Development of myenteric cholinergic neurons in *ChAT-Cre;R26R-YFP* mice

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Abstract:

Cholinergic neurons are the major excitatory neurons of the enteric nervous system (ENS), and include intrinsic sensory neurons, interneurons and excitatory motor neurons. Cholinergic neurons have been detected in the embryonic ENS, however, the development of these neurons has been difficult to study as they are difficult to detect prior to birth using conventional immunohistochemistry. In this study, we used *ChAT-Cre;R26R-YFP* mice to examine the development of cholinergic neurons in the gut of embryonic and postnatal mice. Cholinergic (YFP+) neurons were first detected at embryonic day (E)11.5, and the proportion of cholinergic neurons gradually increased during pre- and postnatal development. At birth, myenteric cholinergic neurons comprised less than half of their adult proportions in the small intestine (25% of myenteric neurons were YFP+ at P0 compared to 62% in adults). The earliest cholinergic neurons appear to mainly project anally. Projections into the presumptive circular muscle were first observed at E14.5. A subpopulation of cholinergic neurons co-express calbindin through embryonic and postnatal development, but only a small proportion co-expressed neuronal nitric oxide synthase. Our study shows that cholinergic neurons in the ENS develop over a protracted period of time.

Introduction:

The enteric nervous system (ENS) is a network of neurons and glia located in the wall of the gastrointestinal tract that controls or modulates many gastrointestinal functions, including motility. Acetylcholine (ACh) is the major excitatory neurotransmitter of the ENS (Furness, 2000; Galligan et al., 2000; Gwynne and Bornstein, 2007; Harrington et al., 2010). In adult mice and guinea pigs, around 60% of myenteric neurons are cholinergic, and they include intrinsic sensory neurons, interneurons, and excitatory motor neurons to the longitudinal and circular muscle layers (Costa et al., 1996; Sang and Young, 1998; Qu et al., 2008). It is therefore important to investigate the development of cholinergic neurons as they are vital for the control of gastrointestinal motility.

All the neurons and glia of the ENS arise from neural crest cells, most of which originate from the caudal hindbrain region of the neural tube and migrate into the gastrointestinal tract during embryonic development (Sasselli et al., 2012; Obermayr et al., 2013a). The small and large intestines are colonized by a rostral-to-caudal wave of migrating vagal enteric neural crest-derived cells (ENCCs), which differentiate into neurons, and later, glia (Baetge and Gershon, 1989; Kapur et al., 1992; Young et al., 2002; Young et al., 2003). Sacral neural crest-derived cells also migrate into the gut from the developing pelvic primordia. The sacral ENCCs migrate rostrally, contributing to a smaller population of neurons and glia in the caudal colon (Burns and Le Douarin, 1998; Kapur, 2000; Wang et al., 2011; Erickson et al., 2012). In the developing mouse, ENCCs first enter the developing small intestine at E10.5 (Kapur et al., 1992). Approximately 10-20% of ENCCs express pan-neuronal markers at this age (Baetge and Gershon, 1989; Young et al., 1999). By E11.5, a sub-population of these early enteric neurons are also immunoreactive for the subtype specific markers, calbindin and neuronal nitric oxide synthase (nNOS; Hao et al., 2010), and transcripts encoding the cocaine- and amphetamine regulated transcript (CART) have also been detected (Heanue and Pachnis, 2006).

Studies of the prenatal development of enteric cholinergic neurons have been hampered by poor sensitivity of antibodies to choline acetyltransferase (ChAT). In both mice and rats, ChAT-immunoreactive (IR) neurons cannot be detected until late embryonic and early postnatal stages (Vannucchi and Faussone-Pellegrini, 1996; Hao and Young, 2009). Likewise, in the zebrafish, ChAT-IR neurons are not detectable in the gut of embryos or larvae (Olsson et al., 2008; Uyttbroek et al., 2010), despite pharmacological studies showing control of gut motility by endogenous ACh from an early age (Holmberg et al., 2004).

However, cholinergic neurons were identified in E11 mouse gut by the synthesis of radio-labeled ACh following uptake of radio-labeled choline, showing that ChAT is present at this age (Rothman and Gershon, 1982). The presence of cholinergic neurons in the stomach was also reported in E13.5 *ChAT^{BAC}-eGFP* mice (Tallini et al., 2006).

In this study, we used *ChAT-Cre;R26R-YFP* mice to examine cholinergic neurons of the myenteric plexus. *ChAT-Cre;R26R-YFP* mice were generated by crossing *ROSA26^{YFP}* reporter mice with a *ChAT-Cre* driver line. We found that the earliest age at which YFP+ cholinergic neurons could be detected in *ChAT-Cre;R26R-YFP* mice was E11.5. We also investigated changes in the proportion, and the co-expression of nNOS and calbindin in YFP+ cholinergic neurons through embryonic and postnatal development. We show that YFP+ cholinergic neurons do not reach adult proportions until after P10 in the small intestine.

