Distinct subpopulations of enteric neuronal progenitors defined by time of development, sympathoadrenal lineage markers and *Mash-1*-dependence

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SUMMARY

Enteric and sympathetic neurons have previously been proposed to be lineally related. We present independent lines of evidence that suggest that enteric neurons arise from at least two lineages, only one of which expresses markers in common with sympathoadrenal cells. In the rat, sympathoadrenal markers are expressed, in the same order as in sympathetic neurons, by a subset of enteric neuronal precursors, which also transiently express tyrosine hydroxylase. If this precursor pool is eliminated in vitro by complement-mediated lysis, enteric neurons continue to develop; however, none of these are serotonergic. In the mouse, the *Mash-1*$^{-/-}$ mutation, which eliminates sympathetic neurons, also prevents the development of enteric serotonergic neurons. Other enteric neuronal populations, however, including those that contain calcitonin gene related peptide are present. Enteric tyrosine hydroxylase-containing cells co-express *Mash-1* and are eliminated by

the *Mash-1*$^{-/-}$ mutation, consistent with the idea that in the mouse, as in the rat, these precursors generate serotonergic neurons. Serotonergic neurons are generated early in development, while calcitonin gene related peptide-containing enteric neurons are generated much later. These data suggest that enteric neurons are derived from at least two progenitor lineages. One transiently expresses sympathoadrenal markers, is *Mash-1*-dependent, and generates early-born enteric neurons, some of which are serotonergic. The other is *Mash-1*-independent, does not express sympathoadrenal markers, and generates late-born enteric neurons, some of which contain calcitonin gene related peptide.

Key words: enteric neurons, sympathetic neurons, *Mash-1*, rat, cell lineage

INTRODUCTION

The enteric and sympathetic nervous systems are derived from different levels of the neural crest (Le Douarin, 1986) and are very different from one another (Gershon et al., 1994). The vagal and sacral regions of the crest provide the cells that give rise to the enteric nervous system (ENS; Le Douarin and Teillet, 1973; Le Douarin and Teillet, 1974; Pomeranz and Gershon, 1990; Pomeranz et al., 1991; Serbedzija et al., 1991; Yntema and Hammond, 1954), while intervening levels supply sympathetic precursors (Le Douarin, 1986). The ENS is far larger and more phenotypically diverse than the sympathetic nervous system (Gershon et al., 1994); moreover, while the ultrastructure of sympathetic ganglia resembles that of other regions of the PNS, that of the ENS is unique and similar to the CNS. Despite these differences in their origins and functional organization, it has been proposed that sympathetic and enteric neurons derive from a common immediate progenitor (Carnahan et al., 1991). This idea was first suggested by the observation that some cells in the developing gut of rats (E11-14; Baetge et al., 1990a; Cochard et al., 1978; Teitelman et al., 1978) and mice (E10-13; Baetge and Gershon, 1989) are transiently catecholaminergic and express neurofilament proteins,

and p75^{LNTR}, the low affinity neurotrophin receptor. Although these transiently catecholaminergic (TC) cells exhibit all of the properties that mark mature sympathetic neurons, including catecholamine storage, expression of tyrosine hydroxylase (TH), dopamine β-hydroxylase (DBH), and the specific uptake of ^{3}H-norepinephrine (^{3}H-NE) (Gershon et al., 1984; Jonakait et al., 1989, 1979), they are proliferating (Baetge et al., 1990a; Teitelman et al., 1981); therefore, TC cells are not neurons but their precursors. At least some of the neurons of the mature ENS, including all of those that are serotonergic, develop from TC cells (Baetge et al., 1990a). In addition to their catecholaminergic properties, TC cells transiently express other sympathoadrenal fetal differentiation antigens (Carnahan et al., 1991; Carnahan and Patterson, 1991), including SA and B2. The expression of these traits in common raises the possibility that TC enteric progenitors are lineally related to the similarly catecholaminergic precursors of sympathoadrenal cells (Carnahan et al., 1991).

Transient expression of *Mash-1* during development is another property exhibited by the precursors of both sympathoadrenal cells and enteric neurons (Lo et al., 1991). *Mash-1* is a member of the basic helix-loop-helix family of transcriptional regulators (for review, see Garrell and Campuzano,

1991) and is a mammalian homologue of *achaete-scute* of *Drosophila* (Guillemot and Joyner, 1993; Johnson et al., 1992; Lo et al., 1991). Expression of *Mash-1* has been detected in sympathoadrenal cells and in the developing ENS during the period when TC cells are present (Guillemot et al., 1993; Lo et al., 1991). Development of sympathetic neurons is virtually eliminated in mice homozygous for a targeted mutation in the *Mash-1* locus (Guillemot et al., 1993), suggesting that *Mash-1* expression is critical for the development and/or survival of sympathoadrenal neural precursors. If this is so, then enteric, as well as sympathetic, neurons derived from the putative common sympathoadrenal-enteric progenitor (the cells that exhibit the SA→B2 switch) might be expected to be deficient in *Mash-1*$^{-/-}$ mice. An apparent delay in the appearance of neurons has been observed in the fetal bowel of *Mash-1*$^{-/-}$ animals (Guillemot et al., 1993; Lo et al., 1994); however, this delay could reflect either the selective ablation of a subset of early generated enteric neurons (Pham et al., 1991), or a delayed production of the normal complement of enteric neurons. Enteric neurons are born in successive waves; the earliest wave generates all enteric serotonergic cells, while later waves generate peptidergic neurons, the latest-born of which expresses CGRP (Baetge and Gershon, 1989; Baetge et al., 1990a; Pham et al., 1991).

In the current study, we have examined the consequences of selectively eliminating the subset of enteric neuronal progenitors that expresses sympathoadrenal lineage markers. This has been accomplished either by immunological ablation of rat precursors in vitro, or by genetic ablation of murine precursors in vivo using the *Mash-1*$^{-/-}$ mutation. In both cases, the generation of serotonergic neurons was selectively prevented. Taken together, these two complementary lines of evidence suggest that the ENS is formed by at least two distinct lineages of precursors. One is *Mash-1*-dependent, transiently expresses sympathoadrenal markers, and gives rise to early-generated enteric neurons, some of which are serotonergic. The other is *Mash-1*-independent, never expresses sympathoadrenal markers, and gives rise to later-generated enteric neurons, some of which express CGRP.

MATERIALS AND METHODS

Preparation of cell cultures

Timed pregnant Sprague-Dawley rats (Charles River) were provided with food and water *ad libidum*. Dams were killed at day E12-14 by carbon dioxide intoxication. Fetuses were placed in iced Hanks balanced salt solution (HBSS). The fetal gut was removed, minced, and incubated at 37°C in a calcium- and magnesium-free saline solution containing glucose (0.1%) and collagenase (0.5%). Incubation times were 10 minutes for fetuses removed at day E12, and 25 minutes for fetuses removed at day E14. Tissues were washed with Eagle's minimal essential medium (MEM, Gibco), supplemented with 10% horse serum (Gibco), 10% chick embryo extract, 0.6% glucose and 2.0 mM glutamine. The bowel was then dissociated by trituration through a series of fire polished Pasteur pipettes. Aggregates were removed by passing the cell suspension through a 53 μm nylon mesh filter. Finally, cells were plated onto 12 mm polylysine/laminin-coated coverslips (50,000/coverslip) and cultured under 5% CO_2 at 37°C. Medium was changed every 2 days and cultures were maintained for up to 7 days. Mass cultures were prepared similarly from fetal mouse intestine dissociated at E12.5. These cultures were grown in a defined medium (Stemple and Anderson, 1992) for 6 hours prior to fixation for immunocytochemistry.

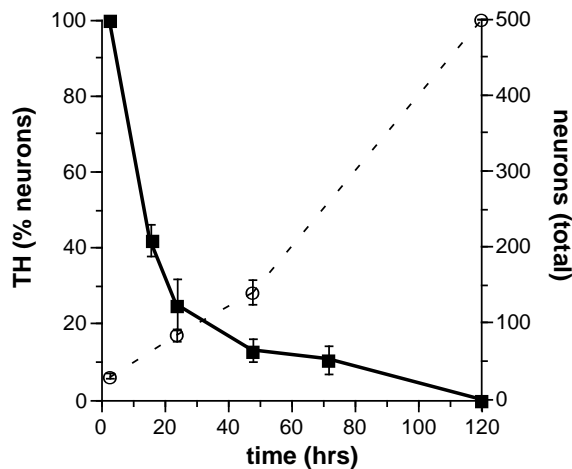### Immunocytochemistry and in situ hybridization

