Isolation of Enteric Ganglia from the Myenteric Plexus of Adult Rats

Christine B. Jaeger

*Center for Paralysis Research and Department of Anatomy
School of Veterinary Medicine
Purdue University
West Lafayette, IN 47907, USA*

SUMMARY

Enteric neurons and glia cells were isolated from adult Sprague Dawley rats. A procedure is described using a combination of microdissection and mechanical dissociation after enzyme treatment which yields large numbers of cell clusters suitable for tissue culture and grafting into the injured spinal cord. Differentiated enteric ganglia remained viable for at least 5 days *in vitro*. Cultured neurons expressed histochemical reactivity for acetylcholinesterase and nicotinamide adenine dinucleotide phosphate diaphorase. Nestin positive glia, which represented a population of non-myelinating enteric Schwann cells, could also be identified in cultures maintained 5 days or longer *in vitro*. The myenteric plexus of adult rats can provide a readily available source of neurons and Schwann cells for grafting to the central nervous system.

KEY WORDS

myenteric plexus, cluster culture, acetylcholinesterase, NADPH-diaphorase, nestin

INTRODUCTION

The enteric nervous system contains a large population of neurons /15,42/ which in humans may approach 10^8 , a similar number to the neurons in the

spinal cord. Enteric neurons are functionally independent from the central nervous system (CNS) /3,14,17/. Motor neurons and sensory neurons coordinate and regulate various aspects of the digestive process including gut motility. Neurons in the myenteric plexus exhibit a remarkable plasticity that persists into adulthood. Injury of myenteric ganglia results in extensive regeneration and regrowth of cell processes *in situ* /11/ as well as reorganization of remaining ganglia in areas of damage to the intestinal wall /38/. Explanted ganglia undergo complex reorganization in tissue culture /23/ where they form interconnecting clusters that resemble myenteric plexuses *in vivo*. Enteric ganglia that are transplanted to the injured spinal cord maintain their synthesis of acetylcholinesterase (AChE) /20/ and may provide novel targets for regenerating spinal afferents /18/. These attributes make enteric neurons useful candidates for potential therapies in spinal injury and other types of CNS repair /20,26,37/. Numerous previous studies have demonstrated development of neo-pathways originating from implants of embryonic neurons /10,16,19,21/. However, mature neurons rarely survive as neural grafts with the exception of enteric ganglia and other types of mature peripheral ganglia /40/. Grafts from such sources consist of fully differentiated neurons and glia cell types which may provide important alternatives to fetal tissue in their application to modify the microenvironment of injured CNS.

Tissue and explant cultures of the myenteric plexus have been used previously to study physiology and neurochemistry of the enteric nervous system. For most of this work enteric ganglia were isolated from immature mammals, such as newborn guinea-pigs /2,6,22,23,33/, neonatal hamsters /25/ or newborn rats /30,31,39/. More recently, important physiological and

Reprint address:

Christine Jaeger
Department of Anatomy
Purdue University
School of Veterinary Medicine
West Lafayette, IN 47907, USA

pharmacological characteristics of enteric neurons and glia were established using preparations from mature guinea-pigs [1,12,41]. Isolating the enteric ganglia from the gut wall musculature of mature animals requires difficult and time consuming dissections which typically yield only small quantities of intact ganglia [1,41]. The need to harvest large quantities of enteric ganglia for transplantation to injured CNS [20,26,37] has provided an incentive to simplify available protocols. A method is described here which allows relatively rapid isolation of sufficient quantities of enteric ganglia from the myenteric plexus of adult rats.

METHODS

Twenty adult rats (150-250 g) of the Sprague Dawley strain were used in separate experiments for the isolation of enteric ganglion cell clusters.