Materials and Methods

Animals

Embryonic, postnatal and adult gut tissue were collected from *Chat-Cre;R26R-YFP* mice, which were generated by mating *Chat-Cre* mice with *ROSA26^{YFP}* reporter mice (both obtained from the Jackson Laboratory, Bar Harbor, ME, USA). *Chat-Cre* mice were on a mixed C57BL6/J and 129S5/SvEv background (nomenclature: B6;129S6-Chattm1(cre)Lowl/J) and *ROSA26^{YFP}* mice were on a C57BL6/J background. Midday of the day on which a plug was found was designated as E0.5. All adult mice were killed by cervical dislocation, or CO₂ followed by cervical dislocation. Postnatal day (P)0, P10 and embryonic mice were killed by decapitation. All experiments were approved by the Animal Ethics Committee of the Departments of Anatomy and Neuroscience, Pathology, Pharmacology and Physiology at the University of Melbourne.

Tissue preparation and immunohistochemistry

All tissue was fixed and processed for immunohistochemistry as described previously (Hao et al., 2010). For immunohistochemistry on postnatal and adult tissue, the middle 1/3 of the small intestine and middle 1/3 of the colon were dissected from animals in 0.1 M phosphate buffer (PB; pH = 7.3 – 7.4). The gut was opened along the mesenteric border, pinned out on sylgard-coated dishes and fixed using 4% paraformaldehyde in PB for 1 – 4 hrs at room temperature or at 4°C overnight. After washing in PB, the mucosa and submucosa were removed from all preparations prior to permeabilization in 0.5% Triton X-100 in PB for 30 minutes. Segments of the circular muscle layer were also removed from P10 and adult gut. For embryonic tissue, the entire gut was dissected, fixed (as above) and then processed for immunohistochemistry as an intact gut tube. All tissue was incubated in primary antisera at 4°C overnight, washed in PB and then incubated in secondary antisera for 1.5 – 4 hours at room temperature. All antibodies were diluted in 0.1 M PB.

Antibody characterization

The primary antisera used are shown in Table 1. Rabbit anti-calbindin was produced against recombinant rat calbindin D-28k protein. The specificity of the antibody was

confirmed by the absence of staining in the brain of calbindin null mutant mice (manufacturer's information), and in myenteric neurons following pre-absorption with calbindin protein (Reiche et al., 1999). Goat anti-calretinin was raised against human recombinant calretinin. The specificity of the antibody was confirmed by the absence of staining in the cerebral cortex of calretinin null mutant mice (manufacturer's information), and the absence of staining in the visual cortex after pre-absorption with recombinant human calretinin protein (Disney and Aoki, 2008). Goat anti-ChAT was raised against the human placental enzyme; this antibody recognizes a protein of 70-74 kDa in mouse brain lysates on Western Blot (manufacturer's information), and has been used in many previous studies of the mouse ENS (Sang and Young, 1998; Qu et al., 2008; Mongardi Fantaguzzi et al., 2009; Hotta et al., 2013). Chicken anti-GFP was raised against the recombinant full length GFP protein. Goat anti-GFP was produced against GST-GFP fusion protein corresponding to the full length amino acid sequence (246aa) derived from *Aequorea victoria*. Rabbit anti-GFP was raised against GFP isolated from *A. victoria*. The specificities of the three anti-GFP antibodies were tested on wholemount preparations of small intestine from adult *ROSA26^{YFP}* mice, where no staining was observed. Human anti-Hu was collected from a patient. This antiserum recognizes a single band of 41 kDa on extracts of chicken brain (Erickson et al., 2012). Sheep anti-nNOS was raised against rat brain nNOS (Norris et al., 1995). In mouse enteric neurons, immunoreactivity is eliminated following pre-absorption of the diluted antibody with purified nNOS protein (Young and Ciampoli, 1998). A list of secondary antisera used is shown in Table 2.

Imaging

All images were taken on either a Zeiss Pascal laser-scanning confocal microscope, or a Leica SP5 laser-scanning confocal microscope. The brightness and contrast were modified slightly during the production of some figures.

Quantification of neurons in embryonic gut

For the quantification of Hu⁺, YFP⁺, calbindin⁺ and nNOS⁺ neurons, one field of view at 20x magnification (with an area of 390 μm $\times$ 390 μm) was imaged at each region of interest:

(i) the rostral small intestine (SI), which was defined as the gut immediately adjacent to the

stomach at E11.5 and E12.5, and a region in the rostral duodenum at E14.5 and E16.5; (ii) the caudal SI, which was defined as the gut immediately rostral to the caecum at all 4 embryonic ages; and (iii) the proximal colon, which was the gut immediately caudal to the caecum. For each region, a z-series spanning the depth of the stained neurons was collected and projected for cell counting. Cells were manually counted in ImageJ, using the “Cell Counter” plugin, which ensured that each stained cell was counted, but only once. Cells were counted if they contained a Hu-IR nucleus. Cells that were not completely within the field of view were only counted if >50% of their nucleus was identifiable. A minimum of 4 gut preparations from at least 2 different litters was analyzed for each data set. The number of YFP-IR and Hu-IR neurons examined at each embryonic and postnatal age are shown in Table 3.

Quantification of neurons in postnatal and adult gut

Hu+, YFP+, calbindin+ and nNOS+ neurons were quantified in the middle 1/3 of the small intestine and colon at P0, P10 and adult mice (>6 weeks of age). For each field of view, a z-series spanning the entire depth of the myenteric plexus was imaged and projected for cell counting. A minimum of 4 fields of view in each region were analyzed for each animal and the data pooled to calculate the overall count for that animal. Data were obtained from a minimum of 3 mice from 3 different litters. The same cell counting method was used as for embryonic gut. ChAT+ neurons were examined in adult gut only.

