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

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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.

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Keywords

cholinergic neuron; enteric motoneurons; green and yellow fluorescent protein; calcium-binding proteins; vasoactive intestinal polypeptide

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 et al., 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 of inhibitory neurons, immunoreactive for nitrogen monoxide synthase (NOS), vasoactive intestinal polypeptide (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 regulatory mechanism (Paton and Vizi, 1969; Vizi, 1977). Two types of intestinal secretomotor neurons, cholinergic and non-cholinergic, have been identified (Furness et al., 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 et al., 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.

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 9–14 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 et al., 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).

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.

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 (Gábel 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; Montgardi 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 motoneurons 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 of the primary antibodies are summarized in Table 1. Final dilutions of the primaries were made with the antibody diluent. After overnight incubation on a shaker at room temperature, the samples were washed in PBS for 6×10 min and the secondary antibodies were applied as follows: donkey anti-chicken Alexa 488 (1:400; Life Technologies); donkey anti-rabbit Alexa 647 (1:200; Life Technologies) and donkey anti-goat Alexa 350 (1:200; Life Technologies). Controls were made by omitting the primary antibodies, in which case the relatively weak intrinsic fluorescence of the genetically modified cholinergic neurons was seen only. Cross-reactivity of the non-corresponding secondary antibodies have also been checked in pairs (the chicken primary against the anti-goat and anti-rabbit secondary, the goat primary against the anti-rabbit and anti-chicken secondary and rabbit primary against the anti-chicken and anti-goat secondary). No cross-reactivity was observed in these experiments.

Imaging

Confocal images have been taken with the Fluoview1000 confocal microscope (Olympus Imaging America, Center Valley, PA, USA) with lasers and the filter settings optimized for the above described Alexa dyes. Individual and serial optical slices (1–3 µm thick) were taken using the Fluoview 3.1 program. Images were adjusted for contrast only. Further

processing (assembling tables and labeling individual figures) were made with the functions in Adobe Photoshop 7.0.

Results

General description of the ENS morphology in the two transgenic constructs

The characteristic pattern of the enteric plexuses can be recognized in both transgenic constructs after amplification with an anti-GFP antibody. This was consistent with the pattern expected for ENS cholinergic neurons. In the ChAT-tauGFP construct, the MP (Fig. 1a) and in serial optical sections the underlying SMP (Fig. 1b) is clearly visible. The individual ganglia in both plexuses are seen to contain labeled cell bodies (Fig. 1c and d) and among these ganglia the fiber tracts run innervating the muscle layers (Fig. 1a, c). Note that the labeling of cell bodies and the dendrites of the individual neurons is not optimal, often only the cytoplasm around the cell nuclei and the axons are stained. The axonal processes of neurons in the ChAT-tauGFP construct could be well resolved. Very often these processes were seen to run through the entire length or width of our preparations. The varicosities of these axons were prominent features of the images taken (Fig. 1c, d).

When one examines the gut plexuses in the ChATcre-YFP construct, a similar pattern can be recognized if compared to the ChAT-tauGFP animals. The ganglia and the fiber tracts are clearly visible both in the MP (Fig. 2a) and the SMP (Fig. 2b). However the characteristic shape of the labeled MP neurons (their Dogiel type I cell morphology) is much better resolved in these preparations, especially if one can also perfectly remove the circular muscle layer (Fig. 2c). Axonal processes were satisfactorily stained, however their varicosities were less prominently featured (Fig. 2c, d). The difference between the two mouse lines may also be due to the different nature and intracellular distribution of the reporter gene products (i.e. diffusible nature and the cytoplasmic localization of the creYFP). The morphology of SMP neurons could also be better revealed than in the ChAT-tauGFP construct (Fig. 2d). Therefore, we performed all further experiments in ChATcre-YFP.

Neurochemical identity of the putative cholinergic interneurons

As described above, two triple labeling experiments were carried out on the neurons of both plexuses. The co-localization results are gathered in Table 2. In the MP, GFP/CALR/VIP staining clearly showed that there was a population of large GFP-positive, presumably cholinergic cells (around 48% of all labeled neurons) that did not co-label with CALR. In most of the remaining GFP-positive cells, CALR was present (approximately 35% of the GFP-positive cells); however, in a few CALR-positive cells GFP was absent (Fig. 3a). GFP and VIP, similarly to VIP and CALR, did not co-localize and therefore represented exclusive non-overlapping populations. VIP-immunoreactive cells had a tendency to stand alone often outside of ganglia, while GFP-positive cells were usually grouped together (Fig. 3b). VIP-immunoreactive puncta formed pericellular baskets around the GFP alone, GFP/CALR and CALR alone cells, as well as around non-labeled somata (Fig. 3a, c).

