CHAPTER 2

Growing and Working with Peripheral Neurons

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Cultures of vertebrate peripheral neurons have been used to address a variety of issues related to the cell biology of the neuron. They are particularly amenable to experimental manipulations, such as microinjection, and can be cultured under a variety of different conditions designed to meet the needs of the particular experiment. This chapter focuses on cultures of rat sympathetic neurons from the superior cervical ganglia and on cultures of chick sensory neurons from the lumbosacral dorsal root ganglia. Information is provided on methods for dissection, preparation of culture dishes and substrates, composition of media, relevant growth factors, reduction of nonneuronal contamination, and maintenance of the cultures.

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I. Introduction

Over the past several years various different kinds of neurons from the peripheral and central nervous systems of various different kinds of animals have been cultured successfully. These cultures have been used to investigate a number of different issues related to the cell biology of the neuron. Several factors contribute to the selection of a particular type of neuron for a particular study. Although some studies focus specifically on an issue related to one type of neuron, most studies are open to the use of different kinds of neurons. For example, our laboratory studies mechanisms of cytoskeletal organization and axonal transport, and these mechanisms are likely to be preserved across different types of neurons. In these cases, the main factor in choosing which neuron to use is how amenable a particular type of neuron is to the particular experimental manipulations that are required to carry out the study. In our laboratory, we most frequently culture rat hippocampal neurons, rat sympathetic neurons from the superior cervical ganglion, and chick sensory neurons from lumbosacral dorsal root ganglia. Hippocampal neurons, from the central nervous system, have the advantage of undergoing very stereotypical and well-defined stages of axonal and dendritic development. However, they are more tedious to culture, somewhat finicky, and generally die if microinjected. In contrast, the two types of peripheral neurons are easier to culture, more rugged, survive injection, and can be grown under a much wider variety of culture conditions tailored to specific experimental needs. The reason why we use chick for sensory neuronal cultures and rat for sympathetic cultures is simply because of the backlog of knowledge from previous work. It is certainly possible to culture rat sensory neurons and chick sympathetic neurons, but fewer studies have been performed on these cultures, at least in our field.

The cultures of sensory neurons have the advantage of simplicity, they generate axons but not dendrites, and display relatively simple morphologies. However, sympathetic cultures have the advantage of complexity, they generate dendrites as well as axons, and display a much wider range of axonal and dendritic behaviors depending on the culture conditions. Therefore, if the purpose of the study is simply to measure the effects of an experiment on axonal outgrowth or length, sensory neurons would be the better choice. However, if the purpose of the study is to investigate finer features of axogenesis, branch formation, dendrites, or other more complex issues, then sympathetic neurons would be the better choice. As far as we can tell, both types of neurons are about the same in terms of their viability to experimental manipulations, such as microinjection, axotomy, transfection, and application of anticytoskeletal drugs. The dorsal root ganglia (DRG) of chick embryos are much easier to dissect than the superior cervical ganglia of rat pups, but after dissection is completed, subsequent steps in the preparation and care of the cultures are quite similar.

There are superb chapters in other books that outline in great detail various options for generating these cultures (Bray, 1991; Higgins *et al.*, 1991; Mahanthappa and Patterson, 1998; Smith, 1998; Johnson, 2001). Our main

purpose here is not to be exhaustive with regard to options, but rather to share the methods that we have come to rely on in our laboratory, as well as insights that we have gained over the years.

II. Methods

A. Substrate

One of the most important issues for neuronal cell culture is substrate. Neurons are poorly adhesive cells compared to many kinds of cells that can be cultured, and yet adhesion to the substrate is absolutely essential for their viability and their capacity to extend neurites. In general, neurons do not adhere to plain glass or even to plastic culture dishes (except when methylcellulose is added to the medium; see later). There are several possibilities for treating these surfaces that enhance their capacity to adhere to cells. As with the rest of this chapter, we describe the most commonly used approaches in our laboratory. In theory, with appropriate substrate treatments, cultures can be grown either on the surface of tissue culture dishes or on glass coverslips. Glass coverslips offer a more optimal surface for light microscopy, but are hard to manipulate for experiments if they are simply placed inside petri dishes. To circumvent this problem, we routinely grow cultures on glass coverslips that are adhered to the bottom of a 35-mm petri dish into which a hole has been drilled. This permits the cells to be visualized easily using an inverted microscope, microinjected, and marked for relocation. Using terminology originally coined by the Banker laboratory, we refer to these hybrid dishes as "special dishes." When culturing directly on plastic, it is important to buy dishes that are treated for cell culture, but for the manufacture of special dishes, it is fine to save money by using untreated petri dishes. Sometimes it is useful to prepare special dishes using glass coverslips that have been prephotoetched for easy relocation of particular cells of interest. Details for cleaning glass coverslips and preparing special dishes are provided in Table 1.

The most common method for promoting the adhesion of neurons is to treat the plastic or glass with a series of positive charges, which are attracted to the negative charges on the surface of the cell membrane. We use poly-D-lysine for this. Several different varieties of polylysine are available; details on the one we use commonly are provided in Table 2. It is necessary to use a relatively low concentration of poly-D-lysine and to rinse very extensively after the glass or plastic is treated. Any excess poly-D-lysine that does not attach to the plastic or glass can be extremely toxic to the cells, as can too much poly-D-lysine that has attached. Generally speaking, we use either 0.1 or 1.0 mg/ml, depending on whether any toxicity arises with the higher concentration. The presence of serum in the medium usually permits the use of the higher concentration, while it is often necessary to use the lower concentration if there is no serum in the medium. Poly-D-lysine alone can be a reasonable substrate for chick sensory neurons (as well as cultures of brain neurons) but generally does not support axonal outgrowth particularly well from

Table I
Preparing Special Dishes

I. Cleaning Glass Coverslips

A. Place circular glass coverslips (Carolina Biological Supply Company) or etched grid coverslips (Bellco) into ceramic coverslip holders. Immerse the holders under HNO_3 in a glass container. Let coverslips soak in HNO_3 overnight in the dark.
B. Rinse coverslips once with double-distilled water and wash three more times, 1 hour each time.
C. Dry coverslips in oven.

Note: Keep HNO_3 in dark at all times because it is light sensitive. If HNO_3 becomes brown rather than colorless, stop using it.

