Video Article

Inducing Dendritic Growth in Cultured Sympathetic Neurons

Atefeh Ghogha[1], Donald A. Bruun[1], Pamela J. Lein[1]

[1]Department of Molecular Biosciences, University of California, Davis

Correspondence to: Pamela J. Lein at pjlein@ucdavis.edu

URL: https://www.jove.com/video/3546
DOI: doi:10.3791/3546

Keywords: Neuroscience, Issue 61, Bone morphogenetic proteins (BMPs), Matrigel, dendrite, dendritogenesis, neuronal morphogenesis, sympathetic neurons

Date Published: 3/21/2012

Citation: Ghogha, A., Bruun, D.A., Lein, P.J. Inducing Dendritic Growth in Cultured Sympathetic Neurons. *J. Vis. Exp.* (61), e3546, doi:10.3791/3546 (2012).

Abstract

The shape of the dendritic arbor determines the total synaptic input a neuron can receive [1-3], and influences the types and distribution of these inputs [4-6]. Altered patterns of dendritic growth and plasticity are associated with impaired neurobehavioral function in experimental models [7], and are thought to contribute to clinical symptoms observed in both neurodevelopmental disorders [8-10] and neurodegenerative diseases [11-13]. Such observations underscore the functional importance of precisely regulating dendritic morphology, and suggest that identifying mechanisms that control dendritic growth will not only advance understanding of how neuronal connectivity is regulated during normal development, but may also provide insight on novel therapeutic strategies for diverse neurological diseases.

Mechanistic studies of dendritic growth would be greatly facilitated by the availability of a model system that allows neurons to be experimentally switched from a state in which they do not extend dendrites to one in which they elaborate a dendritic arbor comparable to that of their *in vivo* counterparts. Primary cultures of sympathetic neurons dissociated from the superior cervical ganglia (SCG) of perinatal rodents provide such a model. When cultured in defined medium in the absence of serum and ganglionic glial cells, sympathetic neurons extend a single process which is axonal, and this unipolar state persists for weeks to months in culture [14,15]. However, the addition of either bone morphogenetic protein-7 (BMP-7) [16,17] or Matrigel [18] to the culture medium triggers these neurons to extend multiple processes that meet the morphologic, biochemical and functional criteria for dendrites. Sympathetic neurons dissociated from the SCG of perinatal rodents and grown under defined conditions are a homogenous population of neurons [19] that respond uniformly to the dendrite-promoting activity of Matrigel, BMP-7 and other BMPs of the decapentaplegic (dpp) and 60A subfamilies [17,18,20,21]. Importantly, Matrigel- and BMP-induced dendrite formation occurs in the absence of changes in cell survival or axonal growth [17,18].

Here, we describe how to set up dissociated cultures of sympathetic neurons derived from the SCG of perinatal rats so that they are responsive to the selective dendrite-promoting activity of Matrigel or BMPs.

Video Link

The video component of this article can be found at https://www.jove.com/video/3546/

Protocol

1. Preparation of Culture Medium (C2 medium)

1. Add the following, in the order listed, to a sterile, disposable 500 ml Erlenmeyer flask:

Amount	Component	Final Concentration
190 ml	DMEM (low glucose)	N/A
10 ml	fatty acid free BSA (20mg/ml in DMEM)	50 µg/ml
2.8 ml	L-Glutamine	1.4 mM
4 ml	insulin/transferrin/selenium(100x)	10 µg/ml insulin 5.5 µg/ml transferrin 38.7 nM selenium
0.4 ml	NGF (125 µg/ml)	100 ng/ml
200 ml	Ham F-12	N/A

2. Swirl gently to mix and aliquot 10 ml per tube in 17 x 100 sterile snap cap tubes.
3. Store at -80 °C
4. Important notes:
   - Thaw NGF immediately prior to usage.

- Before aliquoting the NGF stock solution, precoat the interior walls of the tissue culture plastic serological pipet with protein to minimize sticking of NGF to the plastic. Easiest approach is to Pipette the DMEM mixture containing the BSA several times. Rinse the NGF tube several times with the DMEM mixture.
- Add F-12 last because free cysteine may destabilize disulfide bonds in the insulin.
- Don't freeze in volumes greater than 10 ml because $CaPO_4$ crystals may form
- Do not freeze-thaw C2 medium more than 2 times.