Data analysis

Unless otherwise indicated, all data are shown as mean $\pm$ SEM. Statistical analyses were performed in Excel (Microsoft) or Prism (GraphPad). The Kolmogorov-Smirnov normality test was performed on each data set to determine whether it was normally distributed. Normal data sets were analyzed using one-way ANOVA followed by Tukey post-hoc test. Data sets that were not normally distributed were analysed using the Kruskal-Wallis test followed by Dunn's multiple comparison test. Results were considered significant if $p < 0.05$.

Results:

1. Overlap between YFP expression and ChAT-immunoreactivity in myenteric neurons in adult *ChAT-Cre;R26R-YFP* mice

Double-label immunohistochemistry was performed on wholemount preparations of longitudinal muscle/myenteric plexus of small intestine and colon from adult *ChAT-Cre;R26R-YFP* mice using antibodies to ChAT and GFP, which also recognize YFP. As observed previously (Sang and Young, 1998), myenteric ChAT-IR neurons varied considerably in intensity of immunostaining in both the small and large intestines. Neurons were defined as ChAT+ if the staining of their cytoplasm was higher than background such that unstained nuclei could be seen. The vast majority of ChAT-IR neurons were YFP+ ($87 \pm 2\%$ in the small intestine, 445 ChAT-IR neurons; $86 \pm 4\%$ in the colon, 389 ChAT-IR neurons; $n = 4$ animals; Fig. 1). Conversely, only a small proportion of YFP+ cells were not ChAT-IR ($7 \pm 2\%$ in the small intestine; $1 \pm 0.7\%$ in colon; $n = 4$; Fig. 1). All YFP+ cells were also Hu-IR.

As a control, we compared the proportions of myenteric ChAT-IR neurons and nNOS-IR neurons in adult *ChAT-Cre;R26R-YFP* mice to those reported in previous studies. The proportions of Hu-IR neurons that were also ChAT-IR were $65 \pm 3\%$ ($n = 4$ animals, total numbers of neurons counted shown in Table 3) in the small intestine and $58 \pm 4\%$ ($n = 4$) in the colon, which were very similar to previous reports of 63% and 55%, respectively (Sang and Young, 1998). The proportions of Hu-IR neurons that showed nNOS-IR in *ChAT-Cre;R26R-YFP* mice were $29 \pm 2\%$ ($n = 3$) in the small intestine, which is very similar to previous reports (Sang and Young, 1996; Qu et al., 2008; Obermayr et al., 2013b), and $44 \pm 3\%$ ($n = 3$) in the colon, which is slightly higher than previously reported (35%, Sang and Young, 1996; 38%, Hotta et al., 2013).

2. Rostral-to-caudal wave of cholinergic neuron differentiation in the embryonic gut.

The prenatal development of cholinergic neurons was examined in E10.5, E11.5, E12.5, E14.5, E16.5 and E18.5 *ChAT-Cre;R26R-YFP* mice. YFP+ neurons were observed in progressively more distal regions of the gut during embryonic development. Cells expressing pan-neuronal markers also appear in a rostral-to-caudal wave along the gut (Young et al., 1999), but the most caudal YFP+ neuron was always located rostral to the most caudal Hu-IR

enteric neuron at each age. No YFP+ neurons were observed in the gut of E10.5 mice ($n = 9$ embryos examined; Fig. 2), despite the presence of abundant Hu-IR cells in the stomach and small intestine at this age (Fig. 2A,B). The earliest age at which YFP+ neurons were identified was at E11.5, when small numbers of YFP+ neurons were present in both the stomach and the very rostral region of the small intestine (Fig. 2C,D). No YFP+ neurons were observed in the caudal half of the small intestine, or in the colon of E11.5 mice. At E12.5, YFP+ neurons were present along the entire small intestine, but were not observed in the caecum or the colon, whereas the most caudal Hu+ neurons were in the mid-colon (Fig. 2E,F). At E14.5, YFP+ neurons were located in the caecum and proximal colon, whereas Hu+ neurons were present along the entire colon (Fig. 2H-J). YFP+ neurons were rarely observed in the mid-colon and no cells were present in the distal colon. However, YFP+ fibres were present in the distal colon of E14.5 *ChAT-Cre;R26R-YFP* mice (Fig. 2I); these probably arise from extrinsic neurons in the pelvic plexus (Kapur, 2000; Anderson et al., 2006; Anderson et al., 2007; Wang et al., 2011; Erickson et al., 2012). At E16.5, YFP+ neurons were identified in the distal colon, however, they were not observed at the anal end of the colon until E18.5 (Fig. 2K,L).

