UC Davis
UC Davis Previously Published Works

Title

Autonomic and enteric neurons: Cell culture

Permalink

https://escholarship.org/uc/item/0d84p3f1

Authors

Lein, PJ

Fryer, AD

Publication Date

2016

DOI

10.1016/B978-0-12-809324-5.23365-8

Copyright Information

This work is made available under the terms of a Creative Commons Attribution-NoDerivatives License, available at https://creativecommons.org/licenses/by-nd/4.0/

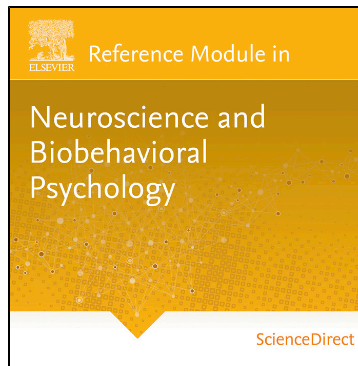
Peer reviewed

eScholarship.org Powered by the California Digital Library
University of California

Author's personal copy

Provided for non-commercial research and educational use.
Not for reproduction, distribution or commercial use.

This article was originally published in the online Reference Module in Neuroscience and Biobehavioral Psychology, published by Elsevier, and the attached copy is provided by Elsevier for the author's benefit and for the benefit of the author's institution, for non-commercial research and educational use including without limitation use in instruction at your institution, sending it to specific colleagues who you know, and providing a copy to your institution's administrator.



All other uses, reproduction and distribution, including without limitation commercial reprints, selling or licensing copies or access, or posting on open internet sites, your personal or institution's website or repository, are prohibited. For exceptions, permission may be sought for such use through Elsevier's permissions site at:
https://www.elsevier.com/about/our-business/policies/copyright/permissions

From Lein P.J., Fryer A.D. Autonomic and Enteric Neurons: Cell Culture. In Reference Module in Neuroscience and Biobehavioral Psychology, Elsevier, 2019. ISBN 9780128093245
ISBN: 9780128093245
© 2019 Elsevier Inc. All rights reserved.
Elsevier

Author's personal copy

Autonomic and Enteric Neurons: Cell Culture☆

PJ Lein[a] **and AD Fryer**[b], [a] University of California, Department of Molecular Biosciences, Davis, CA, United States; and [b] Oregon Health & Science University, Division of Pulmonary and Critical Medicine, Portland, OR, United States

© 2019 Elsevier Inc. All rights reserved.

Introduction	1
Neural Crest Cells	1
Sympathetic Neurons	2
Parasympathetic Neurons	4
Cardiac Parasympathetic Ganglia	6
Tracheal Parasympathetic Ganglia	6
Chick Ciliary Ganglion Neurons	7
Enteric Neurons	7
Conclusions	8
Further Reading	8

Introduction

The autonomic nervous system, which is responsible for maintaining homeostasis, is divided into the sympathetic and parasympathetic nervous systems. Each system is defined anatomically by preganglionic neurons located in the central nervous system whose axons synapse on postganglionic neurons located in the periphery, which in turn directly innervate target organs. While there are published examples of the isolation and maintenance of preganglionic autonomic neurons in culture, it is the peripheral postganglionic neurons that have principally been isolated for tissue culture and these are the focus of this discussion. In addition, we will discuss tissue culture of the enteric nervous system, which is distinct from the autonomic nervous system, but like the sympathetic and parasympathetic nervous systems, is derived from the neural crest.

The culture of autonomic neurons, specifically postganglionic sympathetic neurons, has a long and rich history in neurobiological experimentation. Intact sympathetic ganglia maintained in culture as explants were first described by Lewis and Lewis in 1912, and the observation that a soluble factor produced by a mouse sarcoma stimulated profuse outgrowth of neurites from sympathetic explants in culture led to the discovery of nerve growth factor (NGF) by Levi-Montalcini and colleagues. Since this seminal discovery, sympathetic neuronal cell cultures have proven to be a powerful tool for identifying additional neurotrophic factors and for elucidating the molecular mechanisms by which NGF and other growth factors regulate neuronal survival, differentiation and function. Beyond this application, primary cultures of sympathetic neurons continue to be a popular and robust model system for elucidating basic cell and molecular mechanisms of neuronal cell development and differentiation, and for studying cell and molecular mechanisms of sympathetic physiology and pharmacology. With the exception of the chick ciliary ganglion, primary cultures of parasympathetic and enteric neurons have not been as widely used, and studies employing these culture models have focused largely on the electrophysiological and pharmacological properties of these neuronal cell types.

Neural Crest Cells

All neurons and glia of the sympathetic, parasympathetic and enteric nervous systems are derived from the neural crest. The neural crest also gives rise to cell types other than peripheral neurons and glia, and because of this vast repertoire of cellular fates, and the fact that differentiation of neural crest cells is strongly influenced by environmental factors, neural crest cell differentiation has emerged as a powerful model system for identifying basic developmental principles governing the generation of diverse cell types in not only the peripheral but also the central nervous system. Historically, neural crest cells have been cultured from both rodent and avian species, and detailed descriptions of techniques for isolating and maintaining neural crest cells from these sources have been published (see references listed under Further Reading). Neural crest cell cultures have been critical for confirming that premigratory neural crest cells are pluripotent, and clonal analyses of cultured neural crest cells have yielded much of the experimental evidence upon which is based our current understanding of neural crest cell fate restriction as a function of developmental timing and location along the migratory pathway.