2. Preparation of Glass Coverslips

1. We have found that only fine German glass coverslips work consistently in this protocol (see **Table 1** for recommended source and catalog number).
2. Add 75-100 ml of 70% nitric acid to 300 ml glass specimen bottles with polytetrafluoroethylene (PTFE or Teflon) lid liners (Fisher catalog #09-911-765).
3. Wearing gloves, drop coverslips one by one into the nitric acid solution. Add enough coverslips to make a layer no more than 2-3 coverslips thick on the bottom of the jar.
4. Gently shake the containers with the coverslips for 6-8 hours at room temperature on an orbital shaker set for no more than 185 rpm.
5. Working in a fume hood, carefully decant the acid and dispose according to local regulations. Rinse coverslips once with ultra-pure tissue culture-grade water, then add 75-100 ml water to the container. Coverslips may be rinsed multiple times if the water becomes cloudy. Gently shake at room temperature overnight on an orbital shaker set for no more than 185 rpm.
6. Repeat the above sequence of nitric acid/ tissue culture-grade water daily for a total of 3 days.
7. Decant the final tissue culture-grade water and add enough acetone to barely cover the coverslips.
8. Decant this acetone and dispose properly.
9. Transfer coverslips to a 150 mm glass Petri dish. Add enough acetone to barely cover the coverslips. Ensure the coverslips are in a single layer in the dish.
10. Allow the acetone to evaporate overnight in a fume hood.
11. Bake coverslips in a drying oven at 180 °C for 1.5 hours to sterilize.

3. Preparation of Coverslips for Culture

1. The day before the dissection, add 1 glass coverslip (prepared as described above) to each well of a 24 well tissue culture plate using sterile technique. Add 200 µl of sterile poly-D-lysine solution (100 mg/ml in 0.5 M Tris buffer, pH 8) to each well and store the plate at 4 °C overnight.
2. The day of the dissection, and ideally before starting the dissection, aspirate the poly-D-lysine solution from each well and wash each well 5 times with sterile tissue-culture grade water.
3. Add 200 µl low-glucose DMEM to each plate and store in 37 °C incubator. Approximately 1 hour prior to plating cells, remove low-glucose DMEM and add 250 µl C2 medium per well. Store plates at 37 °C under 5% CO_2 until cells are ready to be plated.