In the other triple-labeling combination (GFP/CALR/CALB), a similarly or even more colorful picture has emerged. Besides single GFP, CALB and CALR labeled cells (Fig. 3d), all possible combinations of double labeling (GFP/CALB, GFP/CALR, CALB/CALR; Fig. 3e, f) and also triple labeling occurred (Fig. 3g). Therefore this labeling procedure alone has defined seven neurochemically distinct types of ENS neurons in the MP.

The same two triple labeling experiments gave partially similar results when the SMP was examined. In GFP/CALR/VIP labeling, we observed single-labeled cells for each marker (Fig. 4a). VIP cells again were seen most often standing again alone (Fig. 4b). Double labeling was observed in the case of GFP/CALR (Fig. 4 a,b) and CALR/VIP (Fig. 4c). We did not observe any GFP/VIP double-stained cells. In the other triple staining (GFP/CALR/CALB), again, a very diverse picture has emerged. Most of the cells contained at least two of these markers (Fig. 4d). There were a minimal number of single labeled cells, though single-labeled GFP+ cells were extremely rare (Fig. 4e). All possible combinations of double staining were verified and we also observed triple-labeled cells (Fig. 4f).

Discussion

In this paper, we have confirmed that the enteric plexuses of ChATcre-YFP and ChAT-tauGFP mice indeed contain a large number of GFP-immunoreactive circuit elements. In the ChATcre-YFP mouse line, the microscopic resolution of the cellular structures is superior to the ChAT-tauGFP mouse line, therefore this line can be preferentially used for studying the fine details of the enteric plexuses and nerve cells, and also it may be more useful when the chemical coding of the enteric neurons is under investigation. With the two triple labeling experiments that we performed, we were able to define more than a dozen distinct ENS neuron types based on their location and neurochemical identity.

Our findings described in the current paper fully agree with Sang and Young (1998) with regard to the fact that a large subpopulation of the gut neurons are cholinergic and that the majority (more than 50%) of the cholinergic neurons contain CALR. These cells contribute robustly to the innervation of the musculature of the gut wall in mouse (Qu et al., 2008) and also in rat (Sayegh and Ritter, 2003) and rabbit (Gábel et al., 1998). This feature may be general in case of non-carnivorous mammals (Furness, 2000), including humans (Beuscher et al., 2014). At the same time we have detected somewhat lower level of co-localization between YFP and CALB than previous studies (Sang and Young, 1998; Qu et al., 2008), but found more cells that contained CALB only. As it was already noted by Furness (2000), there may be a slight variation between different mouse strains. We have to stress that with one exception (YFP/CALR/VIP neurons in the MP; their approximate proportion is about 2–4%) we could identify all formerly described marker combination, therefore our results qualitatively agree with those of other authors (Sang and Young, 1998; Qu et al., 2008; Hao et al., 2013).

During embryonic development, migration of these cells could be observed using the same transgenic approach (Hao et al., 2013). The model is also useful studying aging syndromes (Rayner and Horowitz, 2013) and the different disease conditions associated with altered cholinergic innervation of the gut musculature (Clarke et al., 2007; Alexandrescu et al.,

2013). Co-expression of YFP with CALB was also observed, along with a population of CALB-positive cells that did not express the cholinergic phenotype in the MP (Qu et al., 2008). Although we did not specifically count the strongly CALB-positive neurons, they probably represent more than 2% of the MP neuron population. This difference may have been due to the primary antibody source and/or its lower dilution than in the above cited study.

In addition to the numerous similarities we observed with previous studies, there were some differences also noticed. One striking difference was the total lack of co-localization between GFP labeling and VIP staining in the cholinergic interneurons in our preparations, compared to those of others (Sang and Young, 1998; Qu et al., 2008). This result was observed in both enteric plexuses. There are several possible explanations to this fact. One is that we have not used any method that would enhance peptide localization in the cell bodies of the enteric neurons and thus we failed to stain all VIP-containing cells. Indeed, reports stress the importance of colchicine pretreatment when one uses antibodies against neuropeptides (i.e. Lantos et al., 1995). However it is unlikely that we would have missed an entire subclass of cells, since we observed 38 VIP-positive cells without GFP co-label while detected 16 cells with CALR as a co-localizing marker in the SMP. Alternatively, there may be slight variations in neurochemical identity of MP neurons in different strains of mice, especially transgenic mice. Such examples are well-known in the case of the retina (Haverkamp et al., 2009; Ivanova et al., 2010; Lu et al., 2013).