Cultured neural crest cells remain a popular model for identifying environmental cues that influence the migratory behavior and differentiation of neural crest cells, including those progenitor cells that give rise to the sympathetic and enteric nervous system. For

☆*Change History:* June 2018. P Lein and A Fryer updated the Abstract, the sections "Neural Crest Cells", "Sympathetic Neurons", "Parasympathetic Neurons", and "Further readings". Two new figures were added: Figures 3 and 5.

 https://doi.org/10.1016/B978-0-12-809324-5.23365-8

Author's personal copy

example, the isolation and culture of a sympathoadrenal progenitor from migrating neural crest cells was instrumental in identifying the roles of fibroblast growth factor (FGF) and glucocorticoids in determining whether these progenitor cells differentiate into sympathetic neurons or adrenal chromaffin cells. Studies of cultured sympathoadrenal progenitor cells were also critical for demonstrating that FGF induced NGF responsiveness in these cells, providing some of the first evidence that differentiation signals involve a cascade of responses to multiple signaling molecules. Culture of crest-derived cells immunoselected from within the bowel provided critical confirmation of in vivo observations suggesting that laminin promoted neurogenesis of enteric precursor cells. This model system has since been used to identify additional factors that promote differentiation of both enteric neurons and glia. Other applications of crest cell cultures include determination of the intracellular signaling pathways that mediate the effects of environmental cues on migration and differentiation of sympathetic and enteric precursors, and the identification of genomic and proteomic profiles associated with specific states of determination.

More recently, protocols have been published for the culture and directed differentiation of human epidermal neural crest stem cells (hEPI-NCSC), which are neural crest-derived multipotent stem cells that persist into adulthood in the hair follicle bulge. Following isolation from human hair follicles, these hEPI-NCSCs can be pre-differentiated into proliferating neural stem cell-like cells. At this stage, these cells can be differentiated into purified cultures of dopaminergic neurons, peptidergic nociceptive sensory neurons, or Schwann cells, depending on the specific mixture of growth factors and small molecules to which they are exposed. Protocols for differentiating these cells into sympathetic or parasympathetic neurons have yet to be published; however, this is likely a matter of time, and the availability of such models will provide new, powerful, approaches for drug discovery and patient-specific modeling of diseases characterized by autonomic dysfunction.

Sympathetic Neurons

Sympathetic neurons have been cultured from prevertebral and paravertebral ganglia isolated from diverse species ranging from bullfrogs to humans at varying developmental stages including early embryonic development of the ganglia when a significant percentage of ganglionic neurons are still undergoing cell division as well the mature and even aging organism. However, cultures of sympathetic neurons are most commonly derived from prenatal (19–21 days) or early postnatal (1–14 days) rat superior cervical ganglia or embryonic chick chain ganglia. The easiest type of culture to prepare is an explant in which the intact ganglion is maintained in culture. Because of the profuse outgrowth of neurites from explanted ganglia, this preparation has been used extensively as a bioassay for neurotrophic factors and for studies of axonal growth cone behavior, including the identification of attractive and repulsive axonal guidance cues. Dissociated cultures, however, allow visualization of neurons in their entirety, which facilitates electrophysiological recordings and microinjections, and enables studies of sympathetic structure and function at the subcellular level. A third culture paradigm used with sympathetic neurons are compartmented chambers in which the media bathing distal axons is physically separated from that bathing neuronal cell bodies and proximal processes. Because there is minimal exchange of fluids or solutes between compartments, various factors can be selectively presented to neuronal cell bodies or distal axons and the contents of individual compartments can be independently analyzed. More recently, compartmented chambers have been used to recapitulate the in vivo anatomic relationship between sympathetic neurons and their target tissues in cell culture. In these models, sympathetic neurons are plated in one compartment, and their axons allowed to extend into adjacent compartments into which target cell types, such are cardiomyocytes, have been plated. Data collected using compartmented cultures significantly advanced our understanding of the molecular mechanisms of anterograde and retrograde signaling, and of bidirectional signaling between sympathetic neurons and their peripheral targets. The Campenot chamber (Fig. 1), which was developed by Bob Campenot, is among the earliest examples of compartmented chambers developed for sympathetic neuronal cell cultures. Campenot chambers were set up by attaching a compartmented Teflon insert to

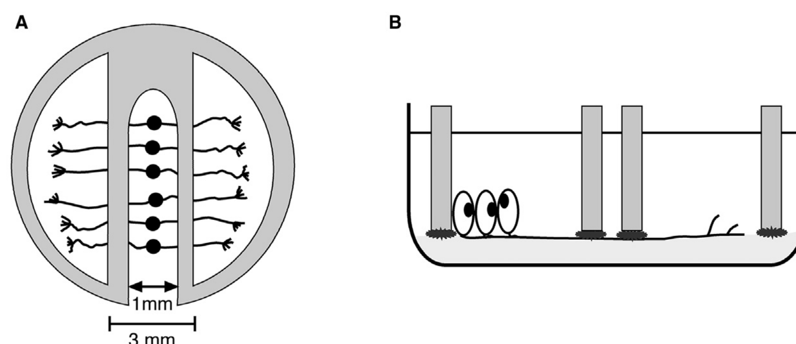


Figure 1 Schematic of Campenot compartmented chambers. (A) Top-view showing neurons plated in the center chamber of the classic Campenot Teflon insert (Camp10, available from Tyler Research Corporation, Edmonton, Alberta). Neurons typically extend axons under the barriers into the adjacent side chambers within 7 days after plating. (B) Side-view showing neurons plated in the far left chamber that have extended axons through the center chamber and into the far right chamber.

Author's personal copy

a collagen-coated substrate using vacuum grease. Neurons were plated in one compartment and axons grew under the grease barriers into adjacent compartments. Axons were encouraged to grow through the grease barrier by scoring parallel tracks in the collagen substrate prior to attaching the Teflon insert such that the tracks ran from the left compartment into the right compartment. These tracks exposed the tissue culture plastic, which axons will not grow across. While some labs still use Campenot chambers, they require considerable skill and experience to set up consistently and reliably. Thus, today, many labs have switched to using microfluidic devices in which fluid exchange between compartments is minimized by differences in hydrostatic pressure between compartments, which results from the maintenance of different levels of fluid (culture medium) in adjacent compartments. Detailed protocols for harvesting, plating and maintaining sympathetic neurons using each of these three culture paradigms are provided in references listed under Further Reading.