4. Dissection and Isolation of the Superior Cervical Ganglion

1. Dissociated cultures of sympathetic neurons are usually prepared by dissociation of superior cervical ganglia (SCG) harvested from prenatal (embryonic days 19-21) rat pups because there are fewer glial cells. However, this same protocol can be used successfully with early postnatal (1 to 3 days old) rat pups or perinatal mouse pups.
2. Euthanize pregnant rat via CO_2 inhalation. Shave the abdomen and wash the skin with 70% ethanol. Remove the uterine horns under sterile conditions and place the uterine horns in a sterile 150 mm Petri dish. Avoid damaging the intestines or other internal organs of the dam during the dissection.
3. Place the Petri dish with the pups onto a pan of ice to anesthetize the embryonic pups. Dissect the embryos free of the uterine horn and amniotic membranes. Cut the spinal cord of each pup along the midline under the shoulder to euthanize the pups. This also severs the vena cava, which reduces bleeding from the carotid artery during dissection of the SCG. Once dissected free of the uterine horn, place embryos in a sterile Leibovitz's L-15 medium (or any air-buffered medium) supplemented with penicillin and streptomycin.
4. Place the embryos on their backs on a 150 mm Petri dish half-filled with Sylgard. Using sterile 20 gauge needles, pin the thorax and the head, gently hyperextending the neck, which facilitates dissection of the SCG.
5. Make a midline incision along the neck at the level of the clavicles to reveal the submandibular glands. Remove the glands using fine forceps (no. 4 or no. 5 Dumont) to expose the muscle layer.
6. Use the fine forceps to cut the sternocleidomastoid muscle near the clavicle. Then gently lift and incise the omohyoid muscle next to the trachea. This muscle is very thin, so avoid cutting too deeply to avoid tearing the underlying carotid artery and SCG. Once these muscle layers are removed, the carotid artery and the vagus nerve should be clearly visible.
7. The SCG lies just below the bifurcation of the carotid artery. Using fine forceps (no. 4 or no. 5), blunt dissect tissue away from both sides of the carotid artery to expose the ganglion. Using 2 fine forceps, grasp the carotid artery just above and below the rostral and caudal ends of the SCG, respectively, to remove the section of the carotid artery lying above the SCG as well as the SCG itself. It is likely that the nodose ganglion, which lies alongside the SCG at this stage of development, will also be removed at this step. The nodose ganglia can be identified by its round shape and the large vagal nerve projecting from it. This lies along one of the branches of the carotid artery. In contrast, the SCG is almond shaped and lies directly under the bifurcation of the carotid artery. Place the dissected tissue into a sterile 35 mm culture dish containing L-15 medium and antibiotics. Remove the SCG from all pups before progressing to the next steps.
8. Using fine forceps, gently separate the SCG from the carotid artery and any other tissues removed during the dissection (particularly the vagus nerve and nodose ganglion). Use fine forceps to remove the capsule from the ganglia. This greatly reduces the numbers of nonneuronal cells in culture. Transfer the ganglia to another sterile 35 mm dish containing L-15 medium.
9. To dissociate the SCG, remove the L-15 medium and replace with 2 ml Collagenase (final concentration 1 mg/ml)/Dispase (final concentration 5 mg/ml) in calcium and magnesium-free Hank's balanced salt solution. Incubate at 37 °C for 40 minutes.

10. Transfer the SCG to a sterile 15 ml conical centrifuge tube and bring the volume up to 10 ml with L-15 supplemented with BSA (final concentration 1-2 mg/ml). Centrifuge at 200 X g at room temperature for 5 minutes. Discard the supernatant and wash once more with L-15 supplemented with BSA.
11. After the second wash, remove all L-15 and resuspend the ganglia in ~2 ml of C2 medium. Triturate the cells using a fire-polished bent-tip pipette, make sure the pipette is coated with BSA/L15 before trituration to minimize sticking of cells to the glass. Triturate gently 3-4 times. Let the large clumps settle for about 1 minute and transfer the cell suspension to a 15 ml conical tube. Add more C2 media to the remaining SCG and triturate. After letting the clumps settle, transfer the cell suspension from the second trituration to that collected after the first trituration. Repeat this process several times until the ganglia are mostly dissociated. Discard any remaining clumps after the fourth trituration.
12. For cells dissociated from one litter of rat pups (typically 12-16 pups), bring the total volume of medium in the cell suspension to 8-10 ml by adding additional C2 medium. Gently resuspend the cells using a pipette and remove a small aliquot to determine the cell density. Adjust the volume of the cell suspension to achieve 2X the cell density desired in the culture, and then, being sure to maintain the cells in suspension, aliquot 250 ml of cell suspension per well. For morphometric studies of dendritic growth, we typically plate cells at low density (~5 x 10^4 cells per well) in 24 well plates.
13. Our laboratory incubates SCG cultures in glass desiccators rather than directly in a CO_2 incubator. We do this because sympathetic neurons grown in serum-free medium perform better with a slightly higher CO_2 concentration (approximately 6.5%). In addition we do not use antibiotics in our media since it inhibits neurite outgrowth and maintaining cultures in the desiccators seems to minimize spread of contamination if it develops in a subset of cultures. Using this system, we routinely maintain SCG cultures for up to 8 weeks. Once plated, SCG cultures are placed on the porcelain desiccator plate in glass desiccators containing sterile water in the bottom. Approximately 120 ml of CO_2 is injected into the desiccator prior to sealing it shut and placing the entire desiccator into an incubator set for 35.5 °C.