3. Projections of embryonic cholinergic neurons

At E11.5 and E12.5, neurites were observed to extend from individual YFP+ neurons and were mostly in the same plane of focus as the neuronal cell bodies (Fig. 2D,G). The vast majority of cells extended a single neurite, which, in some cases, branched at various intervals to produce several processes. A few “bipolar” neurons, with two neurites in opposing directions, were observed. From a total of 118 YFP+ neurons in the E12.5 small intestine, we were able to examine the direction of projection of the neurites of 41 YFP+ neurons, of which 71% projected anally (Fig. 2D,G). All of these neurons had a single neurite that did not branch. However, we could not conclusively determine where the processes terminated. At older ages, the direction of projection of individual neurites could not be determined because of the increased density of YFP+ fibers in the myenteric plexus. As early as E11.5 and E12.5, varicosities were clearly visible at regular intervals along the YFP+ fibres (Fig. 2G, 3A). At E14.5, a few YFP+ neurites were observed at the plane of focus of the presumptive circular muscle layer (Fig. 3B), and the density of YFP+ neurites in the circular muscle of the small intestine increased during development (Fig. 3C).

4. Increase in the proportion of cholinergic neurons through embryonic and postnatal development

In the adult small intestine, $62 \pm 0.8\%$ of Hu+ myenteric neurons were YFP+ ($n = 4$ animals). At E11.5, YFP+ neurons comprised only $2 \pm 0.5\%$ of Hu+ neurons in the rostral small intestine (total of 1572 Hu+ neurons from 5 embryos). A progressive increase in the proportion of YFP+ neurons was observed between E11.5 and E16.5 in all regions examined (Fig. 4). The increase in the proportion of YFP+ enteric neurons continued through postnatal development, with the largest increase occurring between P0 and P10 in the small intestine (Fig. 5). At birth, only $25 \pm 4\%$ ($n = 3$) of Hu-IR neurons in the small intestine was YFP+, which is less than half of the adult proportion.

YFP+ neurons were not present in the colon until E14.5. There were large increases in colonic YFP+ neurons during late embryonic development, as the proportion of YFP+ neurons changed from $2 \pm 0.8\%$ ($n = 4$) at E14.5, to $35 \pm 7\%$ ($n = 3$) at P0. Although there were also postnatal increases in YFP+ neuron proportion, these were not as large as those in the small intestine.

5. YFP expression in relation to calbindin and neuronal nitric oxide synthase (nNOS)

We have previously shown that calbindin-IR and nNOS-IR neurons are also present in the E11.5 gut (Hao et al., 2010). At E11.5, the most caudal YFP+ cell was located rostral to the most caudal calbindin+ and nNOS+ neurons (Fig. 6A). At E11.5, approximately 1/3 of the YFP+ neurons were also immunoreactive for calbindin (Fig. 6B,J). Thereafter the proportion of YFP+ neurons that co-expressed calbindin fluctuated during embryonic development and there were large increases in the proportion of YFP+ neurons that showed calbindin immunoreactivity after birth (Fig. 6J). There was very little overlap between YFP expression and nNOS immunoreactivity at any age (Fig. 6C,G-J).

The proportion of Hu-IR neurons that were nNOS-IR was also examined. We observed a small increase in nNOS+ neurons between P0 and adult in both the small intestine and colon, however, these changes were not significant (Fig. 7).

Discussion:

In this study, we used *ChAT-Cre;R26R-YFP* mice to examine the embryonic and postnatal development of cholinergic neurons. Previous studies have identified the presence of cholinergic neurons at E11 and E13.5 in the mouse (Rothman and Gershon, 1982; Tallini et al., 2006), however, the development of cholinergic neurons has been difficult to examine using conventional immunohistochemical techniques. We showed that *ChAT-Cre;R26R-YFP* mice are a useful tool for labeling enteric cholinergic neurons, especially during embryonic development. YFP+ cholinergic neurons were first identified at E11.5, in accordance with Rothman and Gershon (1982), and the proportion of cholinergic neurons was found to increase incrementally with embryonic age, with further increases in early postnatal development. ACh is the major excitatory neurotransmitter in the ENS, and mediates contraction of the muscle layers during mixing and propulsive motility patterns (Harrington et al., 2010). Surprisingly, although neurally-mediated propulsive motility patterns are present at birth in the small intestine of mice (Roberts et al., 2010), cholinergic neurons comprised less than half of their adult numbers in the small intestine of newborn mice.

YFP expression closely mimics ChAT-immunoreactivity in adult *ChAT-Cre;R26R-YFP* mice

The vast majority of ChAT-IR neurons were YFP+ in adult *ChAT-Cre;R26R-YFP* mice and conversely, the vast majority of YFP+ cells were also ChAT-IR. However, approximately 15% of myenteric ChAT-IR neurons were not immunoreactive for YFP. There are a number of possible reasons for this including incomplete Cre expression and/or recombination in ChAT neurons. Taking this into account, the proportions of cholinergic neurons calculated in this study using the *ChAT-Cre;R26R-YFP* mice are likely to be slightly under-estimated. For the YFP+/ChAT- cells, it is possible that these are neurons that transiently exhibited a cholinergic phenotype during development, as YFP expression would be maintained in these cells after ChAT expression is downregulated. Transient expression of various neurochemical phenotypes during development have been commonly observed in the peripheral and central nervous systems (Moody and Bosma, 2005), and include the transiently catecholaminergic neurons of the ENS (Baetge and Gershon, 1989) and nNOS neurons in submucosal ganglia of mice (Young and Ciampoli, 1998).