II. Attaching Coverslips to the Special Dishes

A. Introduce a circular smooth-edged hole into the bottom of a 35-mm petri dish. This can be done using a vertical drill press with a forstner bit or with a lathe. The diameter of the hole can vary depending on the needs of the experiment; we generally use a diameter of 13 mm.
B. Prepare a mixture of three parts of paraffin and one part of vaseline. Melt in hot water bath.
C. Using a paint brush, paint a ring of wax around the hole in each dish. Take care not to paint too close to the hole and do not use too much wax. Too much wax can leak onto the region of the coverslip where the cells are to be plated.
D. Invert the dish and place the clean coverslip on top of the hole.
E. Put under inverted hot plate, let wax melt, and coverslip fall down evenly. Pull out and let cool before any wax slops into the well.
F. Sterilize the dish and lid by spraying 70% ethanol evenly on the surface and radiating them under UV light in a tissue culture hood for at least 30 min. After the ethanol evaporates completely, the dishes are ready for plating neurons or coating with different substrates.

Note: If the cultures are ultimately to be used for electron microscopy, it is desirable to use a more heat-resistant adhesive such as epoxy to attach the coverslip to the plastic dish.

sympathetic neurons. The reason for this is unclear, but it may be that the poly-D-lysine is actually "too sticky" for sympathetic neurons.

A more favorable and more physiological substrate is provided by introducing laminin after the poly-D-lysine. The application of laminin, a biologically active matrix molecule, promotes better axonal outgrowth from sensory neurons and extraordinarily rapid and robust axonal outgrowth from the sympathetic neurons (Rivas and Goldberg, 1992; Tang and Goldberg, 2000). Laminin can either be applied over the poly-D-lysine prior to plating the cells or be added to the medium together with or after the cells have been plated. The precise morphology and rate of axonal outgrowth will vary somewhat depending on when the laminin is added. The laminin adheres to the positive charges of the poly-D-lysine and provides a robust substrate for the neurons. Even more robust results can be achieved using a partially defined mixture of substrate-related growth factors called Matrigel rather than laminin (Yu *et al.*, 2002). Additional details are provided in Table 2.

Years ago we commonly used a substrate of rat tail collagen to promote the attachment of sympathetic neurons. We rarely do this anymore because the substrate is rather thick, is rather three-dimensional (which is not good for microscopic analyses), and because axonal outgrowth is very slow on collagen.

Table II
Substrate Coating

I. Poly-D-lysine

A. Prepare borate buffer (40 ml)

Ingredient	Amount	Final concentration
Borax (Sigma)	190 mg	4.75 mg/ml
Boric acid (Sigma)	124 mg	3.1 mg/ml
Double-distilled H_2O	40 ml	100% (v/v)

Good for up to 1 month at room temperature.

B. To prepare 1 mg/ml stock solution, dissolve 10 mg of poly-D-lysine (Sigma) in 10 ml borate buffer.
C. Filter sterilize using a 0.2-μm filter. Aliquot desired amount and freeze at -20°C.
D. Add 0.2 ml of poly-D-lysine to the well. Two different methods can be used, one with a higher concentration and the other with a lower concentration of poly-D-lysine. The higher concentration generally provides better adhesion, but sometimes can produce toxicity, particularly if serum-free medium is used. If the latter proves to be the case, then the lower concentration should be used.

Method 1: Coat the glass well with 1 mg/ml poly-D-lysine for 3 h at room temperature. Rinse the dish six times with sterile distilled water, 5 min each time. At the end, add 2 ml of water to the dish and keep the dish in a 37°C incubator overnight. On the next day, rinse the dish with water one more time. Take off water and let dish dry completely. Put the lid back on the dish and keep at room temperature. They are good for up to 1 month.

Method 2: Coat the glass well with 0.1 mg/ml poly-D-lysine (dilute 1 mg/ml stock solution 10 times with borate buffer) overnight at 4°C. On the next day, rinse the dish six times with sterile distilled water, 5 min each time. After the dish dries completely, put the lid back on the dish. They can be kept at room temperature for up to 1 month.

II. Laminin

A. Prepare poly-D-lysine-coated dishes as described previously.
B. On the day of culturing, dilute 10 μl laminin (Invitrogen) in 1 ml Leibovitz's L-15 medium. Add 200 μl diluted laminin into each glass-bottomed well that has been precoated with poly-D-lysine.
C. Incubate the dish in a 37°C, CO_2-free incubator for 3 to 4 h.
D. Take off laminin immediately before plating cells into the well.

III. Collagen

A. Purchase collagen derived from rat tail (BD Biosciences). The product will generally be roughly 4 mg/ml in a dilute acetic acid solution.
B. Collagen can be applied directly to the surface of a plastic culture dish. For glass, precoat with poly-D-lysine as described earlier.
C. The amount of collagen to produce a desirable substrate is determined empirically. Generally, 2–3 drops of the undiluted product is sufficient to coat the glass-bottomed well of a special dish. An insufficient amount of collagen will dry unevenly in patches.
D. Allow to air dry.

However, the collagen substrate has one advantage that is particularly helpful for certain types of experiments. Everyone who works with cultured neurons struggles with the fact that the neurons often lose their attachment and simply "float away" if they are extracted too strongly or exposed to anything that might mechanically or otherwise disturb them. When grown on rat tail collagen, sympathetic neurons are extremely well attached, such that they can be extracted with very high

concentrations of detergents such as Triton X-100, and even with high salt concentrations. This permits a clean separation of soluble and insoluble components of the cytoplasm for biochemical studies and also produces "squeaky clean" cytoskeletal polymers for immunostain analyses. We have found this particularly useful for immunoelectron microscopic analyses of tubulin isoforms within microtubules (Baas and Black, 1990). A collagen substrate is relatively easy to work with on plastic (see Table 2), but is somewhat harder to deal with on glass. The problem is that while the neurons are extremely well attached to the collagen, the collagen itself can sometimes roll off the glass or plastic in a sheet, which is more common on glass than on plastic. The glass needs to be pretreated with poly-D-lysine, but even this does not rectify the problem in many cases.