Thousands of articles have been published describing the development and behavior of sympathetic neurons in tissue culture. This system has proven popular for several reasons. First, these neurons are relatively easy to isolate because sympathetic ganglia are prominent, anatomically discrete entities that are accessible at varying life stages ranging from early ganglionic development (embryonic day 14 and 15 in the mouse and rat, respectively) through adulthood. Second, this preparation yields one of the most homogenous neuronal cell populations available in vertebrates. Sympathetic ganglia contain a large population of principal neurons (>20,000 in the rat superior cervical ganglion), a small population (approximately 300 cells) of interneurons or small intensely fluorescent (SIF) cells and ganglionic glial cells. Ganglionic glial cells and other non-neuronal cells can be eliminated in culture using anti-mitotic agents, and interneurons do not survive in dissociated cell culture in standard serum-free culture media. Thus, changes in these cultures in response to an experimental manipulation tend to be uniform throughout the cell population. Third, the growth factor requirements of sympathetic neurons are well-established, which allows the use of defined medium, and a variety of defined media and substrates work well for growing and maintaining sympathetic neurons. These neurons will grow in the absence or presence of ganglionic glia or target cells and can be maintained for up to 6 months in culture. Most importantly, when grown under the appropriate in vitro conditions, cultured sympathetic neurons express many of the properties expected of mature sympathetic neurons in situ, including synthesis and release of norepinephrine, normal electrophysiological properties and responsiveness to appropriate neurotransmitters, and a dendritic morphology that approximates that seen in situ. Finally, a wealth of data in the peer-reviewed literature facilitates correlation of in vitro and in situ sympathetic development, function and plasticity. The most significant limitations of the model historically include the limited amounts of RNA and protein available from sympathetic ganglia, which may restrict biochemical studies, and difficulty in manipulating gene expression in the majority of neurons in culture. Recent advances in techniques for analyzing gene expression in single cells are overcoming the former disadvantage whereas new approaches for expressing cDNA in primary neuronal cell cultures are rapidly overcoming the latter. There are publications reporting the successful application of lipid-based delivery systems (specifically Lipofecatmine 2000), nucleofection, and viral vectors (adenovirus and lentivirus) to cultured sympathetic neurons. The reported transfection efficiencies are relatively low (ranging from 10% to 20% with lipofection and nucleofection up to 30%–50% with viral infection), which can be an advantage for endpoints based on individual cell analyses, such as morphological analyses, but may be problematic for many biochemical endpoints.

Sympathetic cultures offer particular advantages for the study of neuronal development and plasticity. The development of sympathetic neurons is critically dependent on their environment and this plasticity is retained in tissue culture, where mechanisms can be more readily studied. It should be noted, however, that the plasticity exhibited by sympathetic neurons in culture has some practical disadvantages. The morphological, electrophysiological and pharmacological properties of these neurons can be affected by basic cell culture parameters, including cell density, serum, substratum, and media components such as insulin and potassium. Thus, it is necessary to be certain that the appropriate culture conditions chosen are for any given study. Despite these caveats, studies of cultured sympathetic neurons have provided significant insights regarding the identification and mechanistic characterization of environmental factors that regulate some of the most essential properties of these neurons, such as their morphology, receptor profile, and neurotransmitter phenotype. A significant advantage of sympathetic cultures with respect to studies of neuronal morphogenesis is that, unlike cultured central neurons, it is possible to control when dendritic growth occurs. Thus, sympathetic neurons can be grown under conditions (low density cultures in the absence of serum and ganglionic glial cells) in which < 5% of the neuronal cell population extends dendrites, but the addition of the appropriate stimulus (bone morphogenetic proteins or Matrigel) will trigger dendritic growth in >95% of the neuronal cell population (Fig. 2). Other widespread applications of sympathetic neuronal cultures include studies of: (1) growth cone behavior, structure and biochemical composition; (2) synaptogenesis; (3) the cytoskeleton in a variety of cellular functions including axonal and dendritic outgrowth, axonal transport, and subcellular trafficking; (4) factors that regulate transmitter release; (5) signal transduction downstream of receptor activation; and (6) molecular mechanisms of apoptosis. Recently, primary cultures of sympathetic neurons have been proposed as a physiologically relevant model for studying herpes simplex virus latency and reactivation, and for screening therapeutic candidates for neuroprotective efficacy. An application that is gaining in popularity is the use of sympathetic neurons co-cultured with target cells to study neuron–target interactions. Historically, sympathetic neuron and target cell co-cultures were used to study the influence of target on neurotransmitter phenotype. More recently, sympathetic neurons co-cultured with target tissue are being used to study the role of target-derived trophic factors on the formation and function of sympathetic synapses on heart; sympathetic modulation of cardiomyocyte function, sympathetic control of energy homeostasis via modulation of adipose tissue function; and sympathetic regulation of immune responses via innervation of the spleen and lymph nodes. An exciting application is the co-culture of sympathetic neurons with iPSC-derived cardiomyocytes to study the relationship between sympathetic neurons and cardiomyocytes in cardiovascular disease.

