

Neurogenesis in Postnatal Mouse Dorsal Root Ganglia

Michael P. Namaka* Mike Sawchuk,† Stephen C. MacDonald,† Larry M. Jordan,* and Shawn Hochman†¹

*Department of Physiology, University of Manitoba, Winnipeg, Manitoba, Canada R3E 0W3; and †Department of Physiology, Emory School of Medicine, Atlanta, Georgia 30322

Received August 17, 2000; accepted June 13, 2001; published online September 13, 2001

Neurogenesis continues in various regions of the central nervous system (CNS) throughout life. As the mitogen basic fibroblast growth factor (bFGF) can proliferate neuronal precursors of CNS neurons in culture, and is also upregulated within adult dorsal root ganglia following axotomy, it is possible that the postnatal dorsal root ganglia contain bFGF-responsive neuronal precursors. We undertook cell culture of postnatal mouse dorsal root ganglia to demonstrate neurogenesis. Basic FGF induced a cellular proliferative response in dorsal root ganglia cell culture. After 2 weeks in serum-free medium containing bFGF, neurons were rarely observed. However, following removal of bFGF and addition of trophic factors, many cells were observed that morphologically resembled dorsal root ganglia neurons, stained for neuronal markers, and generated action potentials. Furthermore, bromodeoxyuridine, used as a marker of cyto-genesis, was detected in neurofilament-160⁺ and/or microtubule-associated protein-2⁺ cells that morphologically resembled neurons. In addition to bFGF, epidermal growth factor, nerve growth factor, and sonic hedgehog were also capable of generating spherical cell clusters that contained cells that stained for neuronal markers following the addition of trophic factors. These results suggest that early postnatal dorsal root ganglia contain neural precursors that appear to proliferate in response to various factors and can then be induced to differentiate into neurons. In conclusion, the existence of neural precursors and the possibility of neurogenesis in postnatal dorsal root ganglia may provide a greater range of plasticity available to somatosensory systems during growth or following injury, perhaps to replace ineffectual or dying neurons.

© 2001 Academic Press

Key Words: DRG; sensory; pain; plasticity; stem cell; progenitor; precursor; fibroblast growth factor; nerve growth factor.

¹ To whom correspondence should be addressed at Room 362, Physiology Building, Emory University School of Medicine, 1648 Pierce Drive, Atlanta, GA 30322. Fax: (404) 727-2648. E-mail: shochman@physio.emory.edu.

INTRODUCTION

Dorsal root ganglia (DRG) contain the cell bodies of primary afferents of the somatosensory system. These neurons are functionally heterogeneous, signaling receptor-transduced stimuli of diverse sensory modalities that range from touch, temperature, and pain to proprioception. Nerve injuries that axotomize DRG neurons can destroy many neurons (37) as well as induce pronounced phenotypic and physiologic plasticity both within the DRG (26, 27) and within the spinal cord (33, 38, 62). These injuries lead to abnormalities in sensory encoding resulting in the development of neuropathic pain syndromes (2, 9, 61). One example of nociceptive plasticity correlates allodynia with the sprouting of “touch-sensitive” A β fibers to pain-encoding neurons within spinal cord laminae I and II. This occurs following nerve damage or the selective destruction of pain-transmitting C fibers and their projections to this region using the neurotoxin capsaicin (38, 56). Interestingly, this plasticity appears to be temporary (26, 63), suggesting that compensatory changes can reestablish appropriate sensory encoding. One unexplored possibility is that new sensory neurons are generated.

In adult rat DRG, Ljungberg *et al.* (37) observed an inexplicable increase in neuronal numbers following spinal nerve axotomy and subsequent infusion of nerve growth factor (NGF) or neurotrophin-3 (NT-3) at the proximal nerve stump of adult rat. Moreover, Ji *et al.* (28) reported that following sciatic nerve axotomy, mRNA for the mitogen basic fibroblast growth factor (bFGF) is upregulated in DRG neurons. Since bFGF proliferates neuronal precursors and stem cells of the CNS (51, 60) and the DRG-generating neural crest cells of the PNS (45), upregulated bFGF may also contribute to a neurogenic response in DRG following axotomy.

The adult mammal, including humans, contains neural progenitors in various regions of the CNS (13, 15, 21, 23, 29, 51). In adult rat dorsal root ganglia, the first evidence for the existence of neurogenesis and, hence, the presence of neural progenitors, was based

on counting methods (11). While this observation was later challenged (34), other analyses support continuing neurogenesis into adulthood (5, 50). Obvious functions of neurogenesis would be to add sensory neurons during growth (50) or to replace those that die following nerve injury (37, 47).

We have undertaken studies of early postnatal mouse DRG in culture to provide evidence of neural precursors, precursor proliferation, and neurogenesis. Some of these results have been previously reported in abstract form (46).