Isolation of gut neurons from adult animals

Rats were deeply anesthetized with nembutal (50 mg/kg). The abdomen was shaved, disinfected, and draped. The intraperitoneal cavity was opened by a midline incision, the small intestine was excised and the rat was killed. The jejunum was divided into 2-3 cm long segments, rinsed (in and out) with sterile Hank's balanced salt solution (HBSS) and placed in ice-cold HBSS. For microdissection, each segment of gut was placed separately in a 60 mm Petri dish and thoroughly rinsed with HBSS. The longitudinal muscle layer was carefully lifted and separated from underlying circular muscle and mucosa along one end of the segment by teasing the layers apart with fine tweezers avoiding excessive stretching. In many of the segments the outer muscle layer was peeled off like a sleeve. This microdissected tissue contained longitudinal smooth muscle sheets with attached myenteric ganglia (Fig. 1). Tissue was pooled from the segments, placed in 0.1% collagenase in cell dissociation fluid (Sigma) or HBSS and incubated at 37°C for periods from 30 minutes to four hours. Following incubation, the enzyme solution was aspirated and replaced with Dulbecco's modified Eagle's medium containing 5% horse serum, 5% fetal bovine serum and 50 units penicillin-streptomycin per milliliter. Tissue

Fig. 1: AChE stained myenteric ganglia (open arrows) in a whole mount preparation of the jejunum. Darkfield photomicrograph, scale bar = 200 μ m.

pieces were briefly agitated with a vortex and mechanically dissociated by titration with a Pasteur pipet (0.5-0.3 mm tip). Successful cell separations of ganglia from sheets of muscle tissue were monitored by morphological criteria.

In order to test the effects of storage on ganglion cell isolation, some segments of intestine were first stored overnight in cold (4°C) Dulbecco's modified Eagle's medium with 25 mM HEPES buffer (Sigma D1152) prior to microdissection of the myenteric plexus.

Four rats were used to estimate the recovery and survival of ganglion cell clusters over a period of five days. For this a quantitative evaluation of cell clusters was carried out as follows. Tissue was dissected from the jejunum (≥ 60 cm length) as described above. All dissociated ganglia were collected in 2.6 or 3.6 ml tissue culture medium. Identical volumes (40 μ l) of dissociated cell clusters were plated into 13 or 18 compartments of five multiwell (with 24 wells each) plates coated with collagen. Cell clusters were allowed to settle and rinsed once with fresh medium. After 24 hours of incubation at 37°C, clusters were rinsed again with tissue culture medium to remove floating cells and debris. Subsequently, the culture medium was replaced with fresh medium once per day. One of the plates was fixed (see below) each day for five consecutive days. Subsequently, all the cell clusters were counted in each well and added to obtain an

estimate of the total number of cell clusters per plate. The number of attached cell clusters on each day was compared to counts obtained after one day in culture which was set at 100 percent.

Histochemistry and immunocytochemistry

Cultures were fixed in 4% paraformaldehyde dissolved in 0.1 M phosphate buffer (pH 7.4) for 15-20 minutes. The fixation fluid was replaced by 0.1 M phosphate buffer (pH 7.4) in which the fixed cultures were stored at 4°C until staining. AChE histochemistry was carried out for 60-90 minutes at room temperature using a single incubation step /20/. Staining for nicotinamide adenine dinucleotide phosphate (NADPH) diaphorase was done at 37°C in an incubation medium containing 1 mg/ml NADPH, 25 mg/ml nitroblue tetrazolium and 0.3% Triton X-100 dissolved in Tris buffer (pH 8.0) /4/. For immunocytochemistry, cultures were dehydrated with ascending alcohols (70%, 95%, 100% ethyl alcohol) and again rehydrated. The primary antiserum was added after a 30 minute preincubation step in 0.2% Triton X-100 and 0.5% bovine albumin dissolved in 0.1 M Tris saline buffered at pH 7.4. An "ABC" Vector kit (Burlingame, CA) was used for indirect visualization of the antigens in cultured cells. Monoclonal antiserum Rat 401 was a gift of Dr. S. Hockfield. It was used at dilution 1:500 in Tris saline buffer. Monoclonal antisera to galactocerebroside and glia filament protein (GFAP) were purchased from Boehringer and used at dilutions of 1:200. ED 1 antiserum, diluted 1:500 was obtained from Serotech (Indianapolis).