5. Feeding and Maintenance of Cultures

1. Cultures are generally fed 3 times a week, with the first media exchange occurring 24 hours after plating. Using a sterile bent-tip pipette, gently withdraw about half of the media per well. Using a clean sterile bent-tip pipette, replace the volume of medium removed with fresh C2.
2. To eliminate non-neuronal cells from the culture, the anti-mitotic agent cytosine-D-arabinofuranoside (ARA-C, final concentration 1-2 µM) is typically added to the culture medium at the first media exchange (e.g., 24 hours after plating) for 48 hours. This concentration of ARA-C is usually sufficient to remove all non-neuronal cells. If additional ARA-C treatment is required, it is recommended that these be done every other feeding to minimize toxic effects on the neurons.

6. Inducing Dendritic Growth with Matrigel or BMP-7

1. To induce dendritic growth, Matrigel or BMP-7 is added to cultures after non-neuronal cells have been eliminated and ARA-C levels in the cultures are significantly reduced. Typically, we add Matrigel or BMP-7 to the medium on day 5 *in vitro*, but dendritic growth can be induced by Matrigel or BMP-7 added at any time point *in vitro*. Matrigel or BMP-7 is added to the C2 medium and cultures are fed as described above.
2. Matrigel and BMP-induced dendritic growth is time and concentration-dependent. For maximal effects, Matrigel is added to C2 at a final concentration of 50-75 µg/ml; BMP-7 is added to the C2 medium at a final concentration of 50 ng/ml. Matrigel concentrations of greater than 100 µg/ml or BMP-7 concentrations greater than 100 ng/ml have been noted to cause neuronal toxicity. Dendritic processes (as determined by morphological criteria) become apparent approximately 48 hours after initial exposure to Matrigel or BMP-7 at maximally effective concentrations. To induce robust dendritic growth, cultures are fed with BMP-7 containing medium at every feeding. Equivalent results have been obtained using BMPs 2, 4, 5 and 6 at comparable concentrations [20,21].

7. Immunocytochemical Analysis of BMP-7-induced Dendritic Growth

1. When the desired extent of dendritic arborization has been achieved, cultures are fixed using 4% paraformaldehyde in 0.1 M phosphate buffer. The best fixation is achieved by replacing half the medium in the well with 4% paraformaldehyde and repeating this step 3-5 times over 10-15 minutes.
2. Rinse cultures by replacing half the 4% paraformaldehyde with phosphate-buffered saline (PBS) and repeating this step three times over 10-15 minutes.
3. Remove all the PBS and add 200 µl 0.1% Triton X-100 in PBS to each well for 5 minutes at room temperature to permeabilize the cells.
4. Remove Triton solution by aspiration and add 200 µl per well of blocking buffer solution (PBS supplemented with 3-5% BSA and 1% goat serum). Incubate for 20-30 minutes at room temperature.
5. Remove the blocking buffer and add the primary antibody, microtubule-associated protein-2 (MAP2) diluted at 1:2000 - 1:5000 in blocking buffer. Incubate cultures for 1 hour at room temperature or overnight at 4 °C.
6. Remove the primary antibody solution, wash the cultures with PBS three times over 15 minutes at room temperature.
7. Incubate the cultures with the secondary antibody diluted in PBS supplemented with 1% BSA for 1-2 hours at room temperature in the dark.
8. Remove the secondary antibody solution and wash the cultures with PBS three times over 10-15 minutes at room temperature in the dark.
9. Mount coverslips on glass slides cell side down in a drop of aqueous mounting medium at the appropriate pH for the fluorochrome tagged to the secondary antibody. Keep the slides at room temperature overnight to let the mounting medium dry. Store slides at 4 °C until imaging.
10. Allow the slides to equilibrate to room temp before imaging. Acquire fluorescent images using a phase-contrast microscope equipped for epifluorescence. Typically, the entire dendritic arbor of a single neuron can be captured using a 20X objective and appropriate filters.