Cultures were fixed for 1.5 hrs in 4% formaldehyde (from paraformaldehyde) in phosphate-buffered saline (PBS) at pH 7.5. Cultures were examined as whole mounts. Tissues were permeabilized for 30 min with 0.1% Tween-20 in PBS, and then incubated for 12 hours at 4°C with a primary antibody. For all experiments except the demonstration of Mash-1 immunoreactivity, primary antibodies were visualized using appropriate secondary antibodies coupled either to fluorescein isothiocyanate (FITC) or tetramethylrhodamine isothiocyanate (TRITC). For the simultaneous demonstration of two antigens in the same tissue sections, primary antibodies raised in different species were used in combination with affinity purified species-specific secondary antibodies. No immunostaining was observed when non-immune sera or unrelated antibodies were substituted for the primary antibodies. After washing in PBS, the tissues in which immunoreactivity had been demonstrated were mounted in glycerol containing 22 mM 1,4-diazabicyclo-(2,2,2)octane and visualized by vertical fluorescence microscopy (Leitz Ortholux). TRITC fluorescence was viewed with a 'N$_2$' dichroic mirror/filter cube (exciting filter BP 530-560; dichroic mirror RKP 580; barrier filter LP 580). No FITC fluorescence was passed by this dichroic mirror/filter combination. FITC fluorescence was viewed with a narrow band dichroic mirror/filter combination (Leitz, 'L$_2$'; exciting filter BP 450-490; dichroic mirror RKP 510, barrier filter BP 525/20) that passed no TRITC fluorescence. Mash-1 immunoreactivity could not be visualized sufficiently well using fluorescent probes. To demonstrate this antigen, therefore, a more sensitive cobalt/nickel-enhanced peroxidase technique was utilized (Adams, 1981). Fixed cultures were pre-blocked with 1% normal goat serum and 0.1% NP-40 in PBS for 10 minutes. The preparations were then incubated overnight at 4°C with monoclonal antibodies to Mash-1 (undiluted hybridoma supernatant), washed, incubated with goat anti-mouse secondary antibodies coupled to horseradish peroxidase (HRP; diluted 1:100) and visualized with 3,3′-diaminobenzidine (DAB). To demonstrate TH immunoreactivity, the slides were subsequently incubated with monoclonal antibodies to TH- and HRP-labeled goat anti-mouse secondary antibodies. The reaction product was visualized with 3-amino-9-ethyl carbazole (AEC; Sigma), which gives a red reaction product. Since the Mash-1 protein is exclusively intranuclear, and TH is cytoplasmic, the reagents used for double label immunocytochemistry do not interfere with one another. Doubly labeled cells have blue-black nuclei and red cytoplasm. In addition, antibodies covered by the DAB reaction product are no longer immunoreactive. Controls and the demonstration of cells immunostained with anti-TH or anti-Mash-1 alone ruled out the possibility that antibodies used to visualize Mash-1 non-specifically interfered with the subsequent demonstration of TH.

Non-radioactive, digoxigenin-labeled probes were applied to sections of fetal murine bowel for in situ hybridization as described previously (Guillemot et al., 1993). Sites to which the probes hybridized were visualized immunocytochemically with alkaline phosphatase-labeled antibodies to digoxigenin (Boehringer Mannheim). Primary antibodies, probes, their sources, and references concerning their characterization and use are listed in Table 1.

Complement-mediated lysis

Cultures were incubated for 1 hour at 37°C with SA or B2 monoclonal antibodies. A mixture of SA antibodies 1, 2, 3, and 5 (from cells donated by Dr Paul Patterson, California Institute of Technology) were obtained from ascites fluid. The antibodies were purified by passage through a protein A column (Ey et al., 1978). The purified SA IgG was added to cultures of dissociated fetal bowel in a final concentration of 15 μg/ml. The B2 antibodies (from cells contributed by Dr Jane Dodd, Columbia University) were obtained from hybridoma supernatant medium. Cultures were incubated in the hybridoma super-

natant or in a similar control medium, which was not exposed to the hybridoma. The antibody-containing (or control) media were then aspirated and cells were incubated with MEM containing 1% guinea pig complement (Gibco). In order to detect cell death, the cultures were observed by phase-contrast microscopy at 10 and 20 min

following exposure to complement. In addition, an ethidium homodimer (3.75 μM; Live/Dead™ reagent, Molecular Probes) was included in the solution so that cell death could be assessed by fluorescence microscopy. After 20 minutes the complement-containing medium was aspirated and replaced by fresh maintenance medium. Some cultures were fixed and examined immediately, while others were maintained in culture for up to 6 days. In some experiments, the entire complement-mediated lysis procedure was repeated 4 times (at daily intervals). Effectiveness and specificity of complement-mediated lysis were evaluated by fixing several cultures immediately after each procedure and determining the number of cells remaining in each that expressed the neural marker, peripherin (Portier et al., 1984) and/or B2 immunoreactivity. Although both SA and B2 antigens are expressed at the cell surface (Anderson et al., 1991;

Fig. 1. TH-immunoreactive neurons disappear as a function of time in vitro. TH and neurofilament immunoreactivities in dissociated cultures of E12 fetal rat gut were investigated as a function of time by double label immunocytochemistry. Values shown are mean numbers of cells expressing these markers (± s.e.m.), indicating the total number of neurons (neurofilament-immunoreactive cells; dashed line, right ordinate) and the TH-immunoreactive proportion (solid line, left ordinate).
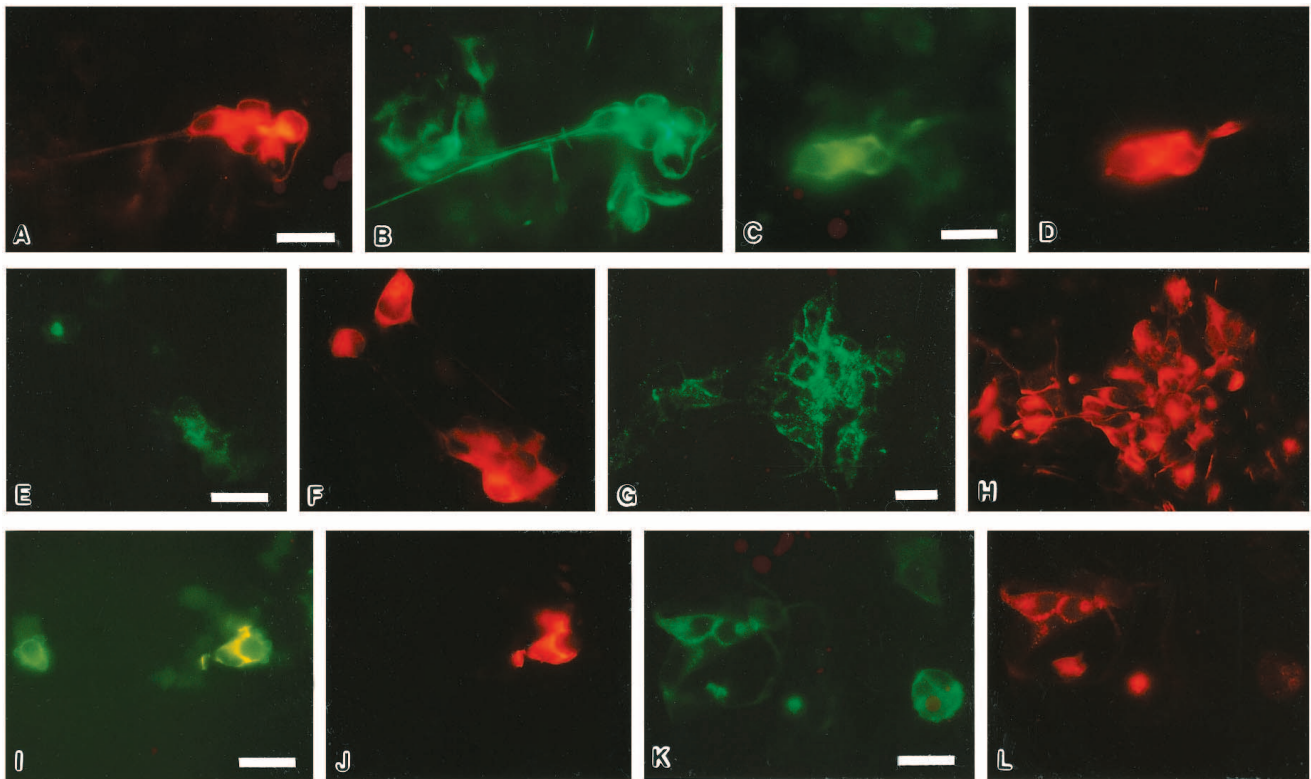

Fig. 2. Identification of neurons (or neural precursors) in vitro. Neurons (or neural precursors) and lineage markers were identified in cultures of dissociated fetal rat gut by double label immunocytochemistry. Bars, 25 μm. (A) TH and (B) neurofilament immunoreactivity after 2 days in vitro. A single field is illustrated: TH, TRITC; neurofilament, FITC. By 2 days some neurons no longer contain TH. (C) SA and (D) TH immunoreactivities after 3 hours in vitro. A single field is illustrated: SA, FITC; TH, TRITC. These two markers are coincidentally expressed. (E) B2 and (F) TH immunoreactivities after 1 day in vitro. A single field is illustrated: B2, FITC; TH, TRITC fluorescence. B2 expression has begun to appear in a subset of cells that express TH. (G) B2 and (H) peripherin immunoreactivities in a mature culture, grown for 6 days. A single field is illustrated: B2, FITC; peripherin, TRITC. B2 expression persists, and all of the B2-immunoreactive cells express peripherin, but the number of peripherin-immunoreactive cells > B2. (I) p75[LNTR] and (J) TH immunoreactivities after 3 hours in vitro. A single field is illustrated: p75[LNTR], FITC; TH, TRITC fluorescence. All TH-immunoreactive cells express p75[LNTR]; however, some p75[LNTR]-immunoreactive cells do not express TH. (K) p75[LNTR] and (L) B2 immunoreactivities after 1 day in vitro. A single field is illustrated: p75[LNTR], FITC and B2, TRITC. All B2-immunoreactive cells also express p75[LNTR]; however, p75[LNTR] > B2.

Table 1. Antibodies and probes used.