Author's personal copy

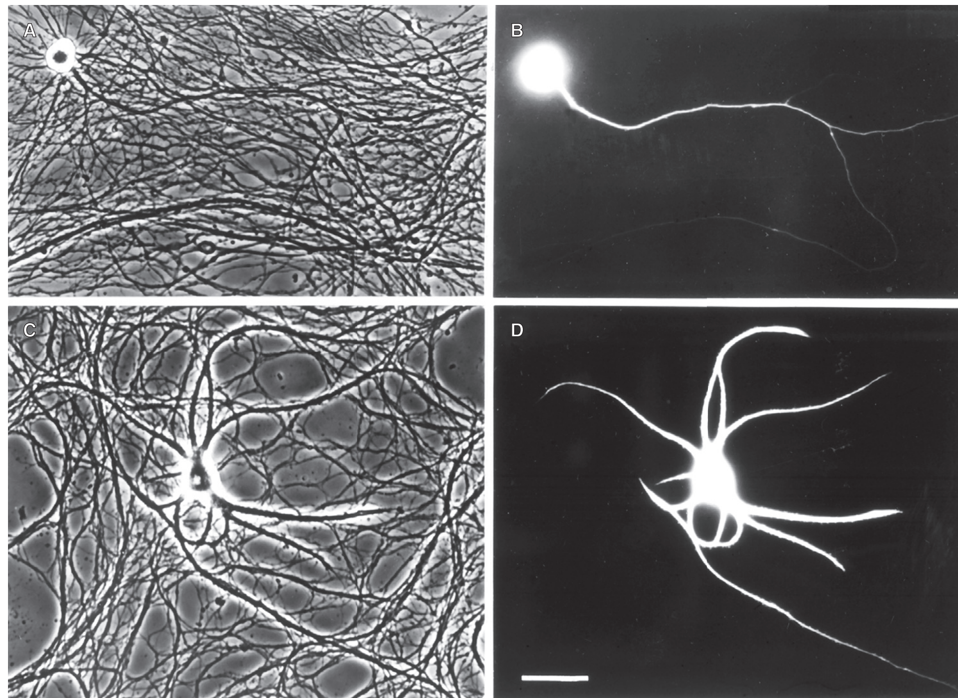


Figure 2 Sympathetic neurons do not extend dendrites in culture unless provided the appropriate environmental stimulus. Phase-contrast (A, C) and fluorescence (B, D) micrographs of rat sympathetic neurons injected with Lucifer yellow during the third week in vitro. Neurons growth in control cultures in the absence of non-neuronal cells and serum (A, B) typically extend only one process, which is axonal in nature. Neurons exposed to bone morphogenetic protein-7 (BMP-7, 50 ng/mL) for 7 days were multipolar, having several tapered dendrites and one axon.

Parasympathetic Neurons

Parasympathetic ganglia are considerably smaller than sympathetic ganglia. They range in size from as few as two or three cells to as many as 30. Although they are usually found within and between muscle layers of end organs they are also located adjacent to and outside of end organs. Fig. 3 shows a parasympathetic ganglion outside the trachea. It is clearly supplied by nerves, and careful examination shows nerve fibers running between the cells as well. It is likely that the parasympathetic ganglia are heterogeneous, with different cells responding to electrical stimulation with different firing patterns. Neurites from these ganglion cells supply airway smooth muscle inside the trachea, but likely also make contact with other ganglia. However, the majority of parasympathetic ganglia are small and because of their location, usually buried within end organs. For these reasons, they have received less attention as in vitro models than sympathetic and sensory ganglia. Professor Geoffrey Burnstock first maintained parasympathetic neurons in cell culture in the early 1980's, initially by explant and later by dissociation of tissues. Parasympathetic neurons have now been isolated from ganglia in many different organs including lungs, heart, bladder and submandibular glands (enteric nerves will be considered separately) and from all species including mice, guinea pigs, rats and humans.

In vitro studies of parasympathetic neurons indicate that factors affecting growth of sympathetic neurons do not automatically transfer to the parasympathetic cultures. For example, NGF is required to maintain sympathetic neurons in culture. While it is commonly added to parasympathetic cultures, many labs do not use it, thus parasympathetic neurons do not require NGF to survive. Cell lines also do not mimic parasympathetic function. Neuroblastoma cells release acetylcholine, but regulation of receptor expression is mediated by different pathways in neuroblastoma versus parasympathetic neurons. To identify the physiology and pharmacology of this branch of the autonomic nervous system, it is necessary to isolate and study the parasympathetic neurons themselves. Since isolation of parasympathetic neurons is not yet routinely done, the technique will be described here. It is not necessary to isolate parasympathetic neurons from fetal animals and there appears to be little difference between cells isolated from young versus older animals. Isolation of parasympathetic ganglia involves dissecting out the tissue containing the ganglia followed by mechanical removal of as many non-neuronal cells as possible. For example, during dissection of parasympathetic ganglia from airways, airway epithelial cells are wiped away with sterile cotton swabs. The tissue is gently minced, and then digested enzymatically. This usually requires incubation with trypsin (0.25%) and collagenase (0.2%) or protease (0.2%) at 37°C for 60–90 min. Thicker tissues can be incubated with the enzymes at 4°C from 1 to 18 h to allow enzyme penetration of tissues, followed by incubation at 37°C to activate the enzymes. Enzymatic digestion is usually accompanied by gentle agitation or pipetting to disperse the cells. This procedure results in suspension of neurons, glial cells, smooth muscle cells, fibroblasts and, depending upon the tissue of origin, endothelial or epithelial cells. Cultures can be enriched for neurons by preplating the cell suspension for 4–18 h on polystyrene dishes to remove fibroblasts and muscle cells. In addition, the anti-mitotic agent cytosine arabinoside (5 μM) can be added