RESULTS

Enzyme treatment and dissociation

Collagenase treatment and mechanical dissociation yielded a population of cell clusters that varied in size containing between 5-60 cells per cluster. Optimal mechanical dissociation of enteric ganglia from microdissected muscularis externa was achieved following enzymatic digestion for 30 to 120 minutes (Fig. 2). Longer times of enzymatic treatment (4 hours) resulted in more complete cell dissociation but limited survival of enteric neurons.

Fig. 2: Freshly isolated ganglion cell clusters after plating into a collagen coated dish. Smooth muscle cells, with long slender shapes (arrow heads), are completely detached from "round cell" containing clusters unlike the preparation shown in Figure 3 below. Scale bar = 180 μ m.

Spindle shapes characterized single smooth muscle cells (Figs. 2,3). Enteric neurons had rounded shapes and they were larger in diameter (Fig. 3). On suitable substrates (such as adjacent non-neuronal cells) the rounded cell clusters grew processes after several days in culture (Fig. 5) and formed extensive networks *in vitro* (Fig. 4). Neurons from such cell clusters were positively identified by their histochemical properties (see below). Under the culture conditions employed, few enteric neurons extended processes after complete mechanical dissociation into single cells. Similarly, few viable enteric

Fig. 3: Two isolated ganglion cell clusters stained positively for AChE. Note attached smooth muscle cell (arrow head). Scale bar = 120 μ m.

Fig. 4: NADPH-diaphorase stained neurons in a large ganglion cell cluster after 19 days *in vitro*. Note radially extending cell processes (open arrows). Scale bar = 200 μ m.

neurons were recovered after cold storage of gut tissue. Cluster cultures from these experiments expressed positive immunocytochemical reaction with Rat 401 similar to enteric glia cultured together with neurons.

Cell clusters were counted in four separate experiments. Each experiment was carried out over a period of five consecutive days (Fig. 8). The data showed that, on average, approximately 80% of the cell clusters remained attached after two days in culture. From the initial plating at least 55% of the cell clusters were recovered after five days. The isolated cell clusters contained living enteric neurons which grew processes (Figs. 4,5) and distributed in colonies that also contained non-neurons which firmly adhered to the culture substrate.

Cell identity: histochemical and immunocytochemical staining

Individual cells in freshly isolated ganglion cell clusters had rounded somata (Fig. 2) similar to those of neurons from other peripheral ganglion cells. Neurons of the myenteric plexus synthesize AChE [20,22]. However, this enzyme was much reduced in smooth muscle and absent in enteric glia cells and fibroblasts. Consequently, AChE histochemistry was useful for positive identification of

Fig. 5: Small AChE positive cell cluster five days after isolation. Neurites are marked by arrows. Darkfield photomicrograph, scale bar = 100 μ m.

enteric neurons in ganglion cell clusters. Following AChE staining, a dark brown reaction product labeled larger cell clusters and a few single cells (Fig. 3). A proportion of small cell clusters with rounded somata and some single cells failed to express AChE positive stain. After three to five days *in vitro* the majority of cell clusters had become flattened in shape but they continued to distribute in cell groups or "colonies". These colonies had variable sizes and they contained AChE positive neurons (Fig. 5) and AChE negative non-neurons or glia cells. A proportion of the myenteric neurons in enteric cell clusters stained with NADPH-diaphorase histochemistry (Fig. 4). The myenteric neurons regrew elaborate processes that extended within and between nearby colonies. Isolation of enteric cell clusters from gut segments that had been stored in cold medium for ten hours or longer (up to 3 days) resulted in fewer cells positive for AChE or NADPH-diaphorase after several days in culture.