Substances	Species	Source	References
Antibodies			
Neurofilament (H, M, L)	Mouse	Boehringer Mannheim	(Baetge and Gershon, 1989; Baetge et al., 1990a)
Tyrosine Hydroxylase	Rabbit	Eugene Tech.	(Baetge and Gershon, 1989; Baetge et al., 1990a)
Tyrosine Hydroxylase	Mouse	Boehringer Mannheim	(Anderson et al., 1991; Carnahan et al., 1991; Carnahan and Patterson, 1991)
SA 1-4	Mouse	Donated by Dr. Paul Patterson	(Anderson et al., 1991; Carnahan et al., 1991; Carnahan and Patterson, 1991)
B2	Mouse	Donated by Dr. Jane Dodd	(Carnahan et al., 1991; Kirchgessner et al., 1988)
Peripherin	Rabbit	Donated by Dr. Ronald Liem	(Baetge et al., 1990a; Portier et al., 1984)
p75^{LNTR} (IgG 192)	Mouse	Donated by Regeneron	(Baetge et al., 1990a; Chao et al., 1986)
5-HT	Rabbit	AMAC	(Baetge et al., 1990a; Costa et al., 1982; Furness and Costa, 1982)
Mash-1	Mouse	Anderson laboratory	
VIP	Rabbit	Dr. Gajanan Nilaver	(Rothman et al., 1984)
CGRP	Rabbit	Peninsula	(Pham et al., 1991)
Probes			
c-ret	Mouse	Dr. V. Pachnis	(Pachnis et al., 1993; Schuchardt et al., 1994)
Mash-1	Mouse	Anderson laboratory	(Guillemot and Joyner, 1993; Guillemot et al., 1993)

Carnahan et al., 1991), SA expression was found to be far more abundant internally than that at the plasma membrane; therefore, TH/SA-immunoreactive cells were not lysed, even when incubated for 1.0 hour in a solution containing 4 of 5 SA antibodies before exposure to complement. The results of complement-mediated lysis with the B2, rather than the SA, reagent were thus analyzed in detail. In order to kill all cells in the TC/SA→B2 lineage with B2 antibodies and complement, cultures must not be treated too early, before cells that will express the B2 antigen do so, or too late, after B2 expression is lost. Since B2 was not expressed by any cells at the time of plating, but was only later acquired in vitro, the first criterion was easily met; nevertheless, although complement-mediated lysis was found, by examining cultures immediately afterwards, to be 100% effective in killing cells that expressed the B2 epitope at the time of exposure, B2-immunoreactive cells reappeared in the treated cultures 24 hrs later, suggesting that B2$^-$ cells are present in 2-day-old cultures that give rise to B2$^+$ successors. A single exposure of cultures to B2 antibodies and complement, therefore, does not eliminate all cells that have the potential to express B2. Complement-mediated lysis was thus carried out repetitively until no more B2$^+$ cells reappeared.

Mice with a targeted null mutation in the *Mash-1* locus

Timed pregnant C57Bl/6J mice carrying a null allele in the *Mash-1* locus were bred at the California Institute of Technology. Since *Mash-1$^{-/-}$* animals die at birth, all studies were carried out with fetal animals at day E17. Dams were killed by cervical dislocation and fetuses were placed in iced Krebs solution. In order to distinguish *Mash-1$^{-/-}$* mice from +/− and +/+ animals, a small piece of liver was removed from each fetus and frozen for later analysis. PCR was used to identify the fetal genotype; the liver was dissolved by incubation for 60 minutes (at 60°C) in 400 µl of buffer (50 mM KCl, 10 mM Tris, pH 8.3, 2 mM MgCl$_2$, 0.1 mg/ml gelatin, 0.45% NP-40, 0.45% Tween-20) that included 0.1 mg/ml proteinase K. Proteinase K was then inactivated by boiling for 10 minutes, after which the solutions were used as templates for PCR. Each reaction solution contained two pairs of primers, one of which identified the presence of an intact *Mash-1* gene, the other the presence of the neomycin resistance gene. Amplifications were carried out for 34 cycles of 1 minute at 94°C, 1 minute at 60°C, and 2 minutes at 72°C. PCR products were identified by size following electrophoresis through a 1.5% agarose gel (468 bp for *Mash-1* and 270 bp for the neomycin resistance gene). Primers for the detection of the mutant allele were neo-1: 5′GATCTCCTGTCATCTCACCT and neo-2: 5′ATGGGTCACGACGAGATCCT. Primers for the detection of the wild-type *Mash-1* were jm-1: 5′CCAACTGGTTCTGAGGAC and jm-3: 5′ CCCATTTGACGTAGTTGG.

Uptake of ^{3}H-5-HT

Segments of small intestine were removed from the animals and divided into 4 sections. Each piece of gut was equilibrated for 5 minutes at 37°C in oxygenated (95% O$_2$/5% CO$_2$) Krebs solution, containing the monoamine oxidase inhibitor, pargyline (100 µM; Sigma) (Fischman and Gershon, 1964; Gershon et al., 1990). ^{3}H-5-HT was then added (0.5 µM; 22.0 Ci/mmol, New England Nuclear Corp.) in the presence or absence of the selective inhibitor of 5-HT uptake, zimelidine (10 µM), and the incubation was continued for 30 minutes. Two of the sections from each bowel were incubated with ^{3}H-5-HT in the presence of the inhibitor and two in its absence. Although zimelidine blocks the action of the 5-HT transporter, it does not antagonize the action of other monoamine transporters (Gershon and Jonakait, 1979; Takaki et al., 1985). Uptake in the presence of zimelidine thus provided an estimate of the non-specific uptake of ^{3}H-5-HT. The reaction was terminated by transferring the tissue to an excess of Krebs solution at room temperature.

In order to evaluate the amount of ^{3}H-5-HT present in whole segments of bowel, specimens were weighed, and extracted overnight in 70% ethanol. Previous experiments have shown that 100% of the ^{3}H-5-HT present in segments of gut is extracted by this procedure; moreover, since radioactive metabolites wash out of the bowel, while ^{3}H-5-HT itself is retained, ^{3}H-5-HT is essentially the only radioactive compound left in the gut when the bowel is thoroughly washed prior to extraction (Gershon and Ross, 1966a,b). The radioactivity of the extracted material was determined by liquid scintillation. In order to normalize the data and correct for non-specific binding, a ratio was obtained in which the total radioactivity per unit weight of tissue determined in the absence of zimelidine was divided by that determined in the presence of the inhibitor. A ratio of 1 would thus be obtained if there was no specific uptake of ^{3}H-5-HT.

For radioautography, specimens were fixed with glutaraldehyde (2%) in PBS containing 3% sucrose for 2 hours (Gershon et al., 1990). Tissues were then washed, dehydrated with ethanol, cleared in propylene oxide and embedded in an Epoxy Resin (Epon 812). Semithin sections (approx.1 µm thick) were cut and collected on slides that had previously been subbed with chromium-alum gelatin. The slides were coated by dipping in a photographic emulsion (Ilford L-4, Polysciences), and exposed for 5 days in an atmosphere of dry CO$_2$. Exposed slides were developed with D-19 (Kodak), washed, fixed and stained with toluidine blue. Slides were examined with a Leitz microscope equipped for bright-field and incident dark-field illumination. The intensity of labeling was quantified by measuring the density of radioautographic silver grains over structures in the tissue. Incident dark-field illumination was employed so that light was reflected only from radioautographic silver grains. The reflected light and the area of labeled tissue were measured by computer-assisted video densitometry (Imaging Research, Ontario, Canada). Background measurements from the submucosa were subtracted from those obtained from ganglia. A labeling index was then calculated for each animal, in which the relative optical density was multiplied by the area labeled.

Visualization of enteric neurons by demonstrating acetylcholinesterase activity

Small intestines were removed from E17 control and *Mash-1*^{-/-} mice and fixed for 4 hours with 4% formaldehyde (from paraformaldehyde) in 0.1 M phosphate buffer (pH 7.4). After washing, the gut was divided sequentially into 5 mm segments along its long axis and each segment was opened by a longitudinal incision. The mucosa was removed and the specimens were processed as floating whole mounts to demonstrate acetylcholinesterase (AChE) activity (El-Badawi and Schenk, 1967). Tetraisopropylpyrophosphoramide (80 µM) was added to inhibit non-specific cholinesterase. Incubation was carried out at 37°C for 4 hours. Tissue was mounted in glycerol:water (9:1) on a glass slide with the serosa facing the coverslip. The number of neurons displaying AChE activity was counted in 8 segments (5 mm in length) of the small intestine from 2 control and 2 *Mash 1*[-/-] mice. Neurons were counted in 3 rectangles (340 µm by 250 µm) placed randomly over the tissue in each segment in order to estimate the mean number of neurons per mm[2]. An average neuronal density was then calculated for each gut.

Electron microscopy

The duodenum was fixed with a mixture of 1% formaldehyde (prepared from paraformalde-hyde), 2% glutaraldehyde, 0.5% acrolein, and 0.5% dimethylsulfoxide in 0.1 M sodium cacodylate buffer (pH 7.4) overnight at room temperature (Tennyson et al., 1986). The tissue was then rinsed in cacodylate buffer for 30 minutes and postfixed with 2% OsO_4 in 0.1 M cacodylate buffer for 1 to 2 hours. Following a buffer wash, the specimens were block-stained in a solution of 1% tannic acid for 1 hour. Following dehydration in a graded series of alcohols, the tissue was embedded in epoxy resin (Durcupan, Electron Microscopy Sciences, Ft. Washington, PA). Semithin sections were cut at 1-3 µm and stained with toluidine blue and used for orientation. Ultrathin sections were then cut and stained with lead citrate and uranyl acetate. Electron micrographs were taken with a JEOL 1200EX electron microscope.