8. Representative Results

Sympathetic neurons dissociated from the SCG of perinatal rats and grown in the absence of serum and ganglionic glial cells fail to extend MAP2 immunopositive processes (**Figure 1**), but rather, typically extend only a single axonal process [14,15]. Exposure to BMP-7 (**Figure 1**) or Matrigel induces the formation of numerous processes that meet the morphological, biochemical and functional criteria of dendrites [17,18]. The dendrite-promoting activity of either Matrigel or BMP-7 is concentration- and time-dependent [17,18,20]. Maximal dendritic growth is observed using

concentrations of Matrigel between 50 and 75 µg/ml or BMP-7 between 30 and 100 ng/ml, and half-maximal effects are typically observed at BMP-7 concentrations of ~2 ng/ml. However, significant changes in dendritic growth can be detected with BMP-7 concentrations as low as 300 pg/ml. The dendritic response to either Matrigel or BMP-7 is relatively slow, with <50% of the neurons forming a second process within 24 hours after exposure to either dendrite-promoting agent. However, within 3 days after the initial BMP-7 exposure, virtually all neurons respond to maximally effective Matrigel or BMP-7 concentrations. The number of dendrites per neurons continues to increase with continuous exposure to BMP-7, with most of the change occurring during the first 10 days of treatment. After 4 weeks, BMP-7-treated neurons typically have 6-8 primary dendrites that exhibit secondary, tertiary and even quaternary dendritic branches [17]. This increase in dendritic arbor occurs in the absence of any significant change in axonal growth, and comparable dendritic responses can be elicited using similar concentrations of BMPs 2, 4, 5 or 6 [20,21]. Dendritic growth can also be elicited by co-culturing sympathetic neurons with endogenous ganglionic glial cells [15,22]; however, the dendritic response is significantly slower under these conditions.

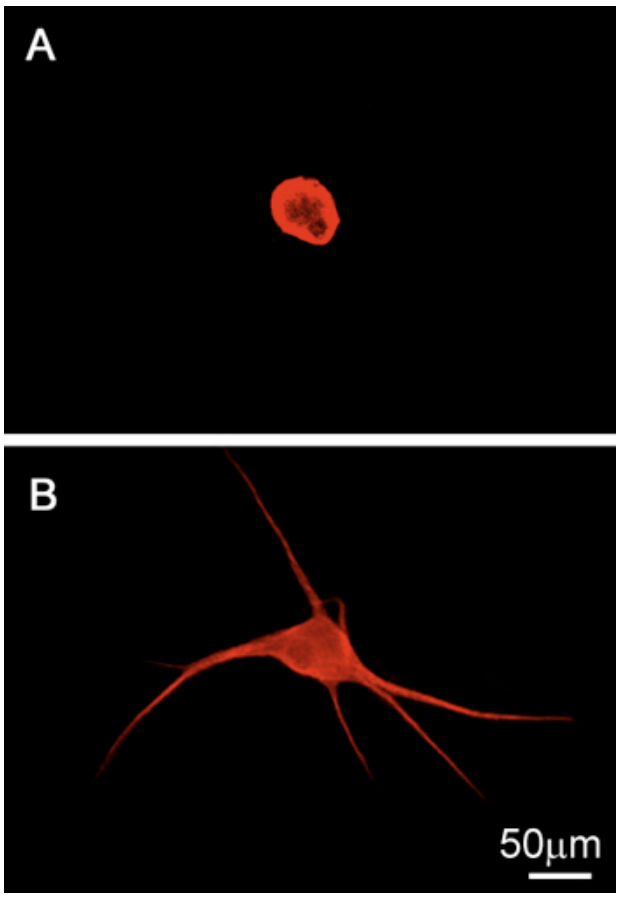

Figure 1. BMP-7 promotes dendritic growth in cultured sympathetic neurons. Non-neuronal cells were eliminated from SCG cultures by treatment with anti-mitotic for 48 hr beginning on day 2 *in vitro*. Beginning on day 5 *in vitro*, cultures were treated with either control medium (A) or medium supplemented with BMP-7 at 50 ng/ml (B). On day 11 in vitro (after 5 days of BMP treatment), cultures were immunostained with mAb against MAP2, a protein found primarily in dendrites and neuronal somata. As shown in representative fluorescence photomicrographs, neurons grown under control conditions lack dendrites as evidenced by the lack of MAP-2 immunopositive processes dendrites (A); in contrast, neurons exposed to BMP-7 (B) typically have several tapered MAP-2 immunopositive dendrites.