Author's personal copy

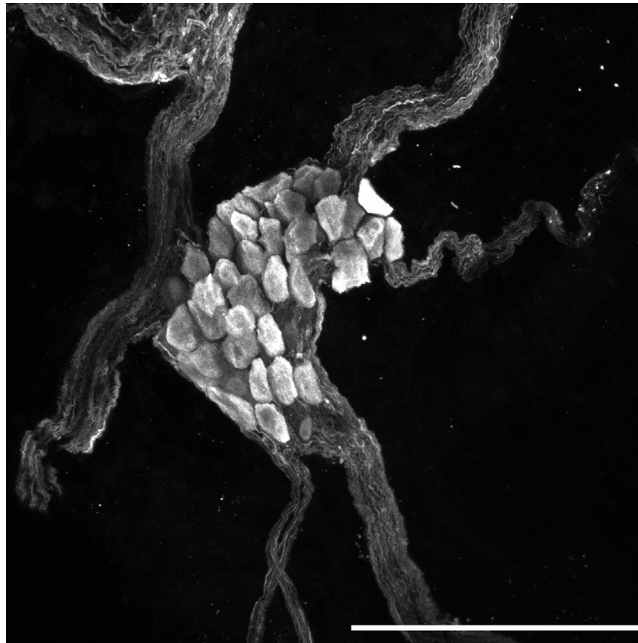


Figure 3 Three dimensional image of a parasympathetic ganglia from the trachea of an adult C57BL/6 mouse. Shown are the cell bodies and nerves that supply the cells and run in between the nerve cell bodies. The image was generated from a mouse lung that was perfused with PBS, before fixing the trachea with Zambonis. The trachea was whole-mounted and immunolabeled with antibody to PGP9.5, which was visualized using Alexa 555 secondary antibody. The immunolabeled ganglia was imaged on a LSM780 microscope using a Plan-Apochromat 63x/1.40 Oil objective. Image provided by Katherine M Lebold, Oregon Health and Science University. Scale bar = 100 μm.

to cultures a day or two after plating to kill dividing cells. Over several days, the cultures consist predominantly of neurons. A major caveat of working with parasympathetic ganglia is that, unlike sympathetic ganglia, cultures derived from parasympathetic ganglia are heterogeneous. This is less an issue in electrophysiological, immunocytochemical or transmitter release studies, but is a major factor when measuring protein or mRNA by western blot or PCR. For these latter endpoints, controls must be added for the presence of non-neuronal cells.

Parasympathetic neurons grow best on extracellular matrix (Matrigel; BD Biosciences)-coated slides or culture dishes. There is no need to use Rose chambers described in the early papers (two coverslips separated by a plastic washer that are clamped together, which can be purchased from Bellco [product 1943-11111]). Cells grow well in serum-free medium. This is made using the following reagents (added in the order listed): 470 mL DMEM, 25 mL 2% bovine serum albumin (Fisher BP1600), 10 mL of 100X insulin-transferrin-selenite (Mediatech 25-800-CR), and 495 mL Ham's F12. This can be aliquoted and frozen at −80°C. Before use, the medium is supplemented with penicillin/streptomycin/fungazone (100 μL of 100X per 10 mL of culture medium), NGF (to final concentration of 100 ng/mL) and cytosine arabinoside (final concentration of 1 μM). Parasympathetic neurons originally appear as spherical cells that settle onto the substrate over 48 h after plating. Neurites initially appear as small protrusions, and extend over the first 3 days. By two or 3 days after plating, neurons with processes are clearly distinguishable. Parasympathetic neurons grow in dense clusters with outwardly radiating and fasciculated neurites (Fig. 4). The choice of plating matrix can influence aggregation of the cells: they form dense clusters if grown on polylysine (this substrate yields fewer cells over all), and smaller clusters (with increased cell survival) when grown on collagen, laminin, or Matrigel. Parasympathetic neurons can be maintained in culture for 7–10 days. It is rare that cultures survive in excess of 14 days, which is another difference from sympathetic cultures.

Parasympathetic neuronal cell cultures have been used to describe receptor distribution, neurotransmitter content as determined primarily by immunocytochemistry and, more recently, to characterize the molecular mechanisms underlying interactions between parasympathetic nerves and inflammatory cells. It is possible to isolate the ganglia from culture and measure mRNA to quantify induction and expression of genes within autonomic nerves (Fig. 5). Acetylcholine release in response to electrical field stimulation or nicotine has also been measured. However, it cannot be assumed that all parasympathetic neurons behave the same way. For example, the distribution of purinoceptors for adenosine 5′-triphosphate (ATP) is different in parasympathetic neurons originating from heart and lung versus those derived from submandibular or otic ganglia. Much is still unknown about the basic cell biology of parasympathetic nerves. No studies have been carried out that characterize the growth and differentiation of parasympathetic nerves; indeed, even the characterization of neurites as axons or dendrites is lacking in this neuronal cell type. Recent work demonstrating that cultured parasympathetic neurons can be transfected (Fig. 6) suggest the exciting possibilities of future studies of the cell and molecular mechanisms regulating the development, growth, and plasticity of parasympathetic neurons.

Author's personal copy

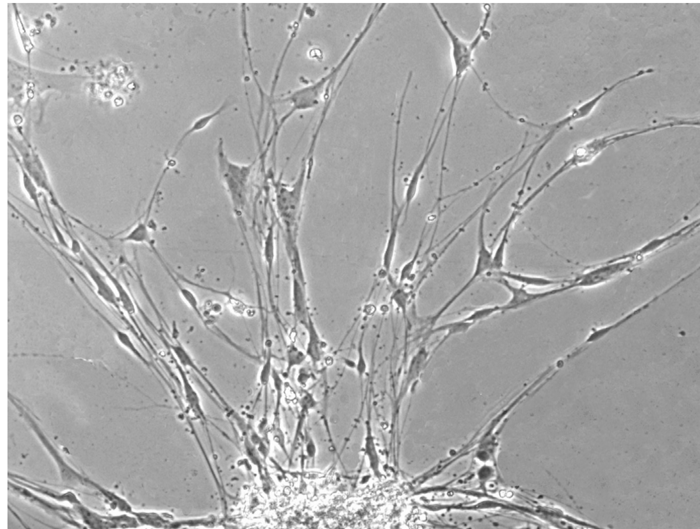


Figure 4 Phase-contrast image of parasympathetic neurons from guinea pig trachea, plated on Matrigel and maintained in serum free medium for 7 days.