Each surviving cluster had at least one, or several, AChE positive cells and a number of negative cells. Unstained cells were mostly enteric glia cells with flattened shapes and elongated processes. Enteric glia cells stained poorly with anti GFAP [20], but weakly GFAP positive cells were noted in the cluster cultures (data not shown). A few isolated cells reacted with antiserum to

galactocerebroside. Positive identification of another non-neuronal cell type was made by immunostaining with ED 1 antisera. ED 1 detects macrophages and other mononucleated cells of the immune system /8/. Cluster cultures from smaller and medium sized cell cluster preparations, similar to those illustrated in Figure 2, were negative for ED 1. Flattened polygonal cells never stained with ED 1 in any of the cultures. ED 1 positive cells maintained their round shapes. A few of these were observed near the surface of rarely noted large colonies, which appeared to be derived from enteric ganglia that had been poorly or not at all dissociated.

Cluster cultures maintained *in vitro* for five days or longer contained cell groups that stained positively with Rat 401 monoclonal antiserum (Figs. 6,7). Immunocytochemical reactivity to Rat 401 was not detected in cell clusters acutely isolated from enteric ganglia and in paraformaldehyde fixed whole mounts of myenteric plexus attached to longitudinal oriented smooth muscle of the muscularis externa.

6

Fig. 6: Ganglion cell cluster immunoreactive with Rat 401 after 5 days *in vitro*. Scale bar = 100 μ m.

Fig. 7: Enteric glia cells, immunoreactive with Rat 401, emerging from an "out-of view" ganglion cell cluster nearby, 12 days *in vitro*. Note unstained layer of flattened cells form a substrate for immunoreactive cells. Scale bar = 100 μ m.

DISCUSSION

These studies demonstrate the feasibility of isolating cell clusters from the myenteric plexus of adult rats. Partially dissociated ganglia were obtained from the intestinal wall after microdissection and subsequent enzymatic treatment of the tissue /1,12,20,30,31,41/. During microdissection the myenteric plexus remains attached to the outer longitudinal layer of the bowel musculature from which it can be extracted by treatment with various enzymes. The present study employed a simple separation of enteric ganglion cell clusters by differential adhesion to collagen coated tissue culture plastic. During this step most of the dissociated smooth muscle cells were removed. The cell clusters that adhered to the dish within one day of culture were separated from floating cells by several changes of the growth medium. After four to five days in serum-containing tissue culture

Cell Cluster Counts

Fig. 8: Histogram of cell cluster counts from three experiments, series 1 to 3, respectively. Day 1 represents counts taken 24 hours after initial plating of the isolated cell clusters.

medium, cells with originally rounded shapes assumed flattened polygonal forms. Enteric neurons reactive for AChE and NADPH-diaphorase extended their processes on enteric glia cells which served as substrates.

Previously, several methods were developed in other laboratories for isolating the immature myenteric plexus /7,22,23,25,30/. The myenteric plexus is free of connective tissue, extracellular collagen and blood vessels /15,17/. Thus, treatment of intact segments of the gut wall with highly purified collagenase allows the isolation of interconnected myenteric ganglia following gentle separation of the muscularis externa of the bowel /22,23,33,35/. The layers of smooth muscle are not dissociated by this method and ganglia of the plexus remain connected causing minimal disruption of myenteric neurons and little, if any, contamination by adherent smooth muscle cells. Other procedures have also relied on enzymatic digestion of connective tissue and muscle following various mechanical operations. For example, mincing the gut wall prior to collagenase treatment was used to isolate myenteric neurons from newborn hamsters

/25/. An efficient microdissection method was introduced by Nishi and Willard /30,31/ by which the outer longitudinal muscle layer of the muscularis externa was separated from the intestine of newborn rats. Stretching the gut segment over a piece of glass tubing aided in the dissection. Subsequently, the tissue was treated with Dispase and mechanically dissociated. This method yields partially dissociated ganglia which contain clusters of enteric neurons and enteric glia cells. In newborn rats the gut wall is thin and transparent and contains relatively few smooth muscle cells which rarely adhere to isolated neuron clusters /30,31/. However, application of this method to mature animals with increased muscle mass in their gut wall poses a greater challenge regarding the elimination of smooth muscle cells. These cells will adhere to the isolated ganglia and grow unless they are damaged by the enzyme treatment or removed from the initial cell isolate. For example, myenteric ganglia from adult rats dissociated by Nishi and Willard's procedure contained large quantities of smooth muscle and connective tissue after transplantation to the spinal cord /20/.