Differentiation of cholinergic neurons lags behind appearance of pan-neuronal markers:

YFP+ neurons appeared along the intestine in a rostral-to-caudal wave. This wave of appearance of YFP+ cells is likely to result from the rostral-to-caudal migration of ENCCs and also the appearance of ENCCs that express pan-neuronal markers (Kapur et al., 1992; Young et al., 1999). It appears that during differentiation, ENCCs first begin to express pan-neuronal markers before expressing subtype-specific markers, as ENCCs expressing pan-neuronal markers are present at E10.5 and include cells at the migratory wavefront (Young et al., 1999), whilst the expression of subtype-specific markers (including calbindin, nNOS and now, ChAT) is not detected until E11.5, and is located rostral to the migratory wavefront. The submucous plexus was not examined in this study as the neurons of the submucous plexus develop later than myenteric neurons (Gershon et al., 1980; Pham et al., 1991; Kapur et al., 1992).

The proportion of cholinergic neurons gradually increases through embryonic and postnatal development

There was a gradual increase in the proportion of cholinergic neurons through embryonic and postnatal development that is probably due to both neurogenesis and to cholinergic differentiation of pre-existing neurons. The interval between exit from the cell cycle and first expression of ChAT for an individual enteric neuron is unknown. However, Pham et al. (1991) showed that cholinergic neurons in the myenteric plexus are born from E8 to E17; therefore, after E17 the increase in cholinergic neurons must be predominantly due to cholinergic differentiation of neurons that have already exited the cell cycle. It is possible that these cells are: (i) cells that do not yet express neuronal markers; (ii) cells that already express pan-neuronal markers, but are yet to express ChAT; or (iii) cells that transiently express other subtype markers that become downregulated through development, while ChAT expression is upregulated. There is evidence for the presence of transient neurochemical phenotypes during the development of the ENS (Baetge and Gershon, 1989; Young and Ciampoli, 1998) and other parts of the nervous system (Dulcis and Spitzer, 2012). Furthermore, recent studies by Laranjeira et al., (2011) and Joseph et al., (2011) showed that Sox10-expressing ENCCs can produce neurons at postnatal stages following injury to the ENS, without cell division.

Studies using ChAT immunohistochemistry to examine the postnatal development of enteric neurons in the rat have reported both increases (de Vries et al., 2010) and decreases (Vannucchi and Fausone-Pellegrini, 1996) in the proportion of cholinergic neurons during postnatal development. In contrast to the increases in the proportion of YFP+ myenteric neurons we observed after birth, we did not detect significant changes in the proportions of nNOS+ myenteric neurons during mouse postnatal development. Postnatal increases in nNOS+ neurons have been shown in the rat (Matini et al., 1997; de Vries et al., 2010), but in the guinea-pig there is a decrease in the proportion of nNOS+ neurons and a decrease in nitregic innervation of the longitudinal muscle during postnatal development (Bian et al., 2009; Patel et al., 2010).

Functional implications

Survival of the newborn animal after birth depends on nutrition from the mother's milk, which replaces the placental nutrient supply. Hence, it is essential that there is efficient mixing and propulsion of the contents of the small intestine to allow digestion and absorption of milk nutrients. Neurally-mediated, propagating motility patterns are present at birth in the duodenum (Roberts et al., 2010), but the role of cholinergic neurons in these motility patterns remains to be investigated. In the colon, activation of muscarinic receptors is vital for neurally-mediated migrating motor complexes at early postnatal ages (Roberts et al., 2007).

ACh is the major excitatory neurotransmitter in the ENS, however, at P0, only 25% of myenteric neurons in the small intestine are cholinergic, compared to 62% in adults. This means that the propagating contraction complexes in the small intestine at P0 can take place with less than half of the key excitatory neurons present. It is possible that at P0 there is increased use of other excitatory neurotransmitters, such as tachykinins, or that propulsion of the gut contents prior to weaning does not require as many excitatory neurons, but this remains to be investigated.

In contrast, the proportion of cholinergic neurons in the colon at birth is not dramatically different from that of adults, but neurally-mediated motility patterns, do not appear until after P6 (Roberts et al., 2007).

The development of cholinergic projections

The direction of projections of cholinergic neurons could only be observed at E12.5. The majority of cholinergic neurons at this age projected anally, which was surprising as the majority of adult myenteric cholinergic neurons project orally in the “ascending excitation” pathways (Gwynne and Bornstein, 2007). There are two possible explanations: first, there are a small number of cholinergic interneurons in the myenteric plexus that project anally (Sang and Young, 1998; Qu et al., 2008), and these might be the first cholinergic neurons to appear (see below). Second, it was only possible to follow the neurites for a short distance, and it is possible that the initial neurite projection direction is not maintained. However, the data are consistent with previous studies that have shown that the majority of enteric neurons at early stages of ENS development project anally, whilst circumferential and oral projections appear at later embryonic ages (Young et al., 2002; Foong et al., 2012; Hao et al., 2013; Sasselli et al., 2013). Projections of cholinergic neurons were also observed in the developing circular muscle layer from E14.5. This is consistent with a previous study that recorded excitatory junction potentials from circular muscle cells at E17.5 (Ward et al., 1997), indicating that transmission between excitatory motor neurons and circular muscle cells commences during embryonic development. Gershon and Thompson (1973) also reported contractions in the fetal rabbit and mouse gut in response to electrical field stimulation, and the contractions were shown to be mediated by ACh in the rabbit. However, because we could not differentiate between cholinergic neurons of the ENS and extrinsic cholinergic innervation, we cannot rule out the possibility that the fibres within the circular muscle arise from extrinsic neurons.