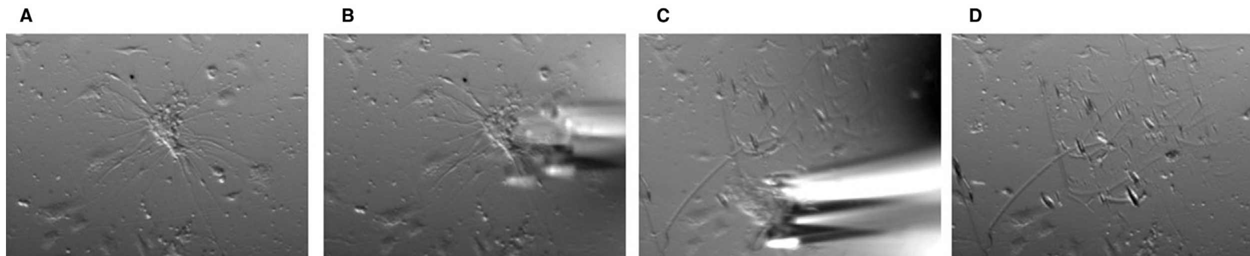


Figure 5 Method for isolating parasympathetic ganglia from cell culture for harvesting nerve-specific mRNA. Replace cell media with ice cold RNAse-free PBS, and identify nerve cluster using an inverted phase contrast microscope (A). Make a glass knife by stretching a capillary tube over a flame, then breaking the tip using fine forceps to create a sharp edge. The capillary tube is used to cut the cells free from their neurites (B). Using connective tubing, attach the untouched end of the glass capillary to a syringe filled with ice cold RNAse-free PBS. Gently suck up the cell bodies by applying suction with the attached syringe (C) and eject nerve cells into an RNAse-free Eppendorf tube. Confirm that the nerve cells are removed (D). All nerve clusters from one dish or treatment are combined in one tube.

Cardiac Parasympathetic Ganglia

Cardiac neurons appear in culture singly or as clusters with neurites extending for hundreds of μm. When isolated from rats, the somata are 25–35 μm in diameter, depending upon the degree of flattening. Nuclei are approximately 5×10 μm. All cells from rat and guinea pig cardiac ganglia express acetylcholinesterase, suggesting that they are all cholinergic, and have functional M_1 and M_2 muscarinic receptors evenly distributed along cell bodies and neurites. However, they are actually a heterogeneous population, with three distinct electrophysiological phenotypes, and a subpopulation immunoreactive for dopamine-β-hydroxylase or tyrosine hydroxylase, suggesting the presence of SIF cells. Half of all neurons in these cultures are immunoreactive for neuropeptide Y, a smaller percentage express vasoactive intestinal peptide, but tachykinin expressing cells have not been detected. Thus, cardiac parasympathetic ganglia in cell culture maintain a considerable degree of neuronal specialization as is seen in vivo.

Tracheal Parasympathetic Ganglia

Parasympathetic ganglia from trachea were initially isolated from young rats, but cultures from mature guinea pigs and adult human organ donors have been isolated and maintained in cell culture for several weeks (see papers from laboratories of Professors Burnstock and Fryer, listed in "Further reading"). Tracheal ganglia behave similarly to parasympathetic nerves in vivo as they release acetylcholine, express nitric oxide synthase and, depending upon the presence of NGF, express substance P. M_2 muscarinic receptors are evenly distributed along cell bodies and neurites, and function to inhibit acetylcholine release. Similar to nerves in vivo, inflammatory cells adhere to parasympathetic nerves in culture, and this effect is regulated by expression of chemotactic cytokines and adhesion molecules by the parasympathetic nerves. Parasympathetic nerves also release some noradrenaline, and have functional

Author's personal copy

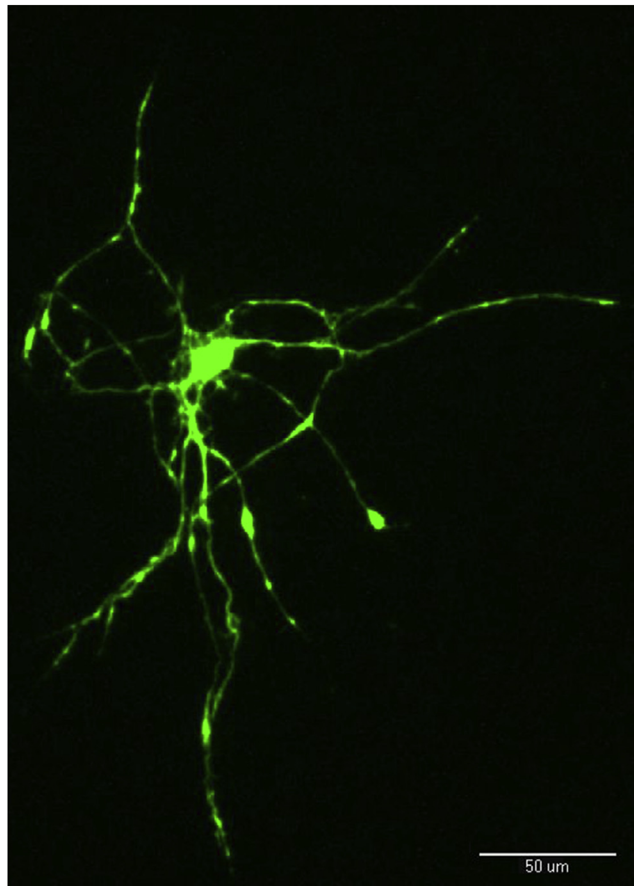


Figure 6 Primary cultures of parasympathetic neurons were plated on Matrigel and maintained in serum free medium for 4–5 days. Cells were transfected with cDNA encoding enhanced green fluorescent protein (eGFP) using the transfection reagent Optifect (Invitrogen). eYFP plasmid (3 μg) was incubated with Optifect (Invitrogen) in 200 μL OptiMEM at room temperature for 5 min. Cells were incubated with 200 μL eYFP/Optifect solution in 1 mL culture medium at. 37°C in 5% CO_2 for 6 h, then rinsed with PBS and fixed with 3.7% paraformaldehyde.