The method described in the present study combined microdissection and enzymatic treatment and it included several modifications of previous protocols. Most importantly, an "adhesion" separation of ganglion cell clusters from dissociated muscle was tested. The initial steps of the isolation procedure were similar to Nishi and Willard's /30/ method. However, separation of the two muscle layers of the muscularis externa in adult rats seemed to be facilitated in low calcium buffer and partly contracted gut segments kept at 4°C. Furthermore, tissue dissociation with purified collagenase and optimal treatment times yielded preparations in which smooth muscle was dissociated into single cells whereas cells of the myenteric plexus remained in clusters. Initially, smooth muscle cells failed to attach to the culture dish. This allowed removal of dissociated smooth muscle cells, by several rinses with tissue culture medium, from ganglion cell clusters, which adhered to the substrate. Significant reduction of smooth muscle cells and enrichment of ganglion cell clusters was achieved. Nevertheless, it remains to be shown whether a small proportion of smooth muscle cells aggregated with the ganglion cell clusters. However, it was observed that contamination of ganglion cell clusters by macrophages was unlikely. Macrophages normally occur in adult intestine /28/. Partial dissociation of enteric ganglia during their isolation appeared to reduce ED 1 reactive macrophages.

Reduced survival of enteric neurons after exposure to anoxia, which occurred during extended storage of the intestine, was an expected finding. Surprisingly, non-neuronal cells that expressed immunoreactivity to Rat 401 antiserum after *in vitro* culture, were isolated from such ganglion cell clusters. Rat 401 antiserum has been shown to label products of the gene named "nestin" /27/. Characteristically neuronal precursor cells were labeled with anti Rat 401. Schwann cells, which form myelin sheaths of peripheral nerves, also react immunocytochemically with Rat 401 /13/. This is of interest because enteric glia do not form myelin in the myenteric plexus /15,17/. However, non-myelinating Schwann cells of the enteric nervous system have the capacity to form myelin after transplantation to chick /9/ or rat spinal cord /18,20/. Expression of Rat 401 in Schwann cells and

cultured enteric glia suggests that these cells share a common ancestor. Absence of staining with Rat 401 in the myenteric plexus *in situ* could indicate low levels of the antigen and conditions which suppress transcription of the nestin gene. Enteric ganglia provide a potential "cell reserve" in the body that could be utilized as a source of fully differentiated neurons and enteric glia cells, with defined properties /14,31,39/, to repair injuries in the central nervous system /20,26,37/. For example, the use of mature neurons has the advantage of supplying already differentiated phenotypes of known functional potential /3,14/. Enteric glia cells may supply myelin sheaths for demyelinated CNS fiber tracts. Although these glia cells do not form myelin in the myenteric plexus /15,17/, they may do so after transplantation to an altered microenvironment.

Separation of nonadherent cells provided an important purification step in the isolation of enteric ganglion cells from dissociated smooth muscle cells. Enzymatic digestion and mechanical dissociation resulted in cell suspensions in which the single muscle cells outnumbered the cell clusters. After plating on an adhesive substrate the nonadherent smooth muscle cells and cell debris were aspirated. However, poor adhesion to the substrate may also have caused some loss of potentially "viable" ganglion cell clusters. An average of 55% of the clusters plated remained attached to the culture dish for at least 5 days *in vitro*. Cell cluster survival will be influenced by culture conditions and by the enzymatic digestion which may potentially injure some cells. Damaged cells would tend to be less sticky, since their ability to replace extracellular matrix components could be impaired. Floating and poorly adherent cell clusters were removed during rinsing and medium replacement procedures. In addition, requirements of adult enteric neurons in culture may differ from those of more immature cells /2,7,32,34/.