For making substrates of rat tail collagen, we used to prepare our own collagen by plucking collagenous tendons from a rat tail and dissolving them in acetic acid. This is relatively simple and there are published methods for doing so (Johnson and Argiro, 1983). However, it is now more time and cost-efficient to simply purchase sterile collagen solutions for cell culture. A few drops of the product are placed on the glass or plastic and are then smeared evenly using a Pasteur pippette into which a 90° bend has been introduced by placing it in a flame. (For glass, it is first necessary to treat with poly-D-lysine.) The collagen is then allowed to air dry. If insufficient collagen is applied, it will tend to dry in patches. The optimal quantity is best determined empirically. As noted earlier, we rarely use collagen substrates for dissociated cultures because they make for cultures with poor optical qualities for light microscopy and because the axons are very slow to grow. Our protocol for collagen coating is provided in Box 2.

B. Media

We generally use two different types of media, both of which are adequate for either sensory or sympathetic cultures. One of these is a modified L-15-based medium, which has the advantage of maintaining pH in air, but has the disadvantage of supporting cultures only for about 1 or perhaps 2 days. The other medium is an N2-based medium, which is excellent for long-term cultures, but requires a 5% carbon dioxide environment to maintain pH. Long-term cultures can be grown in the N2-based medium and then transferred to the L-15-based medium for short-term experiments on the microscope stage. We have found that peripheral neuronal cultures respond much better to L-15-based media than to media buffered with HEPES, although other laboratories have had good luck with HEPES (Gallo *et al.*, 1997). Formulations for the L-15-based and the N2-based media are provided in Table 3. The L-15-based medium requires serum to be present, whereas the N2-based medium contains supplements that permit it to be used either with or without serum. We have found that serum, although not required, appears to make the cells somewhat more resilient to experimental manipulations such as microinjection. Other laboratories have achieved excellent results with adult rat serum (Wang *et al.*, 2000), but we have not tried this. Both media contain

Table III

I. L-15-Based media

A. L-15 Incomplete Medium (250 ml)

Ingredient	Amount	Final concentration
Leibovitz's L-15 medium (GibcoBRL)	242 ml	96.8% (v/v)
D-(+)-Glucose solution (45%) (Sigma)	3.3 ml	0.6% (w/v)
200 m*M* L-glutamine (GibcoBRL)	2.5 ml	2 m*M*
Penicillin (10,000 units/ml) streptomycin (10,000 μg/ml) (Sigma)	2.5 ml	100 units/ml penicillin; 100 μg/ml streptomycin

Filter sterilize. Good for up to 3 weeks at 4°C.

B. L-15 Blocking Medium (50 ml)

L-15 incomplete medium	45 ml	90% (v/v)
Fetal bovine serum (HyClone)	5 ml	10% (v/v)

Filter sterilize. Good for up to 2 weeks at 4°C.

C. L-15 Plating Medium (50 ml)

L-15 incomplete medium	45 ml	90% (v/v)
Fetal bovine serum (HyClone)	5 ml	10% (v/v)
Nerve growth factor (Upstate)	50 μl (from 100 μg/ml stock)	100 ng/ml

Filter sterilize. Good for up to 1 week at 4°C.

II. N2-Based Media For Short-Term Culture

N2 Medium (100 ml)

Ingredient	Amount	Final concentration
DMEM 1 × (Gibco)	49 ml	49% (v/v)
F-12 nutrient mixture (HAM) 1 × (Gibco)	49 ml	49% (v/v)
Bovine serum albumin (BSA) (Calbiochem)	50 mg	0.5 mg/ml
200 m*M* L-glutamine (GibcoBRL)	1 ml	2 m*M*
N-2 supplement (Gibco)	1 ml	1× or 1% (v/v)

Note: BSA is somewhat difficult to dissolve. Initially add the powder to 25 ml DMEM and then pipette up and down several times to dissolve. Combine with the other components and filter sterilize. Then add:

Nerve growth factor (Upstate)	100 μl (from 100 μg/ml stock)	100 ng/ml

Immediately divide into 10- or 30-ml aliquots in sterile polypropylene tubes and freeze at −80°C for future use.

III. N2-Based Media For Long-Term Culture

A. Medium A (100 ml)

Ingredient	Amount	Final concentration
N2 medium	99.2 ml	99.2% (v/v)
B-27 supplement (Gibco)	0.7 ml	1× or 0.7% (v/v)
1-β-D-Arabinofuranosylcytosine (Ara-C) (Calbiochem)	100 μl (from 0.24 mg/ml stock)	0.24 μg/ml

B. Medium B (100 ml)

N2 medium	99.9 ml	99.9% (v/v)
Ara-C (Calbiochem)	100 μl (from 0.24 mg/ml stock)	0.24 μg/ml

(*continues*)

Table III (*Continued*)

C. Medium C (100 ml) Ingredient	Amount	Final concentration
N2 medium	100 ml	100% (v/v)
OP-1 (Curis)[a]	5 μl (from 1 mg/ml stock)	50 ng/ml

[a]To the best of our knowledge, OP-1 is only available by collaborating with Curis (www.curis.com).

IV. Maintenance of Sympathetic Neuronal Culture

A. The day after plating, replace N2 medium with medium A.
B. The next day, replace two-thirds of medium A with medium B. Repeat every other day.
C. One week after plating, start feeding the culture with medium C. Each time, replace two-thirds of the medium with medium C.
D. After 7–10 days in medium C, the cells will have well-developed dendrites.

V. Methylcellulose-Thickened Medium

Inert thickening reagent methylcellulose can be added into either L-15 incomplete medium or N2 medium to facilitate cell attachment to relatively less adhesive substrates, e.g., plain glass. A variety of different types of methylcellulose are available commercially. We use F4M premium grade methocel from Dow Chemical Company (Midland, MI). The procedure for preparing 0.6% methylcellulose containing medium is as follows: Weigh out 0.3 g methylcellulose into a 50-ml polypropylene tube. Autoclave. Add 50 ml L-15 plating medium or N2 medium. Turn the tube upside down and bang the cap against a tabletop to loosen the methylcellulose at the bottom and let it float in the medium. Attach the tube to an electric rotator and rotate at 4°C for at least 12 h to completely dissolve methylcellulose. Warm up when ready to use.

nerve growth factor, which is required for the viability of the cultures, unless replaced with alternative growth factors such as brain-derived neurotrophic factor (Ernst *et al*., 2000).