Discussion

The advantages of this model include: (a) the cultures are comprised of a homogenous population of sympathetic neurons devoid of other cell types [17,23]; (b) 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 [23]; (c) neurons in these cultures respond uniformly to the dendrite-promoting activity of Matrigel, BMP-7 and other BMPs of the dpp and 60A subfamilies [17,18,20,21]; and (d) Matrigel- or BMP-induced dendrite formation occurs in the absence of changes in cell survival or axonal growth [17,18]. This model allows experimental control of when dendritic growth is triggered, which make it possible to obtain synchronized populations of neurons at distinct stages of dendritic growth, e.g., immediately preceding the formation of dendrites, during primary dendritogenesis (the initial formation of primary dendrites) and during later stages of dendritic maturation. The most significant limitations of the model include the limited amounts of RNA and protein available from a typical dissection of a single litter, which may restrict biochemical studies, and some difficulty in manipulating gene expression. Recent advances in techniques for expressing cDNA in primary neuronal cell cultures are rapidly overcoming this latter disadvantage. There are publications reporting the successful application of lipid-based delivery systems, nucleofection, and adenoviral vectors to cultured sympathetic neurons. The reported transfection efficiencies are relatively low (typically ranging from 10-20% with lipofection or nucleofection and up to 30-50% with viral

infection), which is adequate for endpoints based on individual cell analyses, such as morphological analyses, but may be problematic for many biochemical endpoints.

Factors that can interfere with BMP-induced dendritic growth in cultured sympathetic neurons include culturing the neurons at a very high cell density and including serum in the culture media. While the reason that high cell density attenuates the dendrite-promoting activity of BMPs is not known, it is likely that serum attenuates this activity because BMPs bind avidly to serum proteins. Interestingly, serum itself has weak dendrite-promoting activity in cultured sympathetic neurons [24], and our preliminary data suggests that this activity is mediated by BMPs, which are present in most if not all commercial sera. BMPs are also present in Matrigel, and likely mediate its dendrite-promoting activity. A third factor that can impact the dendrite-promoting activity of BMPs is improper handling and storage of BMPs. Do not store BMP stock solutions at a concentration of less than 0.1 mg/ml. It is important to not let BMP stock or working solutions to become very basic and we recommend that buffers used to dilute stock solutions have a pH of 4.5 ± 0.05. Mix all solutions vigorously before aliquoting but not so vigorously that solutions foam, and only use polypropylene tubes because BMPs tend to stick to other plastics. Do not store aliquots in freezers that have an automatic defrost feature because the cycling of temperatures is sufficient to destroy activity of BMP-7. It is strongly advised that BMP solutions be stored at -80 °C and that freeze-thawing be minimized (we do not freeze-thaw more than 2-3 times). Similar precautions should be used in handling Matrigel. Also, it is recommended that Matrigel be slowly thawed at 4 °C. Matrigel will not induce dendritic growth when used to precoat substrates prior to plating neurons; it is only effective when added to the culture medium of established neuronal cell cultures [18].

Disclosures

The authors have nothing to disclose.

Acknowledgements

This work was supported by funding from the National Institutes of Health (grants R21 NS45037 and R01 ES014901).

