localizing cardiac parasympathetic neurons and the distribution of their projections within the myocardium. The original report on the ChAT^{BAC}-eGFP strain suggested a broad utility of these mice for study of the parasympathetic innervation, but no convincing data were reported for the heart and intrinsic cardiac ganglia. Surprisingly, our preliminary experiments did not identify GFP + nerve fibers in the myocardium of ChAT^{BAC}-eGFP mice. Accordingly, our goals for this study were to confirm our initial observation for heart and to perform a qualitative screen of other representative autonomic structures for the presences of GFP in cholinergic innervation of effector tissues. The results demonstrate that GFP is not expressed uniformly throughout the autonomic nervous system. Deficits are prominent in the heart, airway smooth muscle, and terminals of long preganglionic cholinergic nerve fibers.

2. Materials and methods

2.1. Animals

Male and female B6·Cg-Tg(RP23-268L19 EGFP)2Mik/J mice, also known as ChAT^{BAC}-eGFP mice, were obtained from The Jackson Laboratory (Bar Harbor, ME) and bred in house. Adult, male offspring were used for this study (n = 12). Major observations were replicated using tissue from three separate mice. Animal protocols were approved by the East Tennessee State University Animal Care and Use Committee and conformed to guidelines of the National Institutes of Health as published in the *Guide for the Care and Use of Laboratory Animals* (Eighth Edition, National Academy of Sciences, 2011).

2.2. Tissue collection and processing

Mice were euthanized with isoflurane for tissue collection. For experiments using frozen sections, the mice were perfused transcardially (10 ml/min) with 40 ml of 0.1 M phosphate buffered saline (PBS, pH 7.3) containing heparin (1 U/ml) followed by 40 ml of 4% paraformaldehyde (PFA) in PBS. Tissues were collected and stored in fixative overnight at 4°C, rinsed in PBS, and cryoprotected by storage in 20% sucrose in PBS at 4 °C for about 3 days. Tissues were then stored at − 80 °C until sectioning. Frozen 30 μm sections were cut at − 14 °C using a Leica CM 3050S cryostat and collected on charged slides. Slide-mounted sections were stored at − 80 °C in slide boxes wrapped in aluminum foil.

Separate animals were used to prepare whole mounts from the intestines. The gastrointestinal tract (GI) was removed intact from the level of the fundus of the stomach to the rectum and placed in cold