Either type of medium can be supplemented with an inert thickening agent called methylcellulose, which has certain advantages but also has disadvantages depending on the specific needs of the experiment. One main advantage is that cells cultured in methylcellulose will adhere to plain glass coverslips or plastic dishes that are not treated with any substrate or positively charged molecule such as poly-D-lysine. The neurons are not adhered tightly under these conditions, but they are sufficiently adhered to support axonal outgrowth. Cultures of this kind are particularly useful for experiments involving axonal retraction, which is more robust when the axons are initially not adhered so tightly to the substrate (see, e.g., Shaw and Bray, 1977; Ahmad *et al*., 2000; He *et al*., 2002). Methylcellulose presumably promotes attachment to poorly adherent substrates simply by "weighing" the cells down and permitting them to remain stationary long enough to form attachments. Another advantage of methylcellulose is that it tends to dissuade neuronal cell bodies from clumping together. We have encountered two disadvantages of methylcellulose. First, it is more difficult to exchange media or to introduce drugs into the medium when methylcellulose is present; this must also be done using medium containing methylcellulose so that the two media are miscible. Second, we have found that the cells sometimes extract unevenly in the presence of

methylcellulose; for example, in immunofluorescence preparations. Instructions for including methylcellulose in the medium are also provided in Table 3.

C. Dissection

1. Chick Sensory Ganglia

Fertilized chicken eggs are incubated at 37°C in a humidified egg incubator. Lumbosacral DRGs are dissected from the embryos after 10–11 days of incubation. Eggs are wiped with 70% ethanol and are cracked into a sterile petri dish. The chicken embryo is separated from other contents with sterile forceps and scissors. The head is removed, and the rest of the embryo is placed on a dissecting plate with the ventral side facing up. Syringe needles are used to fix the embryo against the dissecting plate. Legs are stretched laterally to flatten the embryo. The next steps are performed under a dissecting microscope. Two pairs of forceps are used to gently open trunk skin and muscle, and the chest wall. Organs in the chest and abdomen are gently removed with forceps, with care not to damage the spinal column area. The tail is cut off to reduce fluid accumulation in the abdomen. We then increase magnification at the microscope for a better view of the area containing the lumbosacral DRGs. The connective tissue is removed carefully to expose clearly the spinal column, the lumbosacral DRGs, and spinal nerves on both sides (see Fig. 1). The chain of sympathetic ganglia sits along the lateral side of the spinal column and on top of (i.e., ventral to) the DRGs. The chain is removed carefully, with care not to damage the DRGs. The lumbosacral DRGs are dissected using two pairs of fine-tip forceps, with one lifting the spinal nerve bundles and the other disconnecting the DRGs from the spinal cord. The DRGs are then transferred immediately to a petri dish containing Leibovitz's L-15 medium. (For this, we use L-15 directly from the bottle, with no supplements.) The DRGs are then separated from spinal nerves and cleared of any covering tissues. Generally speaking, three to five DRGs can usually be obtained from each side of the embryo.

2. Neonatal Rat Superior Cervical Sympathetic Ganglia

Pregnant female Sprague–Dawley rats generally give birth on the 21st or 22nd day postconception. Litters usually consist of 8–14 pups. We dissect superior cervical sympathetic ganglia from postnatal day 0 or day 1 rat pups. Pups are buried under watery ice for at least 15 min to achieve anesthesia. Pups are then sprayed with 70% ethanol and fixed to a wax dissecting plate with three syringe needles (Fig. 2A). A pair of small scissors is then used to cut the heart (entering through the underarm region). This drains blood from the pup, which is critical for achieving a clear dissecting view for the later procedures. The skin is then cut open along the midline from the midthorax to mandible level and from the lower cervical level to the two upper limbs. Under a dissecting microscope, two large salivary glands are clearly seen along the midline of the neck (Fig. 2B). One of the salivary glands (with surrounding connective tissue) is removed with a pair

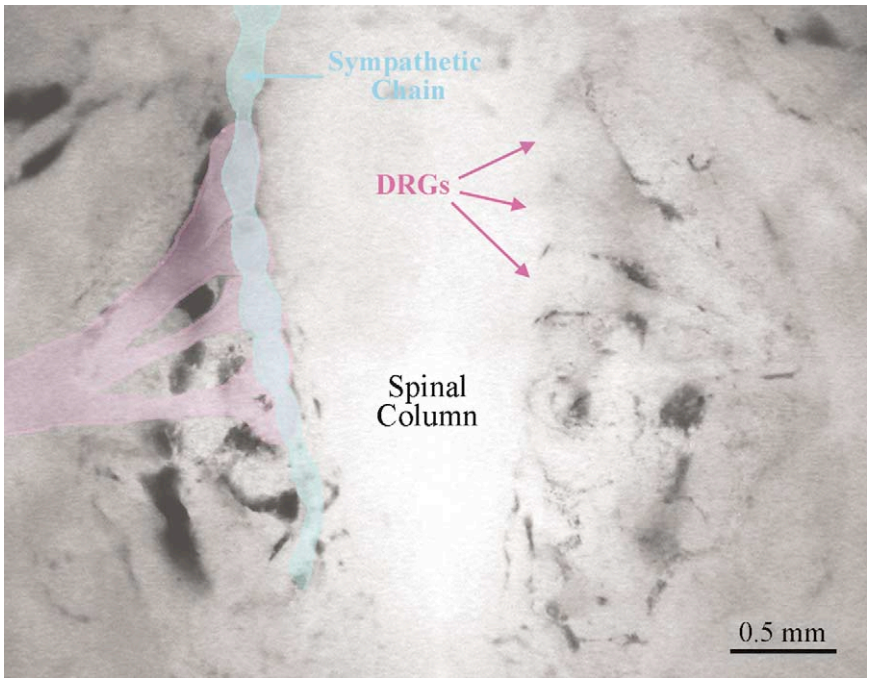

Fig. 1 Dissection of DRGs from a day 10 chicken embryo. After decapitation, the embryo was pinned to the dissection plate with its ventral side up. Organs in the chest and abdomen were removed. Working under a dissecting microscope, connective tissues surounding the spinal column and DRGs were removed carefully. Shown in the middle of the view in this figure is the lumbosacral region of the spinal column (the top of the photo is rostral). On the left side of the figure, lumbosacral DRGs and their roots are highlighted in pink and the sympathetic chain is highlighted in blue. The sympathetic chain has been removed on the right side of the figure to expose the DRGs beneath. Arrows point to three DRG.

