Brain Research Bulletin 111 (2015) 76–83

Contents lists available at ScienceDirect

Brain Research Bulletin

journal homepage: www.elsevier.com/locate/brainresbull

Research report

Enteric plexuses of two choline-acetyltransferase transgenic mouse lines: Chemical neuroanatomy of the fluorescent protein-expressing nerve cells

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ARTICLE INFO

Article history:
Received 30 October 2014
Received in revised form
23 December 2014
Accepted 5 January 2015
Available online 12 January 2015

Keywords:
Cholinergic neuron
Enteric motoneurons
Green and yellow fluorescent protein
Calcium-binding proteins
Vasoactive intestinal polypeptide

ABSTRACT

We studied cholinergic circuit elements in the enteric nervous system (ENS) of two distinct transgenic mouse lines in which fluorescent protein expression was driven by the choline-acetyltransferase (ChAT) promoter. In the first mouse line, green fluorescent protein was fused to the tau gene. This construct allowed the visualization of the fiber tracts and ganglia, however the nerve cells were poorly resolved. In the second mouse line (ChATcre-YFP), CRE/loxP recombination yielded cytosolic expression of yellow fluorescent protein (YFP). In these preparations the morphology of enteric neurons could be well studied. We also determined the neurochemical identity of ENS neurons in muscular and submucous layers using antibodies against YFP, calretinin (CALR), calbindin (CALB), and vasoactive intestinal peptide (VIP). Confocal microscopic imaging was used to visualize fluorescently-conjugated secondary antibodies. In ChATcre-YFP preparations, YFP was readily apparent in somatodendritic regions of ENS neurons. In the myenteric plexus, YFP/CALR/VIP staining revealed that 34% of cholinergic cells co-labeled with CALR. Few single-stained CR-positive cells were observed. Neither YFP nor CALR co-localized with VIP. In GFP/CALB/CALR staining, all co-localization combinations were represented. In the submucosal plexus, YFP/CALR/VIP staining revealed discrete neuronal populations. However, in separate preparations, double labeling was observed for YFP/CALR and CALR/VIP. In YFP/CALR/CALB staining, all combinations of double staining and triple labeling were verified. In conclusion, the neurochemical coding of ENS neurons in these mouse lines is consistent with many observations in non-transgenic animals. Thus, they provide useful tools for physiological and pharmacological studies on distinct neurochemical subtypes of ENS neurons.

1. Introduction

The enteric nervous system (ENS) is contained within the tubular wall of the gastrointestinal tract. It has two ganglionated plexuses. The myenteric plexus (MP) is between the outer longitudinal and circular muscle layers, while the submucosal plexus (SMP) is in the connective tissue below the mucosal layer. These plexuses contain more than a dozen neuron types which have characteristic neurochemical coding in different species and in different gut regions within a species. These neurons form complete reflex arches comprising sensory neurons, motor neurons, and interneurons (Furness, 2000).

Speaking generally, five broad types of ENS motor neurons have been described in these plexuses: (i) excitatory neurons to gut muscle, (ii) inhibitory neurons to gut muscle, (iii) secretomotor/vasodilator neurons, (iv) secretomotor neurons that are not vasodilators and (v) neurons innervating endocrine cells (Timmermans et al., 1997; Furness, 2000; Brehmer et al., 2004; Qu et al., 2008; Freytag et al., 2008). Morphologically, these cells fall into the broad Dogiel type I category (cells with lamellar or filamentous dendrites, elongated cell body and usually one axon). The excitatory motor neurons release acetylcholine (Paton et al., 1971) and are immunoreactive for both the synthesizing enzyme for acetylcholine (choline acetyltransferase–ChAT) and for tachykinins in most species (Costa et al., 1996; Lippi et al., 1998; Furness, 2000), including mouse (Sang and Young, 1998). It seems fairly clear that the different transmitters that are implicated in inhibitory transmission to the muscles arise a single population

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http://dx.doi.org/10.1016/j.brainresbull.2015.01.001
0361-9230/

Table 1
Primary antibodies used in the triple labeling experiments.

Antigen	Host species	Dilution	Supplier	Remark
Green fluorescent protein	Chicken	1:4000	Aves Labs Inc., Tigard, OR, USA	cat # GFP-1020
Vasoactive Intestinal Peptide	Rabbit	1:500	Millipore, Temecula, CA, USA	Synthetic porcine VIP18-28
Calretinin	Goat	1:1000	Swant, Marly, Switzerland	Against human recombinant protein
28 kDa calbindin	Rabbit	1:1000	Swant, Marly, Switzerland	Rat recombinant Calbindin 28 KDa

of inhibitory neurons, immunoreactive for nitrogen monoxide synthase (NOS), vasoactive intestinal polypetide (VIP) and pituitary adenylate cyclase activating peptide (PACAP; Costa et al., 1996; Timmermans et al., 1997). Besides, presynaptic inhibition of acetylcholine release by noradrenalin may also be as regolatory mechanism (Paton and Vizi, 1969; Vizi, 1977). Two types of intestinal secretomotor neurons, cholinergic and non-cholinergic, have been identified (Furness, 2000). The non-cholinergic neurons appear to provide most of the local reflex responses, and employ VIP, or a related peptide, as their primary neurotransmitter (Costa et al., 1996; Timmermans et al., 1997; Mongardi-Fantaguzzi et al., 2009). The latter also holds true for mice (Qu et al., 2008; Mongardi-Fantaguzzi et al., 2009).