RESULTS

Neurons or neural precursors that express SA and B2 antigens develop in vitro

Small intestine from fetal rats was disso-ciated at day E12 of development, the age when TC cells are most abundant (Baetge et al., 1990a). The cells were grown in culture for varying periods of time, up to 1 week. The cultures were then fixed and prepared for the immunocytochemical demonstration of the lineage-specific TH, SA, and B2 antigens. 5-HT immunoreac-tivity and that of neuronal markers, including peripherin (or neurofilament immunoreactivity) and p75[LNTR] immunoreactivities, were also visualized. TH expression was detected from 1 hour in vitro, the earliest time examined, up to

3 days (Figs 1, 2A,D,F). All of the cells that expressed TH immunoreactivity exhibited coincident expression of a neuronal marker (Fig. 2B; compare with 2A). Disappearance of TH-immunoreactive cells was precipitous (Fig. 1), so that by day 3 in culture the number of neurofilament-immunoreactive cells exceeded those displaying TH (Fig. 1, compare Figs 2A and B). No TH immunoreactivity was detectable in the cultures after the third day in vitro. SA immunoreactivity was also seen as early as 1 hour in vitro (Fig. 2C). The SA antigen was only detected in cells that showed coincident expression of TH immunoreactivity (Fig. 2D; compare with Fig. 2C). SA expression, however, disappeared even more rapidly than did TH and could not be found after 1-1.5 days in vitro. The dis-appearance of SA immunoreactivity was accompanied by the appearance of B2 immunoreactivity (Fig. 2E), which could be detected after 1-1.5 days in vitro, again only in cells in which
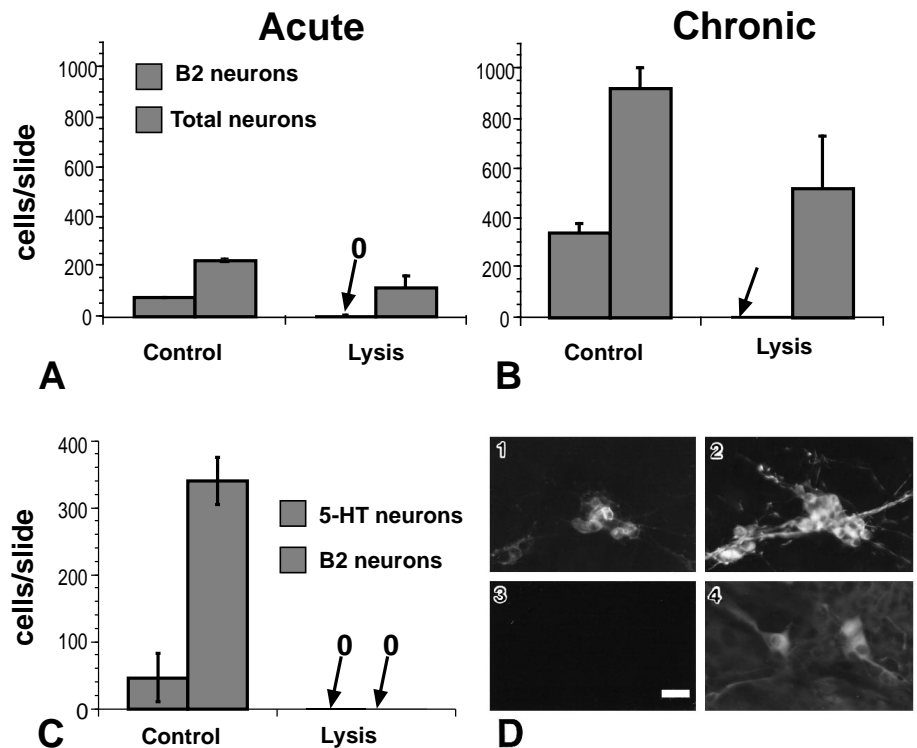

Fig. 3. Complement-mediated lysis of all cells that express B2 prevents serotonergic neuronal development, but other neurons continue to arise in vitro. Cells from dissociated E12 fetal rat gut were cultured for 2 days and exposed to B2 monoclonal antibodies and complement in the presence of an ethidium homodimer to detect cell death by fluorescence. Lysis was observed 10-20 minutes after exposure to complement. B2, peripherin, or 5-HT immunoreactivities were then demonstrated. (A) Cultures were treated once with B2 and complement and examined 2 hours later. Untreated cultures (controls) were exposed to complement, but not to B2. Complement-mediated lysis destroys all B2-immunoreactive cells, but spares others. A typical experiment is illustrated. It was repeated 8 times with identical results. (B) B2-immunoreactive cells were destroyed by complement-mediated lysis 4 times, until no further B2 immunoreactive cells developed. Elimination of all cells that express B2 immunoreactivity did not prevent the appearance of neurons in the cultures. Results are shown of one of 4 experiments, each of which yielded the same data. (C) 5-HT-immunoreactive neurons in control cultures are a subset of B2-immunoreactive cells (approx. 14%). Chronic complement-mediated lysis of B2-immunoreactive cells eliminates both B2-immunoreactive and serotonergic neurons. (D$_1$) B2 immunoreactivity in a control culture. (D$_2$) Peripherin immunoreactivity in the same field. (D$_3$) B2 immunoreactivity in a culture subjected to chronic complement-mediated lysis. No cells fluoresce. (D$_4$) Peripherin immunoreactivity in the same field; neurons are present. Bar for (D$_{1-4}$), 20 µm.

coincident expression of TH immunoreactivity was seen (Fig. 2F; compare with 2E). Although initial expression of B2 always occurred in cells that coincidentally expressed TH, B2 expression outlasted that of TH and persisted for up to 1 week in vitro (Fig. 2G). Coincident expression of a neural marker (peripherin) in B2-immunoreactive cells (Fig. 2H; compare with Fig. 2G) indicated that the cells that expressed this surface antigen were committed to a neuronal lineage. By the end of 6-7 days in vitro, B2-immunoreactive cells represented only about a third of the total of those that expressed peripherin immunoreactivity (compare Fig. 2G with 2H; see also Fig. 3). Taken together, these data indicate that some enteric precursors undergo the same switch in antigenic phenotype, from $SA^+ \rightarrow B2^+$, that is exhibited by sympathetic neurons (Anderson et al., 1991), and that this switch can be recapitulated in vitro.

Neural crest-derived precursors in the developing gut can be defined by their expression of $p75^{LNTR}$ (Baetge et al., 1990a). The number of crest-derived cells that express $p75^{LNTR}$ immunoreactivity in the fetal rat bowel in situ has been reported to be larger than the number of TH-immunoreactive cells (Baetge et al., 1990a). Similarly, the number of $p75^{LNTR}$-immunoreactive cells in dissociated cultures (Fig. 2I,K) was also greater than the number of cells that expressed either TH (Fig. 2J, compare with 2I) or the later, and more stable marker, B2 (Fig. 2L, compare with 2K); however, all TH- or B2-immunoreactive cells displayed $p75^{LNTR}$. These observations suggest that the cells expressing TH, SA, and B2 antigens represent a subset of enteric precursors. This subset is likely to generate enteric serotonergic neurons (Baetge et al., 1990a). In vitro, serotonergic neurons first became detectable by their expression of 5-HT immunoreactivity after about 4 days. At this time, all 5-HT-immunoreactive neurons co-expressed B2 (Fig. 7A,B).

Complement-mediated lysis kills B2-immunoreactive cells

In order to determine directly whether enteric serotonergic neurons are derived from progenitors expressing sympathoadrenal lineage markers, we ablated them by complement-mediated lysis, using the B2 monoclonal antibody, which detects a cell surface antigen. When cultures were exposed (on day 2 in vitro) to B2 antibodies many neurons were lysed by complement, as judged by their morphological appearance and uptake of an ethidium homodimer; moreover, no B2-immunoreactive cells could be detected in cultures examined 2 hours following exposure to B2 antibodies and complement (Fig. 3A). Although some cells that expressed a neural marker (peripherin or neurofilament immunoreactivities) survived this treatment, none expressed B2 or TH immunoreactivities. No lysis was observed in control cultures exposed to complement alone or to B2 antibodies and no complement.