Cholinergic neurons co-express other markers

In the adult myenteric plexus, cholinergic neurons comprise multiple functional subtypes and co-express a variety of other neurochemical markers (Sang and Young, 1998; Qu et al., 2008). We investigated the co-expression of calbindin and nNOS in cholinergic neurons in embryonic *ChAT-Cre;R26R-YFP* mice as both calbindin-IR and nNOS-IR neurons appear early during ENS development (Hao et al., 2010). The maximum overlap with nNOS occurred in the E12.5 rostral small intestine, when approximately 10% of cholinergic neurons were also nNOS+. As the anally projecting cholinergic interneurons in the mouse small intestine co-express nNOS (Qu et al., 2008), this suggests that at least some of the first

cholinergic neurons to develop are descending interneurons. After E12.5, the proportion of cholinergic neurons co-expressing nNOS decreased and was only 2-3% in the adult. This decrease is most likely due to the development of YFP+/nNOS- neurons. In the adult mouse ENS, the cholinergic/nNOS+ descending interneurons make up 3% of myenteric neurons in the small intestine (Qu et al., 2008) and 5% of ChAT+ neurons (Sang and Young, 1998). Similarly, in our study, we found that in the adult small intestine 2% of Hu+ cells were YFP+/nNOS+ and 3% of YFP+ cells were YFP+/nNOS+.

Approximately 60% of adult YFP+ cholinergic neurons in *Chat-Cre;R26R-YFP* mice were found to co-express calbindin, which is similar to what has been previously reported in wild type mice (Sang and Young, 1998). Unlike the guinea pig, calbindin-IR neurons do not represent a single subtype of myenteric neuron, and have not been well characterized in the mouse. During embryonic development, there appeared to be fluctuating changes to the proportion of cholinergic neurons that co-expressed calbindin. In the small intestine, there was initially high co-expression of calbindin in YFP+ neurons at E11.5 (approximately 1/3), this proportion then decreased at E12.5 to 11%, and increased again in postnatal development. These changes probably reflect dynamic changes in the development of subtypes of cholinergic neurons.

In conclusion, some cholinergic neurons differentiate very early during ENS development. However, the development of cholinergic neurons extends over a very protracted period of time and cholinergic neurons do not reach adult proportions until around P10.

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Conflict of Interest:

The authors declare that there are no conflicts of interest.

Role of Authors:

All authors had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. Study concept and design: MH, HY, JB. Acquisition of data and analysis: MH. Drafting of the manuscript: MH, HY, JB. Obtained funding: HY, JB. Study supervisor: HY, JB.

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Figure Legends:

Figure 1. Co-immunolabelling of YFP and ChAT in myenteric neurons in the small intestine (SI, A-C) and colon (D-F) of adult *ChAT-Cre;R26R-YFP* mice. The vast majority of YFP+ neurons are also ChAT-IR in both the small intestine and colon. However, a small number of YFP+ cells do not show detectable levels of ChAT immunoreactivity (*arrows*), and some ChAT+ neurons are YFP- (*open arrows*). Nerve fibers that are both ChAT-IR and YFP-IR can also be observed (*arrowheads*).

Figure 2. Appearance of YFP+ neurons progresses in a rostral-to-caudal direction during embryonic development in *ChAT-Cre;R26R-YFP* mice. Diagrammatic representations of the regions of the E10.5 (A), E11.5 (C), E12.5 (E), E14.5 (H) and E18.5 (K) gut which contain YFP-IR (green) and Hu-IR (magenta) neurons, accompanied by representative confocal micrographs of the Hu+ and YFP+ cells (B,D,F-G,I-J,L-M). **A-B:** At E10.5, Hu+ ENCCs are present in the stomach and intestines, but no YFP+ cells are present in the gut (B). **C-D:** At E11.5, a small number of YFP+ cells were present in the rostral SI. These cells all co-expressed Hu and therefore appear white in the overlay (D, some are shown by arrows). Distinct YFP+ fibres can be observed projecting from some cells (*arrowheads*). **E-G:** At E12.5, YFP+ cells are present along the entire length of the small intestine. Many cells have a neurite that projects anally (G, inset). Varicosities are often observed along neurites (G, *arrowheads*). **Inset:** Polar-plot of the directions of projections of YFP+ neurites at E12.5. Each green arrow represents the direction of projection of a single neuron. The number of neurons in each 45° octant are shown in brackets. A total of 41 YFP+ neurites were examined. The majority of cells projected anally, which was defined as having an angle between -45° and 45° from the longitudinal axis of the gut (*grey region*), where the anal direction = 0°. **H-J:** At E14.5, YFP+ cells are present in the proximal colon and there is an obvious increase in the proportion of YFP+/Hu+ cells in the duodenum (J). In the distal colon, prominent YFP+ fibres are present (I, *open arrowheads*), however, there are no YFP+ cell bodies. **K-M:** YFP+ neurons are present at the anal end of the colon by E18.5 (L, *arrows*), but are few in number. YFP+ neurons are more abundant in the duodenum at this age (M), but are low in proportion compared to the adult intestine. In all micrographs, the

anal direction is to the right of the page, unless otherwise stated. Note: the diagram of the E18.5 gut (K) is not drawn to scale.