The cells that contain the ChATcre-YFP construct show intrinsic fluorescence in living tissue. Therefore they can be targeted with microelectrodes for physiological and pharmacological examinations. The possible experiments range from studying neurotransmitter action and receptor function on individual cholinergic enteric neurons (Harrington et al., 2010) to eventually recording from two cells far from each other but physiologically connected (Thomas et al., 1999). One can easily study their development and migration (Hao et al., 2013; Erickson et al., 2014), eventually even in *in vitro* conditions. We have to note however that according to the above cited papers there is a slight discrepancy between ChAT-immunoreactive cells in ChATcre-YFP mice. However, the ChATcre-YFP mouse line used in our study came from a different source than the ones that were used by them (MMRRC vs. Jackson Laboratories), and does not necessarily imply that the same discrepancy exists. Also, according to our own experience, anti-ChAT antibodies label cholinergic cells with slightly different activities in different tissues.

It might also be interesting to study the transmitter phenotype changes of autonomic neurons coined long time ago (Morris and Gibbins, 1989) but never properly examined experimentally in enteric nerve cells. Further studies might include the examination of selective loss of cholinergic neuron populations in diseases and aging (Wade and Coven, 2004; Chandrasekharan and Srinivasan, 2007; Lourensen et al., 2009; Saffrey, 2013). The neurochemical coding of different cholinergic cell populations described earlier and confirmed in this study for the ChATcre-YFP mouse line makes it possible to follow the fate of individual cholinergic cell types under the above conditions.

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Highlights

- Fluorescent protein expression could be revealed in the enteric plexuses of both the ChAT-tauGFP and ChATcre-YFP transgenic mouse lines
- In the ChAT-tauGFP construct axonal processes are prominently labeled
- In the ChATcre-YFP construct, the shape of neurons is much better resolved
- The neurochemical coding of enteric neurons in these mouse lines is consistent with many observations in non-transgenic animals

Fig. 1. Cell bodies and axons are prominent features in the enteric plexuses in ChAT-tauGFP mice

(a) Ganglia and nerve strands of the MP. Arrows: ganglia and the main strands of the MP; arrowheads: small nerve bundles innervating the muscular layer. (b) In serial optical sections, the underlying SMP is clearly visible. Arrowheads: ganglia of the SMP. (c) GFP-expressing neurons in the MP. Arrow: GFP-positive cell bodies; arrowheads: nerve fibers innervating the circular muscle layer. (d) Submucous plexus: Arrows: labeled cells in SMP ganglia.

Scale bars: 100µm in all Figures.

Fig. 2. Morphology of the enteric plexuses and the individual nerve cells are revealed in ChAT^{Rosa} mice

(a) Ganglia and the fiber tracts are of the MP. G: ganglion; arrows: connective strands; arrowheads: fiber bundles innervating the muscle layer. (b) The SMP. Arrows: ganglia; arrowheads: connective strands. (c) Dogiel type I cells in the MP. (d) Morphology of the SMP.

Scale bars: 100 μ m in all Figures.

Fig. 3. Neurochemical identity of GFP-positive MP neurons in ChAT-Rosa mice
(a-c) GFP/CALR/VIP; **(d-h)** GFP/CALR/CALB staining. **(a)** Several cells are labeled only for GFP, others for CALR only (arrows). Asterisks denote GFP/CALR-negative cells surrounded by VIP-positive pericellular baskets. **(b)** GFP and VIP do not colocalize. Arrow: GFP-labeled cells; arrowhead: VIP-positive cell. **(c)** VIP-immunoreactive puncta formed pericellular baskets around the GFP alone, GFP/calretinin and calretinin alone cells, as well as around (asterisks) non-labeled somata. **(d)** Single GFP, CALR and CALB-labeled cells and several double-labeled cells are observed. Asterisk: **(e, f and g)** All possible

combinations of double labeling (GFP/CALB: orange yellow in **e**; GFP/CALR: pale yellow in **f**; CALB/CALR: pink to lilac in **g**) and a triple-labeled cell (white cell in **h**) are also seen. **Scale bars:** 20 μm in **a** and **f**, 30 μm in **b**, **d** and **h**, 40 μm in **c**, 10 μm in **e** and **g**.