EXPERIMENTAL PROCEDURES

All efforts were made to minimize animal suffering and to reduce the number of animals used. DRG from early postnatal (day 1 or 2) male/female CD1 mice were extracted, minced, and then enzymatically dissociated using 1% trypsin (Gibco) and DNase (10 mg/ml; Boehringer Mannheim) added to Ham's F14 medium (1.58 ml total volume; Gibco) at 37°C for 30–40 min. The reaction was stopped by adding 0.5 ml fetal bovine serum (Gibco) and the tissue was triturated. The DRG-containing medium was then collected and centrifuged in a total volume of 10 ml medium at 250g (1500–1700 rpm) for 5 min. The supernatant was removed and the cell pellet was resuspended in 10 ml medium and centrifugation repeated. The supernatant was again removed and the remaining pellet of cells was resuspended in 4 ml medium and then plated at a fixed cell density on collagen-coated 35-mm petri dishes (Corning) or collagen-coated eight-well plates (Corning) in the presence of culture medium consisting of Ham's F14 or DMEM F12 (Gibco). Collagen solution for coating culture dishes and wells was prepared from adult rat tails (14). Rat-tail collagen was used at a concentration of 50 μ g/ml. Twenty-four hours prior to a culture experiment, and under sterile conditions, culture dishes or wells were coated with 0.1–0.6 ml rat-tail collagen (1:4 dilution in 60% ethanol) and allowed to dry in a culture hood overnight. The dishes were then rinsed twice with 1 ml sterile deionized water and dried and were ready for use.

Unless otherwise stated, in each experimental series, cells were plated at fixed concentrations that ranged from 1600 to 2600 cells per well. The serum-free medium used for the majority of experiments was composed of either Ham's F14 or DMEM F12 supplemented with N2, pyruvate, and glutamine (at 10 ng/ml), if proliferation experiments were being conducted, or with B27, pyruvate, and glutamine (at 10 ng/ml) if differentiation experiments were being conducted. In some experiments 10 ng/ml NGF and 10% horse serum were added to replicate a commonly used medium for culture of DRG neurons (e.g., 7). Under all culture conditions, the medium was changed every third day

by gently removing old medium and adding a fixed volume of new medium to each well.

In some experiments, the thymidine analog bromodeoxyuridine (BrdU) was added at a concentration of 1 μ M to detect DNA synthesis as an assay for cell proliferation. Immunohistochemical procedures were used to detect BrdU and the neuron-specific markers neurofilament-160 (NF-160), microtubule-associated protein-2 (MAP-2), and panaxonal. In order to assess the proliferative capacity of cultured postnatal DRG, the mitogens bFGF and epidermal growth factor (EGF; Upstate Biotechnology) as well as the putative mitogens NGF (Alomone) and sonic hedgehog (Ontogeny) were added individually to serum-free medium for 6 weeks, all at a concentration 10 ng/ml (see Fig. 6 for further details). All primary cultures were fixed in 4% paraformaldehyde (15 min) for subsequent immunohistochemical analysis.

Quantification analysis. Prior to counting, criteria were established for neuronal identification. In order to be counted as a neuron, cells had to display clear immunoreactivity for NF-160 and/or MAP-2, have a spherical cell soma, and possess long axon-like processes. A combination of two neuronal immunomarkers was usually used in order to identify neurons of all size ranges (25, 44, 53). Neuron counts were conducted utilizing a 10 \times fluorescence objective on an Eclipse800 Nikon microscope connected to the NeuroLucida image analysis system (MicroBrightField, VT), which includes a motorized stage controller and marking system that ensures an accurate counting of all neurons in each well examined. All values are presented as means \pm SE.

Electrophysiology. Cultures were perfused with HEPES-buffered recording solution consisting of (in mM): NaCl, 150; KCl, 5; HEPES, 10; MgCl₂, 1; CaCl₂, 2; glucose, 10; at pH 7.4. Whole-cell voltage clamp recordings were obtained using an Axopatch 1D amplifier (Axon Instruments). Patch electrodes were filled with a solution containing (in mM): K-gluconate, 140; ethylene glycol-bis(β -aminoethyl ether)-*N,N,N',N'*-tetraacetic acid, 11; KOH, 35; HEPES, 10; CaCl₂, 1; adenosine 5'-triphosphate, 2; and guanosine 5'-triphosphate, 4. The pH was adjusted to 7.3. Data were acquired and analyzed using pCLAMP (v 6.0; Axon Instruments).

Immunohistochemistry. During immunohistochemical processing, tissue was fixed in 4% paraformaldehyde for 15 min and then washed (6 \times 20 min) in phosphate-buffered saline with Triton X-100 (PBST). Primary antibodies were then added in conjunction with horse or sheep serum (1:100) and allowed to incubate for 2 days at 4°C, then washed (5 \times 20 min) with PBST. Tissue was then incubated in secondary antibody in conjunction with horse or sheep serum (1:100) for 1.5 h and then washed (2 \times 20 min) with