M_1 muscarinic, $alpha_1$ adrenergic and $GABA_A$ receptors. As with cardiac nerves, ganglia cultured from the trachea appear to maintain all characteristics of their in vivo counterparts.

Chick Ciliary Ganglion Neurons

One parasympathetic ganglion that has been well studied in cell culture is the avian ciliary ganglion because it is relatively large, discrete, and easily located outside of the eye. It should be noted that in sharp contrast to the bird, rodent ciliary ganglion are small and dispersed, thus ciliary ganglion isolated from chick may not be an accurate model of the physiology of the mammalian counterpart. Ciliary neurons are large, myelinated cholinergic neurons that innervate striated muscle in the iris and ciliary muscle. Ciliary neurons require neurotrophic factors to support their survival in culture, including ciliary neurotrophic factor and growth promoting activity, which may be different molecules in mammals and birds, and have different receptors and different effects. Neurotransmitter expression in ciliary neurons is controlled by target tissues, in this case the eye, and this interaction may not be representative of parasympathetic neurons as a class. Despite these drawbacks, the ciliary ganglion has been a popular system for neuronal cell culture, and a great deal is known about the growth requirements and behavior of this class of chick parasympathetic neurons.

Enteric Neurons

The enteric nervous system is considerably more complex than other peripheral neural tissue. Neuronal cells bodies reside in the submucosal and myenteric ganglia, and consist of at least 14 different types of neuronal cell types including interneurons, afferent neurons, and both excitatory and inhibitory motor neurons that collectively express diverse neurotransmitters in addition to

Author's personal copy

acetylcholine including substance P, enkephalins, nitric oxide, ATP and γ-amino butyric acid. The enteric nervous system is highly autonomous: nerves form extensive synaptic contacts with one another and are capable of integrated reflex activity in the absence of central connections. Thus, in both its structure and its function, the enteric nervous system bears a greater resemblance to the central nervous system than to other peripheral neural tissues.

Isolation of enteric neurons is similar to that of parasympathetic neurons and involves enzymatic digestion of tissue containing the ganglia with subsequent plating on matrix gels or polylysine. Enteric neurons can survive in culture up to 3 weeks and the diversity of neuronal cell types found in situ is represented in culture. Individual enteric neurons are characterized by markedly diverse morphology, and they retain the variety of neurotransmitters found in situ. Their electrophysiological properties appear to be similar to enteric nerves in the intact animal. Cultured enteric ganglionic glial cells continue to synthesize intracellular glial proteins expressed by their in vivo counterparts, including glial fibrillary acidic protein and glutamine synthetase, suggesting the feasibility of using enteric nerve cultures to study nerve glial cell interactions. A complication of interpreting any study involving cultured enteric neurons is the paucity of information regarding differential effects of environmental factors on different neuronal cell types within the heterogeneous population of neurons that comprise the enteric culture system. Few attempts have been made to discriminate between effects on axons versus dendrites within any individual neuron, although a number of studies have examined factors that promote neurite outgrowth. Glial cells are not required for neurite development in culture, but when they are present, enteric nerves will extend processes along them. Neurite length is enhanced by NGF, consistent with observations that enteric neurons express both the low affinity NGF receptor (p75) as well as TRK receptors. Neurite outgrowth is promoted by cAMP via complementary but independent pathways, as evidenced by observations that inhibitors of cAMP have no effect of NGF-induced neurite growth and that cAMP, but not NGF, enhances neurite branching in the myenteric nerves. Neurite length is also enhanced by ciliary neurotrophic factor, insulin-like growth factors, leukemia inhibitory factor and interleukin 6 (IL-6). The greatest enhancement of neurite length, however, is provided by glial cell line-derived neurotrophic factor (GDNF), which also induces larger clusters of cell bodies and increases neuronal survival in culture. IL-6 induces a greater number of neurites, but GDNF promotes increased neurite length.

While much research on enteric neurons in culture has demonstrated that they retain the characteristics of cells found in situ, there is evidence that at least some neuronal cell types retain the plasticity that has been observed in other neural crest derivatives. Thus, exposure to heart cell conditioned medium induces substantial, but not uniform, changes in their electrophysiological and transmitter-related properties (increasing the percentage of neurons that express 5-hydroxytryptamine, while decreasing the percentage of neurons expressing vasoactive intestinal peptide and substance P).

Conclusions

Almost everything we know about the development and physiology of autonomic nerves has been derived from studies of sympathetic neurons, in large part because of the relative ease with which this neuronal cell type can be isolated and maintained in culture. Recent advances in methods for manipulating gene expression in cultured sympathetic neurons and for culturing sympathetic neurons in the presence of target tissues or ganglionic glia ensure the continued widespread use and popularity of this in vitro model system for discovery-based and mechanistic research. The recent development of techniques for the isolation and maintenance of parasympathetic and enteric neurons in cell culture will enable a more detailed understanding of the basic cell biology of these other peripheral neural systems, thereby refining and extending current paradigms of peripheral nervous system development, physiology and pharmacology.