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Fig. 4. GFP/CALR/VIP (a-c) and GFP/CALR/CALB labeling (d-f) in the SMP

(a) GFP-positive single-labeled (arrowhead) and GFP/CALR double-labeled (double arrowhead) cells. (b) (arrow) CALR, (asterisk) VIP-positive single-labeled and (arrowhead) CALR/VIP double labeled cells. (c) VIP cells (asterisk) tend to be isolated from other ganglionic SMP cells. (d) In case of GFP/CALR/CALB staining, most cells contained at least two neurochemical markers. (e) GFP single-labeled cell (arrow) among several double-labeled cells (pink: CALR/CALB; orange-yellow: GFP/CALB; pale-yellow: GFP/CALR). (f) white: triple-labeled cell.

Scale bars: 10 μm in **a**, **b** and **c**, 20 μm in **e** and 50 μm in **d** and **f**.

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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

Table 2

Numbers and percentages of neurochemically identified enteric nerve cells as counted from the triple labeled preparations from two (A1 and A2) ChATcre-YFP animals.

Object and Staining	MP GFP/CALR/VIP (N=302)		SMP GFP/CALR/VIP (N=116)	
Animals	A1 (n=143)	A2 (n=149)	A1 (n=59)	A2 (n=57)
GFP+ cells (% are in total GFP+ cell number %)				
GFP+/CALR-/VIP-	70 (61.4%)	76 (64.4%)	22 (68.7%)	20 (66.7%)
GFP+/CALR+/VIP-	44 (33.6%)	42 (35.6%)	9 (28.1%)	9 (30.0%)
GFP+/CALR-/VIP+	0 (0%)	0 (0%)	0 (0%)	0 (0%)
GFP+/CALR+/VIP+	0 (0%)	0 (0%)	1 (3.1%)	1 (3.3%)
CALR+ cells (% are in total CALR+ cell number %)				
CALR+/GFP-/VIP-	18 (29%)	20 (32%)	10 (34.5%)	11 (37.9%)
CALR+/GFP+/VIP-	44 (71%)	42 (68%)	9 (31.0%)	9 (31.0%)
CALR+/GFP-/VIP+	0 (0%)	0 (0%)	9 (31.0%)	8 (27.5%)
CALR+/GFP+/VIP+	0 (0%)	0 (0%)	1 (3.4%)	1 (3.4%)
VIP+ cells (% are in total VIP+ cell number %)				
VIP+/GFP-/CALR-	11 (100%)	11 (100%)	8 (44.4%)	8 (47.0%)
VIP+/GFP-/CALR+	0 (0%)	0 (0%)	9 (50.0%)	8 (47.0%)
VIP+/GFP+/CALR-	0 (0%)	0 (0%)	0 (0%)	0 (0%)
VIP+/GFP+/CALR+	0 (0%)	0 (0%)	1 (5.6%)	1 (5.9%)
Object and Staining	MP GFP/CALR/CALB (N=242)		SMP GFP/CALR/CALB (N=116)	
Animals	A1 (n=116)	A2 (n=126)	A1 (n=56)	A2 (n=60)
GFP+ cells (% are in total GFP+ cell number %)				
GFP+/CALR-/CALB-	45 (61.6%)	49 (61.2%)	8 (30.7%)	8 (27.6%)
GFP+/CALR+/CALB-	16 (22.0%)	19 (23.7%)	2 (7.7%)	1 (3.4%)
GFP+/CALB+/CALR-	7 (9.6%)	7 (8.8%)	5 (19.2%)	6 (20.7%)
GFP+/CALR+/CAL+	5 (6.8%)	5 (6.3%)	11 (42.3%)	14 (48.3%)
CALR+ cells (% are in total CALR+ cell number %)				
CALR+/GFP-/CALB-	10 (19.2%)	11 (18.6%)	2 (5.4%)	2 (4.9%)
CALR+/CALB+/GFP-	21 (40.4%)	24 (40.7%)	22 (59.6%)	24 (58.5%)
CALR+/GFP+/CALB-	16 (30.8%)	19 (32.2%)	2 (5.4%)	1 (2.4%)
CALR+/GFP+/CAL+	5 (9.6%)	5 (8.5%)	11 (29.7%)	14 (34.1%)
CALB+ cells (% are in total CALB+ cell number %)				
CALB+/GFP-/CALR-	12 (26.7%)	11 (23.5%)	6 (13.6%)	5 (10.2%)
CALB+/GFP+/CALR-	7 (15.6%)	7 (14.5%)	5 (11.4%)	6 (12.2%)
CALB+/CALR+/GFP-	21 (46.7%)	24 (51.0%)	22 (50.0%)	24 (49.0%)
GFP+/CALR+/CAL+	5 (11.1%)	5 (11.1%)	11 (25.0%)	14 (28.6%)