Despite the apparent efficacy of complement-mediated lysis in killing all cells that express the B2 antigen, new B2-immunoreactive cells developed in cultures one day after complement-mediated lysis, suggesting a protracted generation of these cells from B2⁻ progenitors. Complement-mediated lysis with antibodies to the B2 antigen was therefore repeated until B2-immunoreactive cells failed to reappear one day after treatment. This necessitated a total of 4 cycles of complement-mediated lysis. After such treatment, no B2-immunoreactive cells reappeared in the chronically treated cultures (Fig. 3B); nevertheless, neurons were still detected with pan-neuronal

markers, such as neurofilament or peripherin immunoreactivity (Fig. 3B; see also Fig. 3D1-4) or with antibodies to vasoactive intestinal peptide (VIP; not illustrated). The total number of neurons in the cultures increased between the second and sixth day in vitro, despite the elimination of all cells that expressed B2 (compare Fig. 3A with Fig. 3B); however, 5-HT-
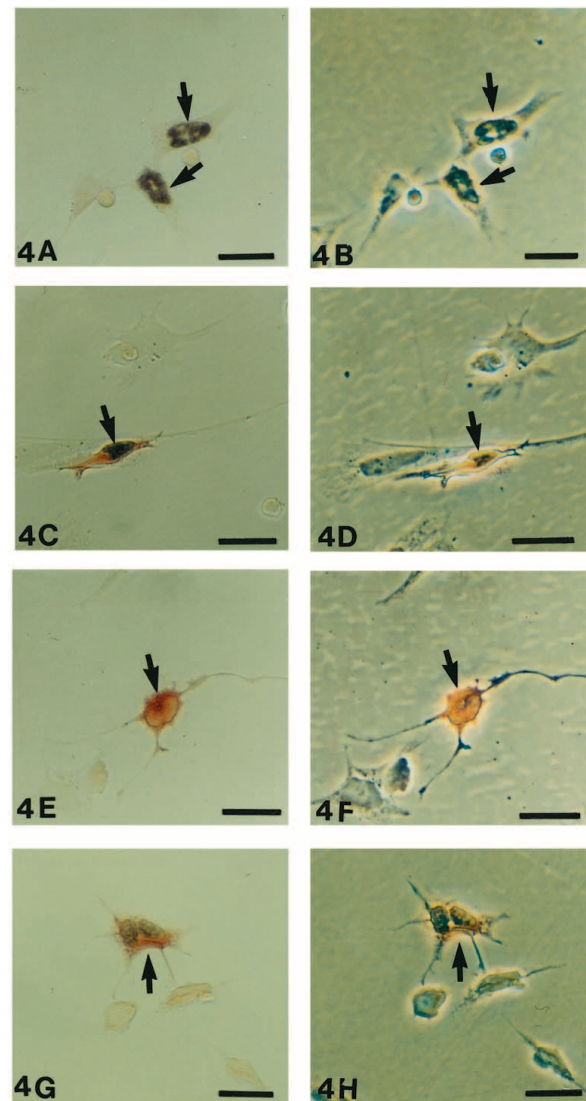
Fig. 4. Coincident expression of Mash-1 and TH occurs in developing neurons cultured from fetal mouse gut. Each pair of figures illustrate the same field photographed with bright-field (A,C,E,G) or phase-contrast (B,D,F,H) illumination. The location of the same cell in each figure is shown by the arrows. Bars, 25 μm. (A,B) Mash-1 immunoreactivity is expressed in the nuclei of cells that appear to be mesenchymal and do not express TH. Note that additional cells with a mesenchymal morphology, which do not express Mash-1 immunoreactivity, can be discerned with phase-contrast optics. (C,D) A small cell with short simple neurites exhibits coincidental expression of Mash-1 (blue-black) and TH (red) immunoreactivities. (E,F) A large cell with a neuronal morphology expresses TH, but not Mash-1 immunoreactivity. The processes of this cell are longer and more complex than those of cells doubly labeled by TH and Mash-1 antibodies. (G,H) The cells that show coincident expression of Mash-1 and TH immunoreactivities are often found in doublets, suggesting that they have recently divided.

immunoreactive cells, which always co-expressed B2, did not develop in cultures subjected to complement-mediated lysis (Fig. 3C). These data suggest that elimination of B2-immunoreactive cells prevents the in vitro development of a subset of enteric neurons, including those that are serotonergic, while other classes of enteric neuron continue to arise.

Serotonergic neurons and TC cells are selectively missing in the gut of *Mash-1*^{−/−} mice

We postulated that the enteric lineage derived from TC progenitors is lost selectively in *Mash-1*^{−/−} mice and that all of the neurons present in the ENS of these animals are derived from other progenitors (which never express a catecholaminergic phenotype). In the rat, TC progenitors can be identified by labeling with the SA and B2 antibodies, as well as by expression of TH. Unfortunately, the SA and B2 antibodies do not cross-react with mouse, precluding their use in the analysis of *Mash-1*^{−/−} mice. As a marker of murine TC cells, therefore, we used TH. This marker was validated by demonstrating that TH-immunoreactive cells in the mouse gut express Mash-1. The fetal mouse bowel was dissociated at E12.5 and cultured for 6 hours. TH and Mash-1 expression were investigated by double label immunocytochemistry. Mash-1 immunoreactivity was exclusively intranuclear and was found in about 2-3% of the total population of cells. Many Mash-1^{+} cells were mesenchymal in appearance and lacked processes (Fig. 4A,B). These cells did not express TH. A second population of Mash-1^{+} cells, which did co-express TH assumed a neuronal morphology and extended short neurites (Fig. 4C,D). All of the TH-immunoreactive cells were process-bearing and most, but not all, contained Mash-1 immunoreactive nuclei. The doubly labeled, TH[+]/Mash-1^{+} cells (Fig. 4C,D) were smaller than the TH[+]/Mash-1^{−} cells (Fig. 4E,F), extended simpler neurites, and appeared to be less mature. In fact, some of the TH[+]/Mash-1^{+} cells (Fig. 4G,H), but none of the TH[+]/Mash-1^{−} cells, were found as doublets, suggesting that the TH[+]/Mash-1^{+} cells had recently divided.

Clear expression of TH immunoreactivity was detected in the bowel of wild-type fetuses examined at day E12.5 (Fig. 5A). In contrast, no TH immunoreactivity could be detected in the gut of fetal *Mash-1*^{−/−} mice at this stage (Fig. 5C; arrowhead), although it was detected in the nearby adrenal gland (Fig. 5C; arrow). The absence of TH from the gut of *Mash-1*^{−/−} fetuses did not reflect an absence of crest-derived enteric progenitors, however, because cells that expressed *c-ret*, previously demonstrated to be a marker for crest-derived enteric neural precursors (Pachnis et al., 1993), were detected in this tissue by in situ hybridization in both wild-type

(Fig. 5B) and in *Mash-1*^{−/−} mice (Fig. 5D; arrowhead), consistent with earlier data (Lo et al., 1994). Taken together, these data indicate that TC cells express *Mash-1* and the *Mash-1* null mutation eliminates TC cells.

Enteric serotonergic neurons were next studied as an example of a neuron likely to be derived from the TC lineage. Serotonergic neurons were detected in the fetal mouse bowel (day E17) by incubating tissue with ^{3}H-5-HT and using radioautography to visualize labeled cells and their neurites. This method is highly sensitive and reveals the presence of serotonergic neurons in the developing bowel 4 days before these cells can be visualized by the immunocytochemical demonstration of 5-HT (Rothman and Gershon, 1982). The radioautographic detection of serotonergic neurites depends on the specific expression in serotonergic neurons of a plasma membrane 5-HT transporter molecule that is not expressed by other types of neuron (Blakely et al., 1991; Hoffman et al., 1991). Uptake of ^{3}H-5-HT has recently been found to be a property of crypt epithelial cells (Wade et al., 1993), which like mast cells, express the same 5-HT transporter that is also found in central and enteric serotonergic neurons (Wade et al., 1993). Neither crypt epithelial cells nor mast cells, however, interfere with the radioautographic detection of ^{3}H-5-HT taken up by serotonergic neurons, because these three cell types are present in distant and non-overlapping locations. Indeed, since crypt epithelial cells and mast cells are not crest-derived, they provide a useful positive control for studies of serotonergic neurons in the bowel of *Mash-1*^{−/−} mice.
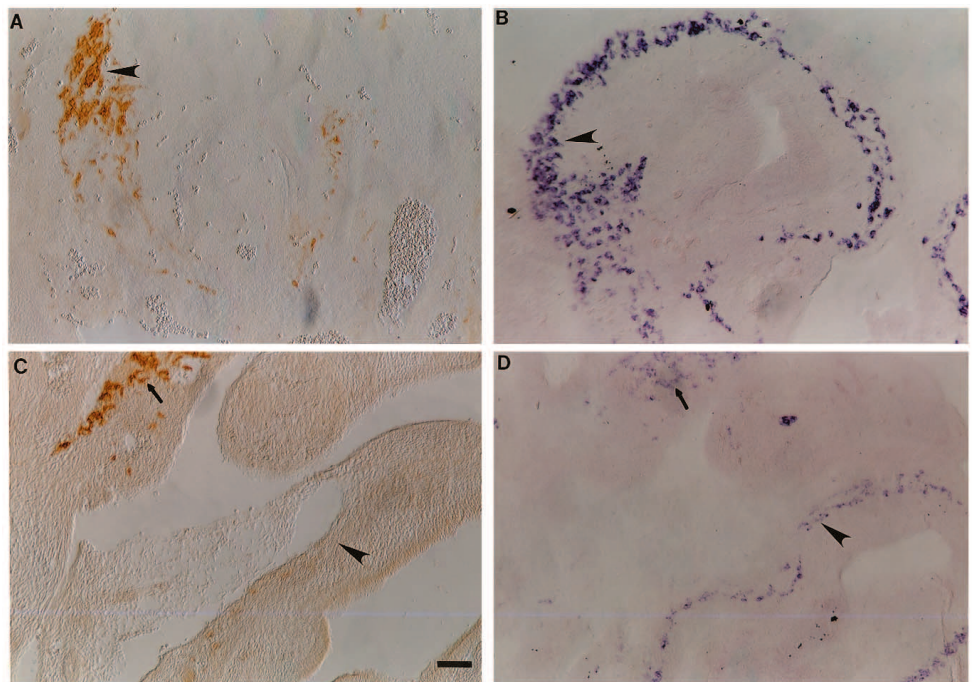

Fig. 5. The *Mash-1*^{−/−} mutation eliminates transiently TH-immunoreactive cells from the fetal gut. E12.5 mouse fetus (sections). (A,B) wild-type fetuses. (C,D) Mash-1^{−/−} fetuses. (A,C) TH immunoreactivity; (B,D) non-radioactive in situ hybridization; probed for *c-ret*. In wild type mice, TH-immunoreactive TC cells (A, arrowhead) can be seen in the same region as cells hybridizing with the c-ret probe (arrowhead) in a near-serial section (B). The wall of the gut of a *Mash-1*^{−/−} fetus (C, arrowhead) lacks TH-immunoreactive TC cells, although in a near-serial section through the same bowel (D), *c-ret*-hybridizing precursors (arrowhead) are present. The adrenal gland (arrow) appears in sections (C and D) through the *Mash-1*^{−/−} tissues. The adrenal is unaffected by the *Mash-1*^{−/−} mutation and serves as an internal positive control for TH expression. Bar, 100 μm.