Figure 3: Projection of YFP+ fibres. **A:** At E11.5, YFP+ cell bodies (*arrows*) give rise to neurites that mostly project anally and varicosities can be observed along their length (*arrowheads*). **B-C:** Projection of YFP+ fibres to the developing circular muscle layer in embryonic gut. At E14.5 (B), several YFP+ fibres (*arrowheads*) can be observed at the level of the circular muscle layer in the duodenum. The number and staining intensity of these fibres in this plane of focus increases at E16.5 (C, *arrowheads*). Some myenteric cell bodies can be seen, but are out of focus (*arrows*).

Figure 4: The proportion of myenteric Hu+ neurons that are YFP+ (cholinergic) increases through embryonic development. In all three regions examined, the rostral SI, caudal SI and proximal colon, the proportion of YFP+ neurons is significantly higher at E16.5 in comparison to E11.5 and E12.5 (* $p < 0.05$; number of embryos examined shown on graph; Kruskal-Wallis test for each region). However, at E16.5, <20% of myenteric neurons were YFP+ in all regions, which is considerably less than the proportion of adult cholinergic neurons.

Figure 5: Postnatal development of cholinergic neurons. **A-F:** Confocal micrographs of neurons co-labelled against Hu and YFP in the SI and colon at P0 (A-B), P10 (C-D) and adult (E-F) ages. The circumferential direction indicated in C applies to all images. YFP+ fibres in the circular muscle are present at P0 in both the small intestine and colon (A-B, *arrowheads*). These fibres become more abundant at P10 and in adults (E-F *arrowheads*). Note: the circular muscle was removed from some preparations, hence, the YFP+ fibres are not present in all preparations. **G:** Changes in the proportion of Hu+ neurons that express YFP during early postnatal development. (* $p < 0.05$; number of animals examined shown on graph; Kruskal-Wallis test for each region).

Figure 6: Co-expression of calbindin and nNOS in YFP+ neurons during embryonic and postnatal development. Examples of co-immunolabelled neurons at E11.5 (A-C), P0 (D-F)

and P10 (G-I). **A:** At E11.5, the most caudal YFP+ neuron is present in the rostral SI (*green arrow*). The most caudal calbindin+ (*blue arrow*) and nNOS+ (*red arrow*) neurons are located in the caudal SI and near the caecum respectively. The most caudal Hu+ neuron (*magenta arrow*) is present in the colon (at the wavefront of ENCCs), indicating that there is probably a delay between the first expression of pan-neuronal markers and the expression of subtype-specific markers. The box indicates the location of the fields of view shown in panels B and C. **B-C:** Many YFP+ neurons are also immunoreactive for calbindin at E11.5 (B, *arrows*; note that 2 are only weakly YFP+), but none are immunoreactive for nNOS (C). **D-F:** An example of a field of view showing calbindin and YFP co-immunoreactive neurons (*arrows*) at P0. Note: a range of intensities of calbindin immunoreactivity was observed, including cells with very dull staining, which were still clearly brighter than background. **G-I:** A YFP+/nNOS+ neuron (*arrow*) in the small intestine of a P10 mouse. YFP+ neurons that were also nNOS-IR were relatively rare throughout development. **J-K:** Proportions of YFP+ neurons that are co-immunoreactive for calbindin (blue) and nNOS (red) at selected embryonic (J) and postnatal (K) ages. Note: the large variation at E12.5 in the caudal SI is due to the small and variable number of YFP+ cells observed in this region at this age.

Figure 7: Postnatal development of nNOS+ myenteric neurons. The proportions of Hu-IR neurons that are nNOS-IR were calculated at P0, P10 and adult, in the SI and colon. There is a small increase in the proportion of nNOS+ neurons between P0 and adult in both regions, however, no significant differences were detected (Kolmogorov-Smirnov test, numbers of animals examined shown on graph).

Table 1. List of primary antibodies used

Antibody	Immunogen	Host	Company; catalogue number	Dilution	Characterization
Calbindin	Recombinant rat calbindin D-28k	rabbit	Swant; CB-38	1:1600	Reiche et al., 1999
Calretinin	Human recombinant calretinin	goat	Swant; CG1	1:100	Disney and Aoki, 2008
ChAT	Human placental ChAT enzyme	goat	Chemicon; AB144P	1:100	Sang and Young, 1998
GFP	Recombinant GFP	chicken	Abcam; ab13970	1:2000	Our data
GFP	GST-Green fluorescent protein (246aa) derived from <i>A. victoria</i>	goat	Rockland; 600-101-215	1:400	Our data
GFP	GFP isolated from <i>A. victoria</i>	rabbit	Molecular Probes; A11122	1:500	Our data
Hu	Patient-derived	human	Dr. Vanda Lennon; (Fairman et al., 1995)	1:5000	Erickson et al., 2012
nNOS	Rat brain nNOS	sheep	Dr. Piers Emson	1:2000	Norris et al., 1995; Young and Ciampoli, 1998