of forceps. This exposes the ipsilateral sternocleidomastoid muscle (Fig. 2C). Transection of this muscle exposes the strap muscles covering the vessels beneath. Dissecting these muscles will then expose the carotid artery. Under higher magnification, bifurcation of the carotid artery can be resolved; the nodose ganglion is located directly lateral to the bifurcation (Fig. 2D). The nodose ganglion is removed to avoid confusion with the sympathetic ganglion later. The superior cervical ganglion sits directly beneath (i.e., dorsal to) the carotid bifurcation. There are two ways to dissect out the sympathetic ganglion. In the first way, the dissector lifts the tail of the ganglion and the carotid artery together with one pair of forceps and then separates the ganglion and the artery from surrounding tissues with another pair of forceps. Once fully detached, the ganglion and the artery are transferred into Leibovitz's L-15 medium in a petri dish. In the second way, the carotid artery and its branches are removed first, which will clearly expose the sympathetic ganglion beneath. Then two pairs of forceps are used to dissect out

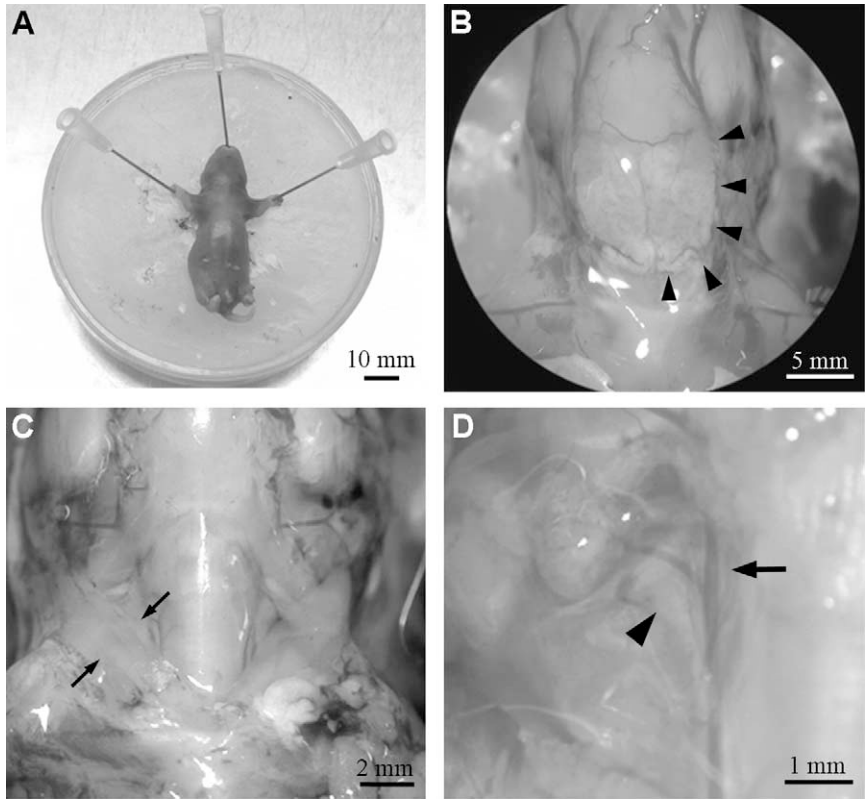

Fig. 2 Dissection of superior cervical ganglion from neonatal rat. (A) A neonatal rat pup was fixed on a dissecting plate with three syringe needles after anesthesia. (B) The two large salivary glands (one of which is outlined by arrowheads) along the midline were exposed after the neck skin was cut open. (C) After the salivary glands were removed, the sternocleidomastoid muscle (bracketed by arrows) was revealed on both sides. (D) Transection of the sternocleidomastoid muscle and strap muscles beneath has exposed the carotid artery and its branch (shown at higher magnification). The nodose ganglion (indicated by the arrowhead) is located directly lateral to the branch point of the carotid artery, and the sympathetic ganglion (indicated by the arrow) is located directly beneath the branch point.

the ganglion and transfer it to Leibovitz's L-15 medium in a petri dish. The ganglion is cleaned of blood vessels and covering connective tissues with two pairs of fine-tip forceps. The nerves from the ganglion are severed from the body of the ganglion. Then, the entire procedure is repeated on the other side of the animal to obtain the second superior cervical ganglion.

D. Preparation of Dissociated Cultures

After dissection from the animals and cleaning of any contaminating tissue, chick DRGs and rat superior cervical ganglia are treated in a very similar way to

obtain cultures of dissociated neurons. The sympathetic ganglia are transferred to a small tube containing 3 ml of 2.5 mg/ml collagenase (Worthington Biochemical), in calcium and magnesium-free phosphate-buffered saline (PBS) and incubated at 37°C for 15 min. The ganglia are then incubated in 2.5 mg/ml trypsin (Worthington Biochemical) in PBS at 37°C for 45 min. (If sympathetic ganglia are dissected from rat fetuses, the collagenase incubation may not be necessary because younger ganglia are easier to dissociate than older ones.) It is important to make sure that all of the ganglia sink to the bottom of the tube, as sometimes they can stick to the edge of the tube or float on a small air bubble. For chick DRGs, we incubate the ganglia with both enzymes at the same time for 20 min at 37°C. At the end of the enzyme treatments, the DRGs or sympathetic ganglia are rinsed twice in L-15 blocking medium (for composition, see Box 3) to neutralize the enzymes. Each rinse is for 5 min. For cultures that will be eventually grown in serum-free N2 medium, the ganglia are rinsed two more times in N2 medium (see Box 3) to remove any residual fetal bovine serum (FBS). We then add about 1 ml of complete L-15 plating medium (see Box 3) or N2 medium. The ganglia are then triturated into a single-cell suspension with a Pasteur pipette whose tip has been fire polished to obtain a narrow opening. The diameter of the pipette opening should be slightly smaller than that of the ganglia so that the ganglia are squeezed through it and dissociated sufficiently. In our experience, trituration should be done roughly five or six times. The concentration of cells in the trituration medium can be counted on a hemocytometer and diluted in L-15 plating medium or N2 medium to the desired concentration for plating. Alternatively, one can determine empirically the number of ganglia required to generate a desired cell density in a certain number of cultures. Cells cultured in L-15 plating medium are kept at 37°C in normal air, whereas cells cultured in N2 medium are kept at 37°C in 5% CO_2. When dividing the cells into dishes, it is important to work quickly because dispersed cells can settle quickly in the test tube or the pipette, resulting in cultures of very different density from one another.