Further Reading

Allen, T.G.J., Burnstock, G., 1987. Intracellular studies of the electrophysiological properties of cultured intracardiac neurons of the Guinea pig. J. Physiol. Lond. 388, 349–366.

Allen, T.G.J., Burnstock, G., 1990. A voltage-clamp study of the electrophysiological characteristics of the intramural neurones of the rat trachea. J. Physiol. Lond. 423, 593–614.

Campenot, R.B., 1992. Compartmented culture analysis of nerve growth. In: Stevenson, B.R., Gallin, W.J., Paul, D.L. (Eds.), Cell-cell Interactions: A Practical Approach. IRL Press, Oxford, pp. 275–298.

Chamley, J.H., Goller, I., Burnstock, G., 1973. Selective growth of sympathetic nerve fibers to explants of normally densely innervated autonomic effector organs in tissue culture. Dev. Biol. 31, 362–379.

Elbon, C.L., Kim, A.L., Xiao, H.Q., Levey, A.I., Jacoby, D.B., 1996. Cultures of airway parasympathetic nerves express functional M2 muscarinic receptors. Am. J. Respir. Cell Mol. Biol. 15, 716–772.

Ghogha, A., Bruun, D.A., Lein, P.J., 2012. Inducing dendritic growth in cultured sympathetic neurons. J. Vis. Exp. 61, 3546. https://doi.org/10.3791/3546.

Grau, C.M., Greene, L.A., 2012. Use of PC12 cells and rat superior cervical ganglion sympathetic neurons as models for neuroprotective assays relevant to Parkinson's disease. Methods Mol. Biol. 846, 201–211.

Higgins, D., Lein, P.J., Osterhout, D.J., Johnson, M.I., 1991. Tissue culture of mammalian autonomic neurons. In: Banker, G., Goslin, K. (Eds.), Culturing Nerve Cells, first ed. MIT Press, Cambridge, MA, pp. 177–205.

James, S., Bailey, D.J., Burnstock, G., 1990. Autoradiographic visualization of muscarinic receptors on rat paratracheal neurons in dissociated cell culture. Brain Res. 513, 74–80.

Jessen, K.R., McConnell, J.D., Purves, R.D., Burnstock, G., Chamley-Campbell, J., 1978. Tissue culture of mammalian enteric neurons. Brain Res. 152, 573–579.

Jessen, K.R., Saffrey, M.J., Bauk, P., Menachem Hanani, M., Burnstock, G., 1983. The enteric nervous system in tissue culture. III. Studies on neuronal survival and the retention of biochemical and morphological differentiation. Brain Res. 262, 17–35.

Kobayashi, M., Kim, J.Y., Camarena, V., Roehm, P.C., Chao, M.V., Wilson, A.C., Mohr, I., 2012. A primary neuron culture system for the study of herpes simplex virus latency and reactivation. J. Vis. Exp. 62, 3823. https://doi.org/10.3791/3823.

Larsen, H.E., Lefkimmiatis, K., Paterson, D.J., 2016. Sympathetic neurons are a powerful driver of myocyte function in cardiovascular disease. Sci. Rep. 6, 38898. https://doi.org/10.1038/srep38898.

Author's personal copy

Lein, P.J., Johnson, M., Guo, X., Rueger, D., Higgins, D., 1995. Osteogenic protein-1 (OP-1) induces dendritic growth in rat sympathetic neurons. Neuron 15, 597–605.
Levi-Montalcini, R., 1987. The nerve growth factor 35 years later. Science 237, 1154–1162.
Lewis, W.H., Lewis, M.R., 1912. The cultivation of sympathetic nerves from the intestine of chick embryos in saline solutions. Anat. Rec. 6, 7–31.
Narytnyk, A., Verdon, B., Loughney, A., Sweeney, M., Clewes, O., Taggart, M.J., Sieber-Blum, M., 2014. Differentiation of human epidermal neural crest stems cells (hEPI-NCSC) into virtually homogenous populations of dopaminergic neurons. Stem Cell Rev. 10, 316–326.
Sakai, K., Shimba, K., Ishizuka, K., Yang, Z., Oiwa, K., Takeuchi, A., Kotani, K., Jimbo, Y., 2017. Functional innervation of human induced pluripotent stem cell-derived cardiomyocytes by co-culture with sympathetic neurons developed using a microtunnel technique. Biochem. Biophys. Res. Commun. 494, 138–143.
Sakaue, M., Sieber-Blum, M., 2015. Human epidermal neural crest stem cells as a source of Schwann cells. Development 142, 1–10.
Smith, C.L., 1998. Cultures from chick peripheral ganglia. In: Banker, G., Goslin, K. (Eds.), Culturing Nerve Cells, second ed. MIT Press, Cambridge, MA, pp. 261–287.
Smith, T.H., Ngwainmbi, J., Grider, J.R., Dewey, W.L., Akbarali, H.I., 2013. An in vitro preparation of isolated enteric neurons and glia from the myenteric plexus of the adult mouse. J. Vis. Exp. 78 https://doi.org/10.3791/50688.
Stemple, D.L., Anderson, D.J., 1992. Isolation of a stem cell for neurons and glia from the mammalian neural crest. Cell 71, 973–985.
Wilson, R., Ahmmed, A.A., Poll, A., Sakaue, M., Laude, A., Sieber-Blum, M., 2018. Human peptidergic nociceptive sensory neurons generated from human epidermal neural crest stem cells (hEPI-NCSC). PLoS One 13, e0199996.
Zareen, N., Greene, L.A., 2009. Protocol for culturing sympathetic neurons from rat superior cervical ganglia (SCG). J. Vis. Exp. 23, 988. https://doi.org/10.3791/988.
Ziller, C., 1986. Development of neuronal properties in neural crest cells cultured in vitro. Curr. Top. Dev. Biol. 20, 177–193.