The uptake of ^{3}H-5-HT was found to be reduced in the whole gut of *Mash-1*$^{-/-}$ mice relative to equivalent regions of bowel from control (*Mash-1* +/+ or +/−) animals (Fig. 6A). The degree of this reduction in whole gut homogenates, however, while significant, was relatively modest. Radioautographic investigation revealed a much more striking difference in the labeling of enteric serotonergic neurons (Fig. 6B). Three cell types became radioautographically labeled by ^{3}H-5-HT in the control bowel, crypt epithelial cells, mast cells, and neurons in the enteric plexuses, particularly the myenteric (Fig. 7C,E,F). Within the myenteric plexus, neuronal cell bodies were labeled, but the bulk of labeling was of neurites in the gan-glionic neuropil (Fig. 7C). In neither *Mash-1*$^{-/-}$, nor control bowel, was labeling of any structure detected when zimelidine, a specific 5-HT uptake inhibitor, was included with ^{3}H-5-HT in the incubation medium (Fig. 7D). In all *Mash-1*$^{-/-}$ mice, the amount of radioautographic labeling in the myenteric plexus was severely decreased (Fig. 7G,H; compare with 7E,F).

A small amount of ^{3}H-5-HT was detectable by radioautog-raphy in the ganglia of some *Mash-1*$^{-/-}$ animals and virtually none in others. A similar pattern was noted in the sympathetic ganglia of the *Mash-1*$^{-/-}$ mice used in this study. Again, a small number of TH-immunoreactive cells and neurites were detectable (always far less than in controls), especially in the prevertebral ganglia. Apparently, a small number of neurons in the *Mash-1*-dependent lineage are able to develop despite the deficiency induced by the targeted mutation in the *Mash-1* locus. In contrast, the labeling of crypt epithelial and mast cells in *Mash-1*$^{-/-}$ mice could not be distinguished from that in controls (compare Fig. 7E,F with G,H). These observations suggest that enteric serotonergic neurons, not the 5-HT trans-porter are selectively deficient in *Mash-1*$^{-/-}$ mice. In contrast to enteric serotonergic neurons, calcitonin gene related peptide (CGRP)-immunoreactive neurons were present at E17 in both control and in *Mash-1*$^{-/-}$ bowel in approximately equal numbers (Fig. 7I-K). The lesion in enteric serotonergic neurons was thus a selective one. Since the foregoing tissue culture data indicate that, in the rat, all serotonergic neurons appear to derive from precursors expressing the B2 antigen, and B2-immunoreactive cells, in turn, derive from TH-expressing TC cells, it is likely that the *Mash-1* mutation affects neurons derived from the analogous set of sympathoadrenal-enteric precursors in the mouse as well.

The morphology of the ENS is abnormal in *Mash-1*$^{-/-}$ mice

The enteric plexuses were examined morphologically in *Mash-1*$^{-/-}$ and control mice. Acetylcholinesterase (AChE) activity was demonstrated in dissected laminar preparations of the bowel wall, because this marker is expressed in almost all enteric neurons. The number of neurons demonstrable by this technique was moderately, but significantly, reduced in the *Mash-1*$^{-/-}$ mice (Fig. 8B) relative to controls (Fig. 8A), to 1650±54 neurons/mm^2 from 2034±69 neurons/mm^2 ($P<0.0001$); moreover, ganglia were more spread apart and interganglionic connectives were longer and more prominent in the *Mash-1*$^{-/-}$ animals. The normal orientation of ganglia perpendicular to the long axis of the gut was lacking in the *Mash-1*$^{-/-}$ bowel, where the ganglia were more randomly arranged. Electron micrographs of myenteric ganglia revealed a difference in the ganglionic ultra-structure between control (Fig. 8C) and *Mash-1*$^{-/-}$ mice (Fig.
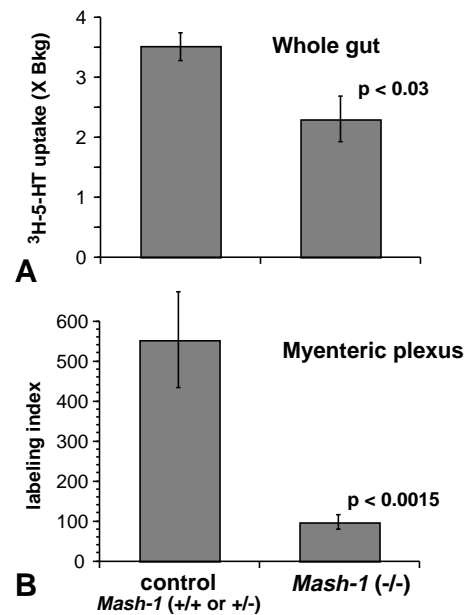

Fig. 6. The specific uptake of ^{3}H-5-HT in the bowel of *Mash-1*$^{-/-}$ mice is reduced relative to that of control animals. Fetal bowel (E17) from *Mash-1*$^{-/-}$ or control (*Mash-1* +/+ or +/−) mice was incubated with ^{3}H-5-HT (0.5 µM). ^{3}H-5-HT was extracted or fixed for the radioautographic visualization of ^{3}H-5-HT uptake sites. (A) Uptake of ^{3}H-5-HT in the absence of zimelidine ÷ uptake of ^{3}H-5-HT in the presence of the inhibitor. The specific uptake of ^{3}H-5-HT by the whole gut (including crypt epithelial cells) is moderately, but significantly, attenuated in *Mash-1*$^{-/-}$ mice. (B) Radioautography enables the uptake of ^{3}H-5-HT by myenteric neurons to be evaluated in the absence of interference by the mucosa or mast cells. The labeling indices (determined by computer-assisted video microdensitometry), indicate that the specific uptake of ^{3}H-5-HT by enteric neurons of *Mash-1*$^{-/-}$ mice is very much less than that of controls.

8D). In the *Mash-1*$^{-/-}$ animals, the ganglionic neuropil was reduced in extent so that the regions in which nerve cell bodies abutted upon one another were longer and more frequently encountered. This difference between the appearance of the ganglia in the two animals appeared to be most severe in a subset of ganglia and thus was not obvious in all; nevertheless, when the area of the ganglionic neuropil was measured in many ganglia (31 control and 75 *Mash-1*$^{-/-}$) and expressed as a pro-portion of the total ganglionic area, a reduction in the size of the neuropil in *Mash-1*$^{-/-}$ mice was clearly demonstrated (from 33.3±2.8% to 21. 3±2.5%; $P<0.01$).

DISCUSSION

The hypothesis that a common progenitor contributes to the formation of both enteric and sympathetic neurons was tested in the present study. In the gut, this common progenitor was postu-lated to be the TC cell (Baetge et al., 1990a), which in the devel-oping rat intestine, exhibits an SA → B2 switch in antigenic phenotype parallel to that in sympathetic ganglia (Anderson et al., 1991; Carnahan et al., 1991). One set of in vitro experiments were carried out with fetal rat intestine in order to permit rat-specific immunological reagents to be used as experimental tools.

These studies were designed to determine whether enteric neurons would continue to develop when the putative sympathoadrenal-enteric progenitor was eliminated by complement-mediated lysis of all cells that express the B2 antigen. A second set of experiments was carried out to ascertain whether the subset of enteric neurons that is derived from the postulated sympathoadrenal-enteric progenitor is selectively eliminated in *Mash-1*⁻/⁻ mice, animals in which the sympathetic nervous system is virtually absent (Guillemot et al., 1993; Lo et al., 1994).

Within 1-3 hours of plating, TC cells, marked by their expression of TH and SA immunoreactivities, were abundant in dissociated cell cultures of E12 fetal rat small intestine, although B2 was not detectable at this time. Expression of both the TH and SA markers was transient in vitro. The disappearance of the SA antigens, moreover, was accompanied by the appearance of the B2 epitope, indicating that the SA → B2 switch, which is seen in situ, also occurs in vitro. Coincident expression of B2 and TH-immunoreactivities, which could be detected because TH expression outlasted that of the SA antigens, indicated that the SA → B2 antigen switch occurs in TC (TH-expressing) cells. Since the B2 epitope was more stable and strongly expressed at cell surfaces than the SA antigens, B2 provided a better target than SA for the complement-mediated lysis of cells in the TC/SA → B2-defined lineage.