E. Maintenance of Cultures

Neurons cultured for more than 2 days must be "fed" roughly every other day or twice a week. For this, we remove two-thirds of the medium and replace this volume with fresh medium. The inclusion of serum in the medium will promote dendritic differentiation in cultures of sympathetic neurons, but even more rapid and robust dendritic differentiation can be induced by including a member of the transforming growth factor β family called OP-1 (see, e.g., Yu *et al.*, 2000). Cytosine arabaninoside (also called Ara-C or 1-β-D-arabinofuranosylcytosine), a deoxycytidine analogue, can be added into the culture to inhibit the growth of dividing nonneuronal cells, which could dominate the culture otherwise. B-27, a commercially available mixture of culture additives, contains a cocktail of antioxidants that reduce reactive oxygen damage and enhance optimal growth and long-term survival of neurons. Details on our standard method for maintaining

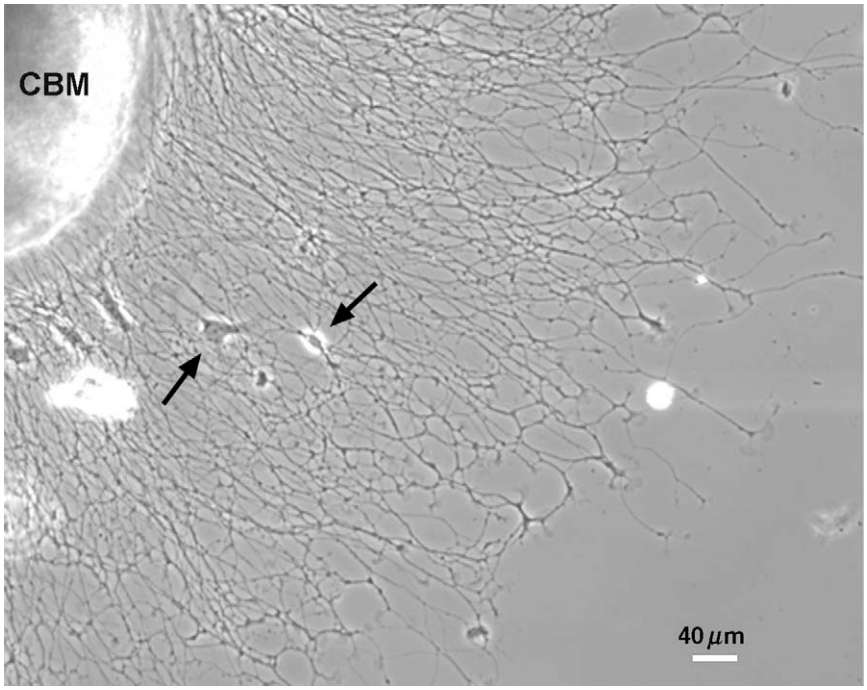

Fig. 3 Sympathetic explant culture. A chunk of neonatal rat sympathetic ganglion was plated on a substrate of poly-D-lysine and laminin. One day after plating, numerous axons had grown radially from the cell body mass (CBM). Arrows point to a few nonneuronal cells.

long-term sympathetic cultures and inducing dendrite formation are provided in Table 3.

F. Preparation of Explant Cultures

Explants are another way to culture neurons. Rather than dissociating the ganglia into individual cells, the ganglia are simply cut into small pieces and cultured as chunks. Axons grow radially away from the "cell body mass." Cultures of this kind are used far less commonly than dissociated cultures because the morphology of individual neurons cannot be fully discerned. However, there are special applications for which explant cultures are ideally suited. For example, explant cultures can be used for biochemical analyses in which one wishes to compare composition of the axons with that of cell bodies and dendrites. The cell body mass can simply be cut away from the axons under a dissecting microscope and then plucked out of the culture after development of the axonal halo. The axonal halos and cell body masses can then be analyzed separately by biochemical means. The axonal halo contains only axons, whereas the cell body masses contain

cell bodies, proximal regions of the axons, and, in the case of sympathetic neurons, dendrites. For a clean separation, it is necessary that there are no loosened cell bodies within the axonal halo. If this happens, the cell body masses can be plucked out and then cultured a second time, which results in a cleaner axonal halo. Most typically, a collagen substrate is used for explants because the explants do not tend to adhere well unless they have a thick substrate into which to "burrow." However, smaller explants can be grown successfully on any of the substrates used for dissociated cultures. Fig. 3 shows a small explant grown on laminin for 1 day; axonal outgrowth is apparent, as are a few nonneuronal cells that have detached from the cell body mass. More details on explant cultures are provided in Peng *et al*. (1986).

G. Reducing Nonneuronal Contamination

For most purposes, it is advantageous to have cultures that are as enriched as possible for neurons. However, ganglia are composed of nonneuronal cells (fibroblasts and glial cells), as well as neurons, and hence primary neuronal cultures consist of a mixture of cell types. Because nonneuronal cells undergo mitosis, whereas neurons do not, ganglia from younger animals contain a higher proportion of neurons to nonneuronal cells than ganglia from older animals. One method for routinely obtaining "cleaner" cultures of neurons is to carefully remove the capsule from each ganglion after it has been dissected from the animal. The capsule surrounding each ganglion is essentially invisible to the eye, but can be pealed away (almost like a banana peel) by a talented dissector with some good luck. An attempt should be made, but if the ganglia begin to tear, it is wise to abandon the attempt to remove the capsule and simply live with additional nonneuronal cells in the culture. Tearing a ganglion can result in the death of many neurons. Some workers have found that they can reduce nonneuronal contamination by plating the cells, agitating the culture, and replating the cells that lose their attachment during agitation (Shaw and Bray, 1977). Neurons are more poorly adhered than nonneuronal cells, and hence replating will result in a richer density of neurons. We have not had good luck with this approach, although we have not put substantial effort into it. For long-term cultures, antimitotic agents such as cytosine arabinoside can be added to the cultures. These agents prevent cells from dividing and slowly kill cells that normally divide, but do not have notable adverse effects on neurons if used at appropriate concentrations. For dissociated cultures, a concentration of 0.24 μg/ml generally works well; this concentration can be increased 10-fold for explant cultures (Peng *et al*., 1986). For experiments on cultures that are grown for less than 3 days, there is no point in adding such agents. However, for sympathetic cultures grown for over a week, exposure to cytosine arabinoside can result in cultures that consist of over 95% neurons. This is particularly useful for biochemical studies. Eliminating nonneuronal cells from DRG cultures tends to be more problematic, for reasons that we do not understand.