Fiorica-Howells and coworkers /12/ used another method of separating intact myenteric ganglia from the intestine of adult guinea-pigs. The tissue suspension was filtered through "nucleopore" filters of just 8 µm pore size. All isolated smooth muscle cells were removed in the filtrate and intact ganglia remained on the filter surface. During the course of the present study a

similar method was tried unsuccessfully and subsequently abandoned in favor of the adhesion procedure. A nitex membrane of 70 μm mesh size was used as a sieve. This membrane retained isolated cell clusters of partial ganglia, which differentiated into enteric neuron and glia cell containing colonies. Single muscle cells were observed in the filtrate. However, cells retained by the nitex membrane, as tested by direct microscopical observation, included a significant number of smooth muscle cells. In their relaxed state smooth muscle cells isolated from the rat jejunum had diameters of less than 10 μm but measured 180-250 μm in length. These different observations may relate to differences in filter properties and variable characteristics of smooth muscle cells in guinea-pig and rat.

The myenteric plexus is subjected to variable stretching and relaxation periods during normal peristaltic movements of the gut musculature. This activity may cause a certain amount of ongoing "natural injury" on enteric ganglia. Thus, the essential ability to reconstitute cell processes and to migrate under suitable circumstances may persist in adult enteric neurons. Moreover, the tissue environment of enteric ganglia, which become embedded in smooth muscle, may promote release of growth factors and discourage the synthesis of growth inhibiting molecules in enteric glia cells. Examples of plastic changes in enteric ganglia have been described following lesions of the myenteric plexus /11/ and reanastomosis of the intestine /38/. These studies showed that individual enteric neurons from intact ganglia migrate to regions of denervated smooth muscle and reestablish functional connections.

Several factors may be generated and stored by enteric ganglia which could regulate expression of plasticity and proliferation. A recent investigation has shown that neurotrophin-3 induces the differentiation of neural crest-derived cells *in vitro* while NGF, BDNF, and neurotrophin-4/5 do not /7/. Other studies have suggested that purines and fibroblast growth factor (FGF) may contribute signals for regulation of cell survival and differentiation of enteric neurons /36/. FGF is known to affect survival and proliferation of CNS neuron precursors /29/ in addition to stimulating process

elongation of neurons and PC12 cells. The source of FGF in the enteric nervous system has not been established but interstitial cells and enteric glia may be involved in its synthesis. Basement lamina proteins that ensheath the plexus in its entirety may also aid in the storage of FGF. The newly developed procedures for isolating enteric glia and enteric neuron enriched cultures from ganglia of immature intestine /2,7,33,35/ and the methods described here will complement each other to allow further studies of the mechanisms that regulate plasticity in the enteric nervous system.

ACKNOWLEDGEMENTS

I thank Bryan Baetsle for his invaluable contribution in testing numerous dissection protocols for the isolation of myenteric plexus. Monoclonal antibody, Rat 401, was generously supplied by Dr. S. Hockfield. Rose Marie Killian's expert editorial assistance was much appreciated during preparation of the manuscript.

REFERENCES

1. Baidan LV, Fertel RH, Wood JD. Effects of brain-gut related peptides on cAMP levels in myenteric ganglia of guinea-pig small intestine. *Eur J Pharmacol Mol Pharmacol* 1992; 225: 21-27.
2. Bannerman PGC, Mirsky R, Jessen KR. Establishment and properties of separate cultures of enteric neurons and enteric glia. *Brain Res* 1988; 440: 99-108.
3. Bartho L, Holzer P. Search for a physiological role of substance P in gastrointestinal motility. *Neuroscience* 1985; 16: 1-32.
4. Blottner D, Baumgarten H-G. Nitric oxide synthetase (NOS)-containing sympathoadrenal cholinergic neurons of the rat IML-cell column: Evidence from histochemistry, immunohistochemistry, and retrograde labeling. *J Comp Neurol* 1992; 316: 45-55.
5. Blottner D, Baumgarten H-G. Basic fibroblast growth factor prevents neuronal death and atrophy of retrogradely labeled preganglionic neurons *in vivo*. *Exp Neurol* 1992; 118:35-46.
6. Broussard DL, Bannerman PGC, Tang C-M, Hardy M, Pleasure D. Electrophysiologic and molecular properties of cultured enteric glia. *J Neurosci Res* 1993; 34: 24-31.
7. Chalazonitis A, Rothman TP, Chen J-X, Lamballe F, Barbacid M, Gershon MD. Neurotrophin-3 induces neural crest-derived cells from fetal rat gut to develop