References

1. Purpura, D.P. In: *The Neurosciences: A Study Program.* Quarton, G.C., Melnechuk, T., & Schmitt, F.O., eds., Rockefeller University Press, 372-393, (1967).
2. Purves, D. Functional and structural changes in mammalian sympathetic neurons following interruption of their axons. *J. Physiol.* **252**, 429-463 (1975).
3. Purves, D. *Body and Brain: A Trophic Theory of Neural Connections.* Harvard University Press, (1988).
4. Miller, J.P. & Jacobs, G.A. Relationships between neuronal structure and function. *J. Exp. Biol.* **112**, 129-145 (1984).
5. Schuman, E.M. Synapse specificity and long-term information storage. *Neuron.* **18**, 339-342 (1997).
6. Sejnowski, T.J. The year of the dendrite. *Science.* **275**, 178-179 (1997).
7. Berger-Sweeney, J. & Hohmann, C.F. Behavioral consequences of abnormal cortical development: insights into developmental disabilities. *Behav. Brain Res.* **86**, 121-142 (1997).
8. Connors, S.L., *et al.* Fetal mechanisms in neurodevelopmental disorders. *Pediatric Neurology.* **38**, 163-176 (2008).
9. Pardo, C.A. & Eberhart, C.G. The neurobiology of autism. *Brain Pathology (Zurich, Switzerland).* **17**, 434-447 (2007).
10. Zoghbi, H.Y. Postnatal neurodevelopmental disorders: meeting at the synapse? *Science (New York, N.Y.)* **302**, 826-830 (2003).
11. de Ruiter, J.P. & Uylings, H.B. Morphometric and dendritic analysis of fascia dentata granule cells in human aging and senile dementia. *Brain Res.* **402**, 217-229 (1987).
12. Flood, D.G. & Coleman, P.D. Hippocampal plasticity in normal aging and decreased plasticity in Alzheimer's disease. *Prog. Brain Res.* **83**, 435-443 (1990).
13. Jagadha, V. & Becker, L.E. Dendritic pathology: an overview of Golgi studies in man. *Can. J. Neurol. Sci.* **16**, 41-50 (1989).
14. Bruckenstein, D.A. & Higgins, D. Morphological differentiation of embryonic rat sympathetic neurons in tissue culture. I. Conditions under which neurons form axons but not dendrites. *Dev. Biol.* **128**, 324-336 (1988).
15. Tropea, M., Johnson, M.I., & Higgins, D. Glial cells promote dendritic development in rat sympathetic neurons *in vitro*. *Glia.* **1**, 380-392 (1988).
16. Lein, P., *et al.* The effects of extracellular matrix and osteogenic protein-1 on the morphological differentiation of rat sympathetic neurons. *Int. J. Dev. Neurosci.* **14**, 203-215 (1996).
17. Lein, P., Johnson, M., Guo, X., Rueger, D., & Higgins, D. Osteogenic protein-1 induces dendritic growth in rat sympathetic neurons. *Neuron.* **15**, 597-605 (1995).
18. Lein, P.J. & Higgins, D. Laminin and a basement membrane extract have different effects on axonal and dendritic outgrowth from embryonic rat sympathetic neurons *in vitro*. *Dev. Biol.* **136**, 330-345 (1989).
19. Higgins, D., Lein, P.J., Osterhout, D.J., & Johnson, M.I. In: *Culturing Nerve Cells*. Banker, G. & Goslin, K., eds., The MIT Press, 177-295 (1991).
20. Beck, H.N., Drahushuk, K., Jacoby, D.B., Higgins, D., & Lein, P.J. Bone morphogenetic protein-5 (BMP-5) promotes dendritic growth in cultured sympathetic neurons. *BMC Neurosci.* **2**, 12 (2001).
21. Guo, X., Rueger, D., & Higgins, D. Osteogenic protein-1 and related bone morphogenetic proteins regulate dendritic growth and the expression of microtubule-associated protein-2 in rat sympathetic neurons. *Neurosci. Lett.* **245**, 131-134 (1998).
22. Lein, P.J., *et al.* Glia induce dendritic growth in cultured sympathetic neurons by modulating the balance between bone morphogenetic proteins (BMPs) and BMP antagonists. *J. Neurosci.* **22**, 10377-10387 (2002).
23. Higgins, D., Lein, P., Osterhout, D., & Johnson, M.I. In: *Culturing Nerve Cells*. Banker, G. & Goslin, K., eds., MIT Press, 177-205 (1991).
24. Bruckenstein, D. A. & Higgins, D. Morphological differentiation of embryonic rat sympathetic neurons in tissue culture. II. Serum promotes dendritic growth. *Dev. Biol.* **128**, 337-348, [pii] 0012-1606(88)90296-5 (1988).