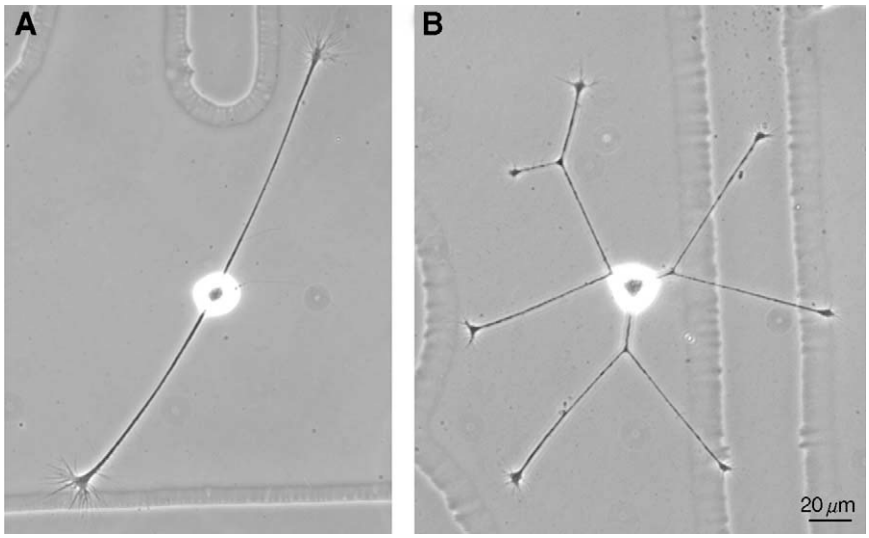

Fig. 4 Dissociated embryonic chick DRG culture. Embryonic day 10 chick DRG neurons were plated on plain glass coverslips in L-15-based medium thickened with methylcellulose. By 16 to 20 h after being plated, DRG neurons had generated axons tipped with growth cones. Some neurons generate several simple axons without branches (A), whereas others generate axons that bifurcate (B).

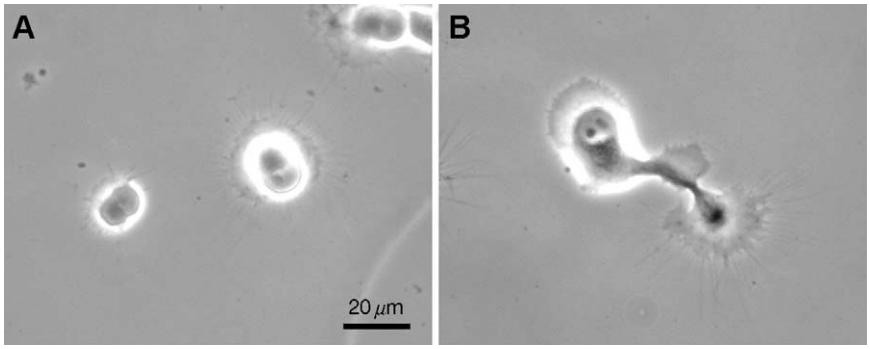

Fig. 5 Rat sympathetic neuronal culture. (A) When plated in L-15-based medium on a poly-D-lysine-coated coverslip, neurons attach firmly to the substrate and generate small lamella and numerous filapodia. Most neurons do not grow processes under these conditions. (B) When plated in N2 serum-free medium, sympathetic neurons show somewhat more robust outgrowth, with broader lamellae and short stumpy processes with large growth cones.

III. Cultures

In closing, this section is devoted to describing the cultures that result from the efforts described earlier.

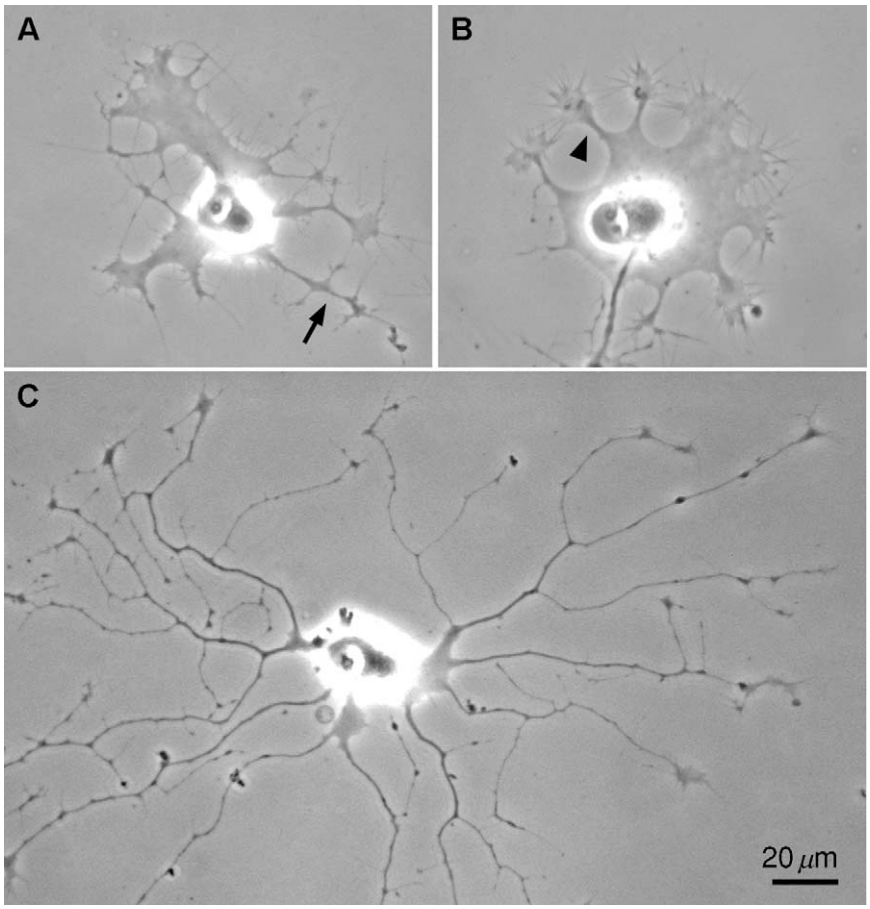

Fig. 6 Laminin-induced axogenesis from sympathetic neurons. Sympathetic neurons were treated with laminin in N2 serum-free medium. (A and B) Thirty minutes after laminin treatment, broad lamellae have extended from the base of the cell bodies. Early processes tipped with large growth cones have begun to protrude from the periphery of the lamellae (arrowhead) and some short axons are already seen (arrow). (C) By 3 h after laminin treatment, sympathetic neurons had generated long, thin axons with complex branches.