Neurons continued to develop in dissociated cell cultures of fetal rat intestine, albeit in diminished numbers, even when the putative sympathoadrenal-enteric progenitor was eliminated by complement-mediated lysis of all cells that express the B2 antigen. This observation suggests that enteric neurons originate from at least two lineages, one that is derived from a progenitor that expresses TC/SA → B2 markers, and one that does not. None of the neurons developing after the elimination of B2-immunoreactive precursors, expressed 5-HT, although some expressed a peptide, VIP. Since 5-HT-immunoreactive cells in control cultures always expressed B2 and were absent from cultures in which B2-expressing cells were eliminated, it is likely that enteric serotonergic neurons develop from a subpopulation of enteric progenitors that express sympathoadrenal lineage markers (SA → B2).

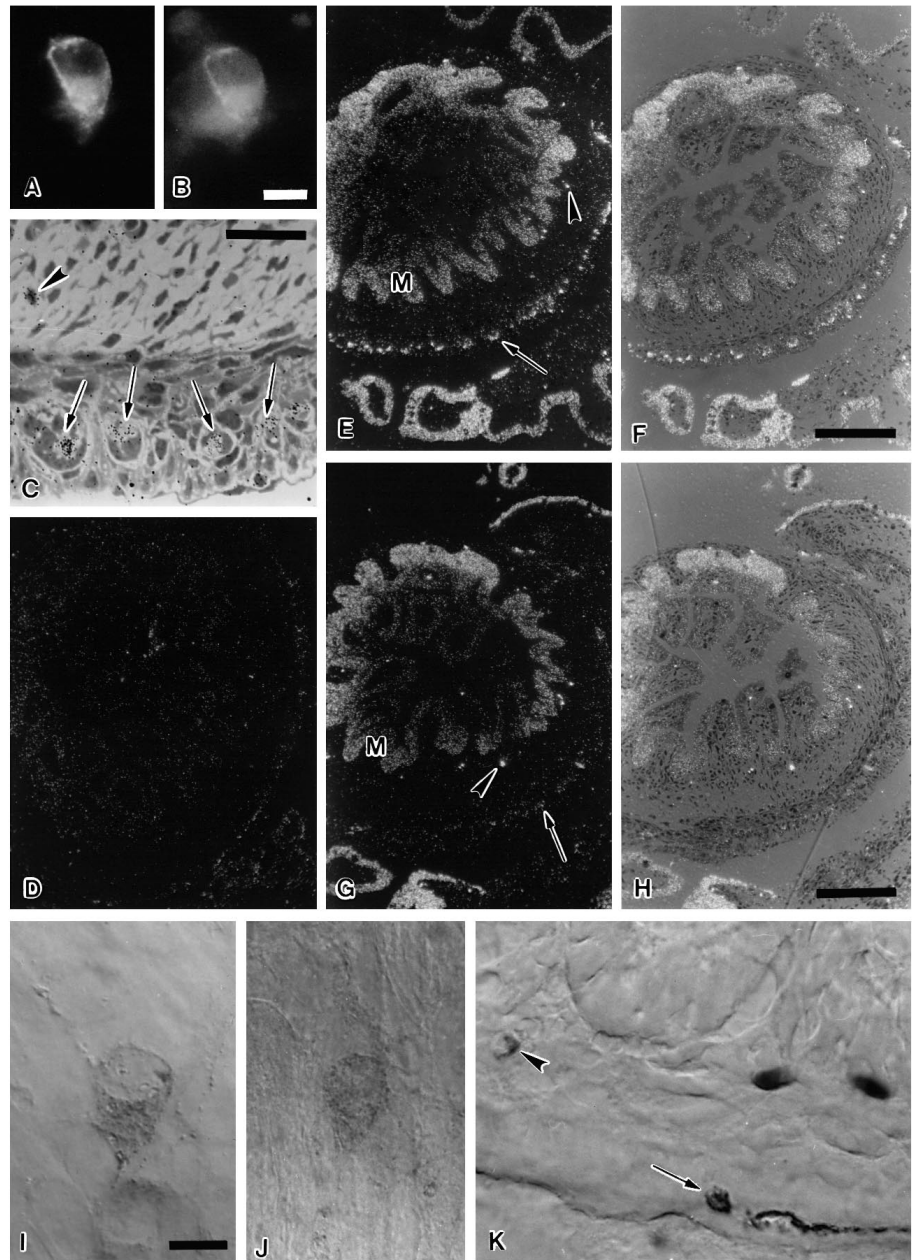The development of the ENS in *Mash-1*⁻/⁻ mice provided a second model in

Fig. 7. Serotonergic neurons express the B2 antigen in vitro and are selectively missing from the ENS of *Mash-1*⁻/⁻ mice. (A,B) Dissociated rat gut after 4 days in vitro. Coincident expression of the B2 antigen (A) in a 5-HT-immunoreactive cell (B). Double label immunocytochemistry; B2, FITC; 5-HT, TRITC. Bar, 10 μm. (C-H) E17 fetal mice, incubated with ³H-5-HT similarly to those in Fig. 5. (E) Control mouse, incident dark-field illumination. Crypt epithelial cells in the developing mucosa (M), mast cells in the connective tissue under the epithelium (arrowhead) and myenteric ganglia (arrow) are labeled. (F) The same field as in E; however, bright-field and dark-field illumination are combined to permit underlying tissue to be visualized. The bar, 100 μm. (C) Higher magnification (bright-field illumination). The radioautographic silver grains in the myenteric plexus are predominantly found over the intraganglionic neuropil (arrow). Mast cells (arrowhead) are also labeled. Bar, 20 μm. (G,H) *Mash-1*⁻/⁻ mouse, incident dark-field (G) and combined dark-field/bright-field illumination (H). Labeling of crypt epithelial cells in the developing mucosa (M) and mast cells in the connective tissue under the epithelium (arrowhead) is as prominent as in the control; however, there is almost no labeling by ³H-5-HT of myenteric ganglia (arrow). (D) Control mouse, incubated with ³H-5-HT in the presence of zimelidine. Labeling has essentially been abolished. (I-K) CGRP-immunoreactive neurons are present in the control (I) and *Mash-1*⁻/⁻ bowel (J, K). I and J are dissected laminar preparations showing myenteric neurons; K is a section showing submucosal CGRP immunoreactive cells (arrowhead), as well as myenteric neurons (arrow). Bars, 20 μm.
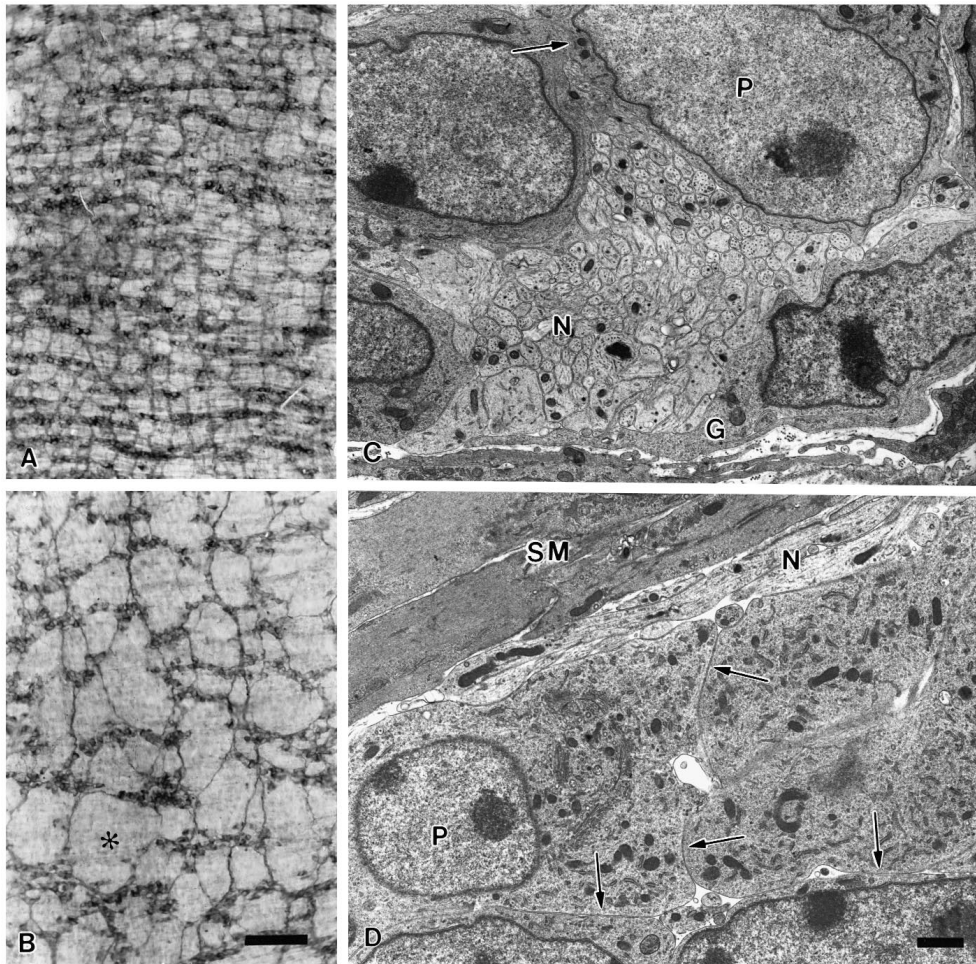
Fig. 8. The structure of myenteric ganglia is abnormal in *Mash-1⁻/⁻* mice. (A,B) AChE activity in whole mounts of bowel wall. In the control tissue (A) myenteric ganglia are circumferentially arrayed (long axis perpendicular to that of the gut). This regular pattern is not seen in the gut from *Mash-1⁻/⁻* fetuses (B). The intervening space (*) between ganglia is greater in the *Mash-1⁻/⁻* tissue and interganglionic connectives are longer. The size of ganglia and number of neurons (relative to controls) are also moderately reduced in the *Mash-1⁻/⁻* preparation. Bar, 100 μm. (C,D) The ultrastructure of ganglia of the duodenal myenteric plexus of control (*Mash-1* +/+ or +/−) mice was compared with that of *Mash-1⁻/⁻* fetuses. (C) Control ganglion. The neuropil (N) consists of many naked neurites, which are not individually enclosed by glial processes (G). Neuronal perikarya (P) are in contact with the neuropil and make limited contact with one another (arrow). (D) *Mash-1⁻/⁻* ganglion. Note the paucity of the ganglionic neuropil (N). Neurites tend to be situated at the periphery of ganglia between neuronal perikarya (P) and the smooth muscle (SM). Domains of contact between neuronal perikarya (arrow) are greatly expanded relative to those seen in control ganglia (C). Bar for C and D, 1.0 μm.