- in vitro as neurons or glia. *J Neurosci* 1994; 14: 6571-6584.
8. Dijkstra CD, Dopp EA, Joling P, Kraal G. The heterogeneity of mononuclear phagocytes in lymphoid organs: distinct macrophage subpopulations in the rat recognized by monoclonal antibodies ED1, ED2 and ED3. *Immunology* 1985; 54: 589-599.
 9. Dulac C, Le Douarin NM. Phenotypic plasticity of Schwann cells and enteric glial cells in response to the microenvironment. *Proc Natl Acad Sci USA* 1991; 88: 6358-6362.
 10. Dunnett SB, Rogers DC, Richards S-J. Nigrostriatal reconstruction after 6-OHDA lesions in rats: combination of dopamine-rich nigral grafts and nigrostriatal "bridge" grafts. *Exp Brain Res* 1989; 75: 523-535.
 11. Ekblad E, Ekman R, Hakanson R, Sundler F. Return of nerve fibers containing gastrin-releasing peptide in rat small intestine after local removal of myenteric ganglia. *Neuroscience* 1988; 24: 309-319.
 12. Fiorica-Howells E, Wade PR, Gershon MD. Serotonin-induced increase in cAMP in ganglia isolated from the myenteric plexus of the guinea pig small intestine: mediation by a novel 5-HT receptor. *Synapse* 1993; 13: 333-349.
 13. Friedman B, Zaremba S, Hockfield S. Monoclonal antibody Rat 401 recognizes Schwann cells in mature and developing peripheral nerve. *J Comp Neurol* 1990; 295: 43-51.
 14. Furness JB, Bornstein JC, Murphy R, Pompolo S. Roles of peptides in transmission in the enteric nervous system. *Trends Neurosci* 1992; 15: 66-71.
 15. Gabella G. Innervation of the gastrointestinal tract. *Int Rev Cytol* 1979; 59: 129-193.
 16. Gerloff C, Knappe UJK, Hettmannsperger U, Duffner TK, Volk B. Intrastriatal cerebellar grafts: differentiation of cerebellar anlage and sprouting of Purkinje cell axons. *Dev Brain Res* 1993; 74: 30-40.
 17. Gershon MD. The enteric nervous system. *Ann Rev Neurosci* 1981; 4: 227-272.
 18. Jaeger CB. Adult enteric neurons implanted in the spinal cord. *Soc Neurosci Abst* 1993; 19: 86.
 19. Jaeger CB, Lund RD. Efferent fibers from transplanted cerebral cortex of rats. *Brain Res* 1979; 165: 338-342.
 20. Jaeger CB, Toombs JP, Borgens RB. Grafting in acute spinal cord injury: Morphological and immunological aspects of transplanted adult rat enteric ganglia. *Neuroscience* 1993; 52: 333-346.
 21. Jaeger CB, Wolf AL. Neo-pathway formation of dissociated neural grafts demonstrated by immunocytochemistry, fluorescent microspheres, and retrograde transport. *Brain Res* 1989; 487: 225-237.
 22. Jessen KR, Saffrey MJ, Baluk P, Hanani M, Burnstock G. The enteric nervous system in tissue culture. III. Studies on neuronal survival and the retention of biochemical and morphological differentiation. *Brain Res* 1983; 262: 49-62.
 23. Jessen KR, Saffrey MJ, Burnstock G. The enteric nervous system in tissue culture. Cell types and their interactions in explants of the myenteric and submucous plexuses from guinea pig, rabbit and rat. *Brain Res* 1983; 262: 17-35.
 24. Karnovsky MJ, Roots L. A 'direct-coloring' thiocholine method for cholinesterases. *J Histochem Cytochem* 1964; 12: 219-221.
 25. Korman LY, Nylen ES, Finan TM, Linnoila RI, Becker KL. Primary culture of the enteric nervous system from neonatal hamster intestine. Selection of vasoactive intestinal polypeptide containing neurons. *Gastroenterol* 1988; 95: 1003-1010.