Table 2. List of secondary antibodies used

Target species	Conjugated to	Host	Company; catalogue number	Dilution
Chicken	FITC	Donkey	Jackson; 703-095-155	1:100
Human	Alexa 594	Donkey	Jackson; 709-585-149	1:500
Rabbit	FITC	Donkey	Jackson; 711-095-152	1:200
Rabbit	Alexa 647	Donkey	Molecular Probes; A31573	1:400
Sheep	FITC	Donkey	Jackson; 713-095-147	1:100
Sheep	Alexa 594	Donkey	Molecular Probes; A11016	1:100
Sheep	Alexa 647	Donkey	Molecular Probes; A21448	1:500

Table 3. Numbers of cells counted for each age and gut region

	Small intestine		Colon	
Age	# Hu-IR neurons (# embryos or animals)	# YFP-IR neurons (# embryos or animals)	# Hu-IR neurons (# embryos or animals)	# YFP-IR neurons (# embryos or animals)
E11.5	1572 (5)	66 (12)	(12)	(12)
E12.5	4551 (5)	100 (7)	(7)	(7)
E14.5	3454 (4)	1119 (13)	1209 (4)	100 (13)
E16.5	9181 (8)	1606 (8)	5434 (8)	552 (8)
P0	4925 (3)	1249 (3)	4186 (3)	1409 (3)
P10	8418 (3)	4608 (3)	4219 (3)	2072 (3)
Adult	2993 (4)	1853 (4)	4317 (4)	2229 (4)

Note: Because no YFP+ cells were observed in the colon at E11.5 and E12.5, only the number of embryos examined are shown.

Figure 1. Co-immunolabelling of YFP and ChAT in myenteric neurons in the small intestine (SI, A-C) and colon (D-F) of adult *ChAT-Cre;R26R-YFP* mice. The vast majority of YFP+ neurons are also ChAT-IR in both the small intestine and colon. However, a small number of YFP+ cells do not show detectable levels of ChAT immunoreactivity (arrows), and some ChAT+ neurons are YFP- (open arrows). Nerve fibers that are both ChAT-IR and YFP-IR can also be observed (arrowheads).
166x82mm (300 x 300 DPI)

Figure 2. Appearance of YFP+ neurons progresses in a rostral-to-caudal direction during embryonic development in *Chat-Cre;R26R-YFP* mice. Diagrammatic representations of the regions of the E10.5 (A), E11.5 (C), E12.5 (E), E14.5 (H) and E18.5 (K) gut which contain YFP-IR (green) and Hu-IR (magenta) neurons, accompanied by representative confocal micrographs of the Hu+ and YFP+ cells (B,D,F,G,I-J,L-M). **A-B:** At E10.5, Hu+ ENCCs are present in the stomach and intestines, but no YFP+ cells are present in the gut (B). **C-D:** At E11.5, a small number of YFP+ cells were present in the rostral SI. These cells all co-expressed Hu and therefore appear white in the overlay (D, some are shown by arrows). Distinct YFP+ fibres can be observed projecting from some cells (arrowheads). **E-G:** At E12.5, YFP+ cells are present along the entire length of the small intestine. Many cells have a neurite that projects anally (G, inset). Varicosities are often observed along neurites (G, arrowheads). **Inset:** Polar-plot of the directions of projections of YFP+ neurites at E12.5. Each green arrow represents the direction of projection of a single neuron. The number of neurons in each 45° octant are shown in brackets. A total of 41 YFP+ neurites were examined. The majority of cells projected anally, which was defined as having an angle between -45° and 45° from the longitudinal axis of the gut (grey region), where the anal direction = 0°. **H-J:** At E14.5, YFP+ cells are present in the proximal colon and there is an obvious increase in the proportion of YFP+/Hu+ cells in the duodenum (J). In the distal colon, prominent YFP+ fibres are present (I, open arrowheads), however, there

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170x189mm (300 x 300 DPI)

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Figure 3: Projection of YFP+ fibres. **A:** At E11.5, fibres YFP+ cell bodies (arrows) give rise to neurites that mostly project anally and varicosities can be observed along their length (arrowheads). **B-C:** Projection of YFP+ fibres to the developing circular muscle layer in embryonic gut. At E14.5 (B), several YFP+ fibres (arrowheads) can be observed at the level of the circular muscle layer in the duodenum. The number and staining intensity of these fibres in this plane of focus increases at E16.5 (C, arrowheads). Some myenteric cell bodies can be seen, but are out of focus (arrows).
103x110mm (300 x 300 DPI)

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118x75mm (300 x 300 DPI)

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82x190mm (300 x 300 DPI)

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168x201mm (300 x 300 DPI)

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82x67mm (300 x 300 DPI)

Graphical abstract

Using *ChAT-Cre;R26R-YFP* mice, the authors were able to examine the embryonic and postnatal development of myenteric cholinergic neurons. The authors show that although some cholinergic neurons differentiate very early during the development of the enteric nervous system, they do not reach adult proportions until around post-natal day 10.

70x70mm (72 x 72 DPI)

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