which the hypothesis that enteric and sympathetic neurons arise from a common progenitor could be tested. In these mice, the subpopulation of enteric neurons that is serotonergic was selectively eliminated, as was the TH-immunoreactive population (TC cells) in the fetal gut. Despite the absence of TC cells from the fetal *Mash-1⁻/⁻* gut, cells that express *c-ret* were present, and non-serotonergic enteric neurons, such as those that contain CGRP, ultimately developed. Expression of *c-ret* characterizes crest-derived cells colonizing the fetal bowel (Pachnis et al., 1993) and enteric ganglia are completely absent in mice homozygous for a targeted mutation in *c-ret* (Schuchardt et al., 1994). Mash-1 and TH immunoreactivities, moreover, were found to be colocalized in developing neurons in dissociated cultures of fetal mouse gut, confirming that Mash-1 is indeed expressed by TC cells. The *Mash-1*-dependent enteric neurons, thus appear to be a subset of a larger set of *c-ret*-dependent enteric neuronal precursors. The evidence that the *Mash-1*-dependent enteric serotonergic neurons are derived from the sympathoadrenal-like progenitors in mice is indirect; nevertheless, the fact that TH-immunoreactive cells (TC) express *Mash-1* and are eliminated by the *Mash-1* mutation allows us to link these experiments in mice to previous evidence from rat, suggesting that enteric serotonergic neurons are derived from TC cell progenitors (Baetge et al., 1990a). The present study, furthermore, has indicated that all of the serotonergic neurons that arise in dissociated cultures of rat intestine derive from B2-immunoreactive precursors,

which themselves derive from cells that express TH. Taken together, therefore, these lines of evidence suggest that in the mouse as in the rat, enteric serotonergic neurons derive from progenitors in the sympathoadrenal lineage, which are the same as the TC cells.

The conclusion that the *Mash-1* mutation selectively affects the subset of enteric neurons (including serotonergic neurons) that are derived from a progenitor expressing sympathoadrenal lineage markers is consistent with the fact that the *Mash-1* null mutation affects precursors of sympathetic neurons as well (Lo et al., 1994). Serotonergic cells, moreover, are among the first enteric neurons to be born and the timing of their birth encompasses the period in murine development when the bowel contains TC cells (Pham et al., 1991). This period, when TC cells are normally present (Baetge and Gershon, 1989; Baetge et al., 1990b) and serotonergic neurons are born, corresponds in time to the window of development in *Mash-1⁻/⁻* mice when enteric neurons cannot be seen (Guillemot et al., 1993; Lo et al., 1994). The delay in the appearance of enteric neurons during the ontogeny of the ENS in *Mash-1⁻/⁻* mice (Guillemot et al., 1993; Lo et al., 1994) could thus be explained if the development of lineages of early- but not later-developing enteric neurons were arrested in these animals. While these data indicate that sympathoadrenal progenitors are similar or identical to precursors of the early-born enteric serotonergic neurons in terms of their antigenic phenotype and *Mash-1* dependence, they cannot exclude the possibility that in vivo

these precursors represent two distinct lineages. Transplantation or culture experiments should reveal whether TC precursors in the bowel have the capacity to differentiate into sympathetic neurons, and whether sympathetic precursors can give rise to enteric neurons. Recent studies of developing avian bowel provide support for the notion that enteric crest-derived cells can give rise to sympathetic neurons. Crest-derived cells from recently colonized segments of quail or mouse gut migrate to sympathetic ganglia and differentiate as catecholaminergic neurons when the bowel is back-transplanted into younger chick host embryos (Rothman et al., 1993, 1990).

Examination of the bowel of late fetal *Mash-1*^{−/−} mice revealed that the ENS in these animals not only appeared late, but was abnormal as well. In comparison to control, the distribution of myenteric ganglia was distorted, the ganglia were less densely packed, and the number of neurons was moderately reduced. At the ultrastructural level, the proportion of the area within each ganglion occupied by neurites was decreased. These changes in ganglionic and neuronal morphology suggest that the normal development of later-born enteric neurons may be dependent on the presence of early-generated neurons, which are eliminated by the *Mash-1* mutation.

In summary, our data are consistent with the idea that a subset of neural crest-derived cells, which are identifiable by their transient expression of SA and B2 antigens and of TH, populates both the primordia of the sympathetic ganglia and the gut (Fig. 9). In both locations, the catecholaminergic cells express *Mash-1* and proceed to extinguish SA and express B2 antigens. In the sympathetic ganglia, however, expression of TH is maintained, while in the bowel it is lost. While expression in common of individual genes and antigenic markers cannot prove a lineage relationship, the fact that both neuronal populations are also dependent upon *Mash-1* function indicates that, at the very least, they share a similar genetic program. This correlation, however, should not imply that all cells in the sympathoadrenal lineage are *Mash-1*-dependent, nor that all *Mash-1* expressing cells in the gut are in the sympathoadrenal-enteric lineage. In the first case, it has previously been shown (Guillemot et al., 1993), and confirmed in the current study, that adrenal chromaffin cells, which also derive from the sympathoadrenal lineage and express SA antigens, are only weakly affected by the *Mash-1* mutation. In the second case, it remains possible that the late-born enteric neurons, which can develop when *Mash-1* function is disrupted, nevertheless derive from *Mash-1*-expressing precursors. It has been shown that *Mash-1*-expressing neuronal progenitors in the CNS are unaffected by the loss of function of this gene (Guillemot et al., 1993). *Mash-1* expression itself, is thus not an absolute lineage marker for the sympathoadrenal-enteric progenitor, but rather a gene the function of which is essential for the development of this lineage in several different fetal environments.

While our data suggest that *Mash-1*-dependent progenitors in the gut are similar or identical to progenitors of sympathetic neurons, they also suggest that these progenitors give rise to only a subset of enteric neurons, specifically those that are born early. This implies that neural crest-derived precursors that colonize the bowel give rise to at least two successor lineages (Fig. 9). One, for which *Mash-1* expression is obligatory, is born early, expresses SA and B2 antigens and is transiently catecholaminergic. Enteric serotonergic neurons are an example of a mature neuron that is derived from this lineage. The other lineage(s) can
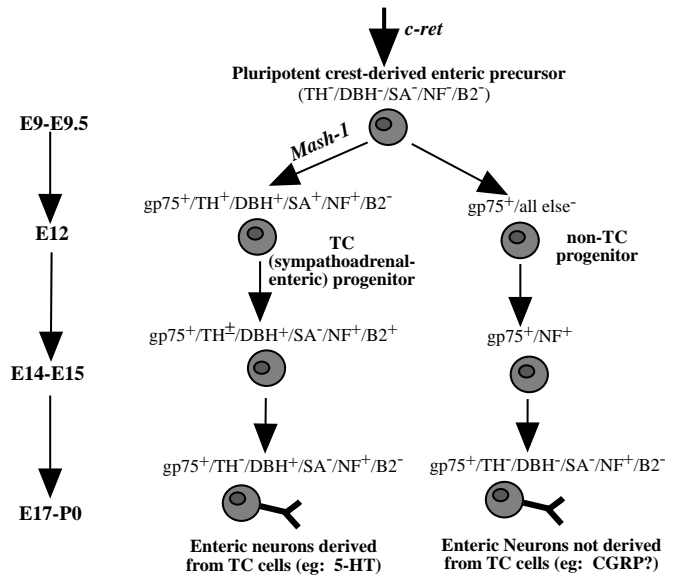

Fig. 9. A heuristic model outlining a possible scheme for enteric neuronal development (see text).

develop even in the absence of *Mash-1* function, does not express sympathoadrenal markers, and thus far can only be detected in the early bowel by general markers such as *c-ret* or $p75^{LNTR}$ expression. Neurons that are born later and express neuronal markers after those that are *Mash-1*-dependent are derived from this/these lineage(s). It is suggested that CGRP-containing neurons, which are among the last to be born in the ENS (Pham et al., 1991), are derived from *Mash-1*-independent progenitors. The sequential waves of enteric neurogenesis thus reflect the existence of two distinct enteric progenitor populations with different antigenic phenotypes and genetic programs.

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