 26. Lawrence JM, Raisman G, Mirsky R, Jessen KR. Transplantation of postnatal rat enteric ganglia into denervated adult rat hippocampus. *Neuroscience* 1991; 44: 371-379.
 27. Lendahl U, Zimmerman LB, McKay RDG. CNS stem cells express a new class of intermediate filament protein. *Cell* 1990; 60: 585-595.
 28. Mikkelsen HB, Rumessen JJ. Characterization of macrophage-like cells in the external layers of human small and large intestine. *Cell Tissue Res* 1992; 270: 273-279.
 29. Murphy M, Drago J, Bartlett PF. Fibroblast growth factor stimulates the proliferation and differentiation of neural precursor cells *in vitro*. *J Neurosci Res* 1990; 25: 463-475.
 30. Nishi R, Willard AL. Neurons dissociated from rat myenteric plexus retain differentiated properties when grown in cell culture. I. Morphological properties and immunocytochemical localization of transmitter candidates. *Neuroscience* 1985; 16: 187-199.
 31. Nishi R, Willard AL. Neurons dissociated from rat myenteric plexus retain differentiated properties when grown in cell culture. II. Electrophysiological properties and responses to neurotransmitter candidates. *Neuroscience* 1985; 16: 201-211.
 32. Nishi R, Willard AL. Conditioned medium alters electrophysiological and transmitter-related properties expressed by rat enteric neurons in cell culture. *Neuroscience* 1988; 25: 759-769.
 33. Saffrey MJ, Bailey DJ, Burnstock G. Growth of enteric neurons from isolated myenteric ganglia in dissociated cell culture. *Cell Tiss Res* 1991; 265: 527-534.
 34. Saffrey MJ, Burnstock G. Growth factors and the development and plasticity of the enteric nervous system. *J Auton Nerv Syst* 1994; 49: 183-196.
 35. Saffrey MJ, Hassall CJS, Allen TGJ, Burnstock G. Ganglia within the gut, heart, urinary bladder, and airways: Studies in tissue culture. *Int Rev Cytol* 1992; 136: 93-144.
 36. Saffrey MJ, Schafer KH, Burnstock G. Effects of 2-Chloroadenosine and FGF on myenteric neurons in dissociated cell culture. *Soc Neurosci Abst* 1994; 20: 1690.

37. Tew EMM, Anderson P, Saffrey MJ, Burnstock G. Transplantation of the postnatal rat myenteric plexus into the adult rat corpus striatum: an electron microscopic study. *Exp Neurol* 1994; 129: 120-129.
38. Tokui K, Sakanaka M, Kimura S. Progressive reorganization of the myenteric plexus during one year following reanastomosis of the ileum of the guinea pig. *Cell Tiss Res* 1994; 277: 259-272.
39. Willard AL, Nishi R. Neurons dissociated from rat myenteric plexus retain differentiated properties when grown in cell culture. III. Synaptic interactions and modulatory effects of neurotransmitter candidates. *Neuroscience* 1985; 16: 213-221.
40. Wrathall JR. Spinal cord injury models. *J Neurotrauma* 1992; 9: S129-S134.
41. Yau WM, Dorsett JA, Parr EL. Characterization of acetylcholine release from enzyme-dissociated myenteric ganglia. *Am J Physiol* 1989; 256: (Gastrointest Liver Physiol 19) G233-G239.
42. Young HM, Furness JB, Sewell P, Burcher EF, Kandiah CJ. Total numbers of neurons in myenteric ganglia of the guinea pig small intestine. *Cell Tiss Res* 1993; 272: 197-200.