The intrinsic primary afferent neurons comprise at least three major categories: stretch-sensitive large cells in the MP, the mucosal chemoreceptors, and the mucosal mechanoreceptors. Most sensory neurons fall into the Dogiel type II category (large cells often with several axons and quite different neurochemical coding across species (e.g. Timmermans et al., 1997; Furness et al., 2004). In mice, these cells also contain ChAT (Qu et al., 2008); however, they were not found in the SMP (Mongardi-Fantaguzzi et al., 2009). One type of orally ('ascending') and three types of anally ('descending') directed interneurons have been identified in the guinea-pig small intestine. Both the ascending and the descending interneurons contain ChAT in most species, including mouse (Costa et al., 1996; Furness, 2000; Sang and Young, 1998; Qu et al., 2008).

From the above description, it is clear that ChAT-positive neurons constitute a large proportion of all ENS neurons in all mammalian species, including mouse. Transgenic mouse lines have been developed to investigate the anatomy, physiology and pharmacology of circuit-specific cholinergic neurons in the brain (Gong et al., 2007; Grybko et al., 2011; von Engelhardt et al. 2007). However, similar studies on the ENS are only now becoming an area of focus (Gautron et al., 2013; Hao et al., 2013; Erickson et al., 2014; Foong et al., 2014). In fact, most studies regarding the ENS of transgenic mice relate to disease conditions (Aubé et al., 2007; Kuo et al., 2010;) and not designed to investigate the neurochemical identity of ENS neurons per se. Here, the putative cholinergic elements of the small intestine of two ChAT transgenic mouse lines are examined from the viewpoint of the use of these animals for microanatomical, physiological and pharmacological research. Using immunocytochemistry and these transgenic mice, we have also tested the hypothesis that neurons in the MP and SMP of choline-acetyltransferase transgenic mouse lines form the same diverse populations that can be divided into a number of neurochemically distinct cell groups as has been done on non-transgenic (Sang and Young, 1998; Qu et al., 2008) and transgenic (Hao et al., 2013; Erickson et al., 2014; Foong et al., 2014) mouse lines. This approach will also enable further studies on the fate of individual cholinergic neurons during development and aging.

2. Materials and methods

Transgenic mice. All procedures were performed in accordance with the University of Montana Institutional Animal Care and Use Committee (AUP 026-11). After wean, mice were socially housed in gender-specific groups of 4–5 littermates to a cage. They were kept under standard lighting conditions (12:12 h light:dark cycles), fed and watered *ad libitum*. Animals used in these experiments were between 9 and 14 weeks of age ($n = 11$). ChAT-CRE mice (GM24 founder line, MMRRC 017269-UCD; Ivanova et al., 2010; Gong et al., 2007) were genotyped for zygosity and using qPCR (Tesson et al., 2002), and maintained as a homozygous CRE mouse line as described previously for parvalbumin-CRE mice (Yi et al., 2014). Cre recombinase is a tyrosine recombinase enzyme derived from a bacteriophage that is able to perform site-specific recombination events. Homozygous ChAT-CRE mice were crossed with homozygous Rosa26-YFP (yellow fluorescent protein) mice (Jackson Labs stock number 007920; Soriano, 1999; Madisen et al., 2009) to yield ChAT-CRE/Rosa-YFP heterozygous mice (ChATcre-YFP mice; Hao et al., 2013; $n = 8$ was used in this study). ChAT-tauGFP (green fluorescent protein) mice ($n = 3$) were also employed (Grybko et al., 2011). In this construct GFP is coupled to the cytoskeletal protein tau; so whenever the tau gene is transcribed GFP will also be transcribed with it. Cells containing the transgene expressed endogenous YFP (ChATcre-YFP) or GFP (ChAT-tauGFP), and these signals could be amplified with post hoc immunocytochemistry using an anti-GFP antibody (Table 1).

2.1. Tissue preparation

Animals were euthanised with an overdose of isoflurane, the abdomen opened. We dissected out the small intestine in warm (37 °C) physiological saline, discarded the duodenum and the upper 20 cm of the jejunum was used for our study. After ligation of the distal part, the gut lumen was gradually extended with warm (37 °C) physiological saline as described by Gamage and coworkers (Gamage et al., 2013) and then fixed in ice-cold 4% paraformaldehyde dissolved in 0.1 M phosphate buffer overnight. Samples were then cut into 1–2 cm long rings, and thoroughly washed in phosphate buffered saline (PBS, pH 7.4; 0.9% NaCl) for 4–6 h. The rings were cut open along the mesenteric border and the mucosal layer was removed by using fine forceps. In some cases, the muscular layer and the submucous layers were dissected from each other and processed separately for immunohistochemistry.

2.2. Immunohistochemistry

Tissues were treated with 1% Triton X-100 dissolved in PBS for 1 h and then preincubated for 1 h in an antibody diluent solution as described previously (Gábriel et al., 1992). This antibody diluent contains 1% bovine serum albumin dissolved in PBS and supplemented with 1% Triton-X 100 to diminish nonspecific primary antibody binding and non-specific fluorescence. Two triple labeling experiments were designed based on the results obtained by the Furness group on non-transgenic mice (Qu et al., 2008; Mongardi-Fantaguzzi et al., 2009) and carried out with the following primary antibody combinations (tissue samples from $n = 6$ animals were used for both): (i) anti-GFP to label the ChAT-expressing putative cholinergic neurons, anti-calretinin as a secondary marker for several cholinergic cell types and anti-VIP for inhibitory motorneurons in the MP and secretomotor neurons in the SMP; (ii) anti-GFP, anti-calretinin (CALR) and anti-calbindin 28 kDa (CALB), the latter is to identify a cholinergic cell population that is potentially different from the ChAT/CALR population. The sources and dilutions