A. Short-Term Chick Dorsal Root Ganglia Culture

Chick DRG neurons are generally used for short-term experiments in our laboratory. We typically plate DRG neurons in methylcellulose-thickened L-15 plating medium on plain glass coverslips. Because plain glass is a relatively less adhesive substrate, these conditions are ideal for studying axonal retraction (Ahmad *et al.*, 2000; He *et al.*, 2002). By 16 to 20 h after plating, most DRG neurons have grown several distinguishable axons tipped with growth cones. The axonal identity of these processes is evidenced by their long, thin, and

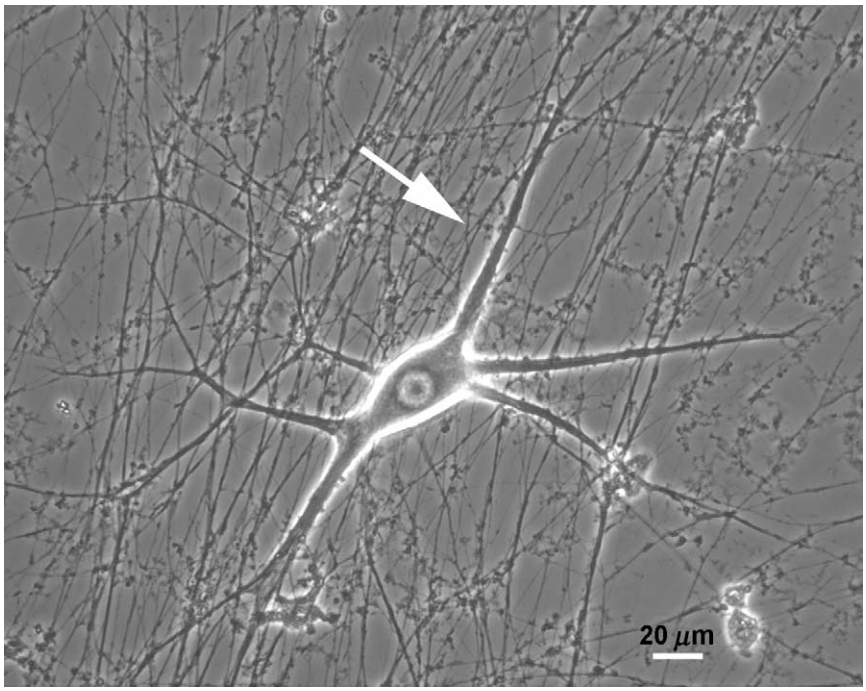

Fig. 7 Long-term rat sympathetic culture. Neonatal rat sympathetic neurons were plated and maintained as described in the text. A typical neuron is shown. After roughly 2 weeks in culture, the neuron has developed several short, thick and tapering dendrites, one of which is marked by an arrow.

uniform-diametered morphology, and also by their expression of axonal marker proteins (Smith, 1998). Some DRG neurons generate several simple primary axons (Fig. 4A), whereas some generate primary axons that bifurcate (Fig. 4B). When cultured for a longer period of time, a typical DRG neuron undergoes significant recrafting of the axonal arbor to produce a morphology that better reflects that of the DRG neurons inside the animal.

B. Short-Term Rat Sympathetic Culture

For most of the short-term experiments in our laboratory, we plate sympathetic neurons on poly-D-lysine-coated glass coverslips in L-15 plating medium. Neurons attach to the bottom of the dish shortly after being plated. Within the first 1 to 2 h after plating the neurons have generated a modest lamella from the base of the cell body, with numerous filopodia extending from the edge of the lamella (Fig. 5A). By this time, neurons have attached to the coverslip firmly. The morphology of the neuron remains essentially the same over the next several hours and even by the next morning. The rapid outgrowth of axons can be induced by the addition of laminin to a final concentration of 25 μg/ml (Invitrogen) to the culture (Rivas *et al.*,

1992; Tang and Goldberg, 2000). A commercially available partially defined mixture of growth factors (which contains laminin as well as other factors) termed Matrigel matrix (BD Biosciences) can be used at 1:40 dilution instead of pure laminin for an even more rapid and robust response (Slaughter *et al.*, 1997; Yu *et al.*, 2002). Alternatively, laminin can be applied directly onto the poly-D-lysine substrate prior to the plating of the cells (see Box 2). This approach produces a rapid outgrowth of axons that begins almost immediately after the firm attachment of the cell body to the substrate (roughly 30 min after plating; see, e.g., Ahmad *et al.*, 1999). When grown in the presence of laminin or matrigel, neurons generate thin and extremely long axons, as shown in Fig. 6. It is our impression that an even broader, more robust lamella is produced on the poly-D-lysine and that an even more robust response to laminin or matrigel can be obtained by using the N2-based medium as opposed to the L-15-based medium (see Fig. 5B). We generally use the L-15-based medium, however, because its capacity to maintain pH in normal air is advantageous for experimental manipulations on the microscope stage.

C. Long-Term Rat Sympathetic Culture

Neurons in culture generate dendrites much later than axons. Therefore we need to maintain rat sympathetic neurons in culture for a longer period of time to study dendrites. Generally speaking, after 7 to 10 days in medium containing OP-1, neurons will have developed robust dendrites (Yu *et al.*, 2000). Each neuron has several dendrites, which are distinguishable from axons on the basis of their short, thick, and tapering morphology (Fig. 7). The axons have become extremely long at this point and have formed a dense network on the culture substrate. It is impossible to discern at this point which axons arise from which cell bodies, but interestingly, the number of axons has been pruned such that each individual neuron typically has a single axon. This can be revealed by microinjecting a fluorescent dye into an individual cell body to reveal its detailed morphology (Higgins *et al.*, 1991).

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