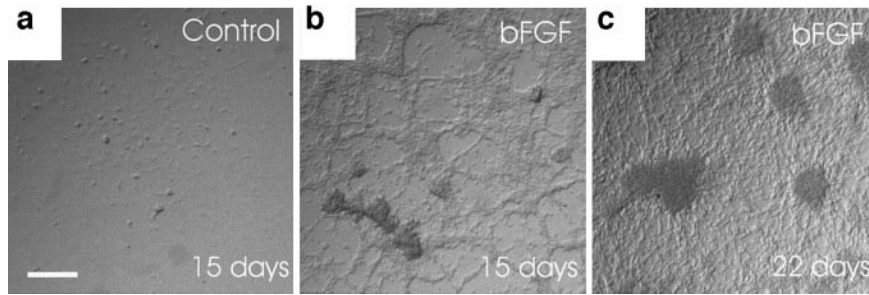


FIG. 1. bFGF supports robust cell proliferation. DRG were cultured in serum-free medium in the absence (a) and in the presence of bFGF (b and c). In this experiment collagen-coated flasks contained 8 ml of serum-free medium containing 10 ng/ml bFGF and were cultured at an initial density of 5000 cells/ml. Scale bar, 250 μ m.

PBST. The immunohistochemical processing for BrdU was similar to that described by others (59): Following two washes in PBST, the tissue was incubated in a final mixture containing 50% formamide and (2 \times) saline sodium citrate (SSC) for $\frac{1}{2}$ h at 65°C. It was then washed for 5 min in (2 \times) SSC at room temperature before incubation (30 min, 37°C) in 2 N HCl, followed by a 10-min rinse with 0.1 M sodium borate buffered to a pH of 8.5. It was then washed (5 \times 20 min) with Tris-buffered saline followed by a 30-min wash in Tris-buffered saline with 0.1% Triton X-100 (TBST) and 3% horse serum, before being incubated overnight in sheep anti-BrdU (Fitzgerald; 1:100) and sheep serum (1:100). After this incubation period, the tissue was washed (3 \times 30 min) with TBST and incubated in the secondary antibody (anti-sheep IgG FITC; Jackson; 1:50) for 1.5 h. Finally, it was washed (1 \times 20 min) in TBST and 50 mM Tris-HCl (2 \times 20 min) before being cover-slipped with Vectashield mounting medium containing 4',6-diamidino-2-phenylindole (Vector Laboratories, CA).

The following primary and secondary antibody combinations were also used for immunohistochemical processing of cultured tissue: mouse anti-NF-160 (Sigma; 1:100) and donkey anti-mouse CY3 (Jackson; 1:250), mouse anti-MAP-2 (Sternberger; 1:1000) and donkey anti-mouse CY3 (Jackson; 1:250), and mouse panaxonal (Sternberger; 1:1000) and donkey anti-mouse CY3 (Jackson; 1:250).

RESULTS

When DRG were cultured in serum-free medium, few cells survived plating in the absence of bFGF after 15 days (Fig. 1a); however, in the presence of bFGF, cells attached to the collagen-coated substrate and proliferated, to form clusters after 15 days (Fig. 1b). By day 22, these cells completely covered the bottom of the dish and cell clusters became more evident (Fig. 1c). Some spherical clusters lifted off the substrate and were suspended in the medium while others remained adherent to the culture dish substrate.

We examined whether the actions of bFGF included promoting the survival and/or proliferation of neural precursors that could then be induced to differentiate into neurons following the addition of trophic factors. To study the differentiation of putative neural precursors, DRG were first cultured for 7 or 14 days on collagen-coated eight-well plates in the presence of serum-free medium enriched with bFGF. This was then followed by further culture for 4 days in the absence of bFGF but in the presence of various individual trophic factors: NGF, glial-derived neurotrophic factor (GDNF), ciliary neurotrophic factor (CNTF), brain-derived neurotrophic factor (BDNF), and NT-3. Control plates were fixed immediately after the 7- or 14-day bFGF incubation period and hence received no additional trophic support. Similar numbers of NF-160/MAP-2⁺ cells were observed after 7 days in bFGF alone as well as after further incubation in trophic factors (Figs. 2a, bottom, and 2b1). In comparison, NF-160/MAP-2⁺ cells were rarely detected in these control plates after 14 days in bFGF (Fig. 2a, top). However, significant increases in NF-160/MAP-2⁺ cells were observed in wells with subsequent addition of trophic factors after 14 days (Fig. 2a, top). These cells had a characteristic DRG neuron morphology and extensive process formation (Fig. 2b2–6). Wells with applied NGF appeared to contain the greatest number of neurons following both 7- and 14-day bFGF incubation periods. Thus, most neurons die when cultured for 14 days in serum-free bFGF-containing medium but bFGF sustains and may promote the proliferation of neural precursors that then differentiate into neurons following subsequent addition of trophic factors.

Whole-cell recordings were performed to determine whether the bFGF-sustained precursor cells induced to differentiate into NF-160/MAP-2⁺ cells with DRG morphology also displayed electrical properties characteristic of neurons. Dissociated DRG were cultured for 14 days in serum-free medium containing bFGF. As shown above, this procedure virtually eliminates the DRG neurons while sustaining (and possibly proliferating) neuronal precursors present at the time of plat-

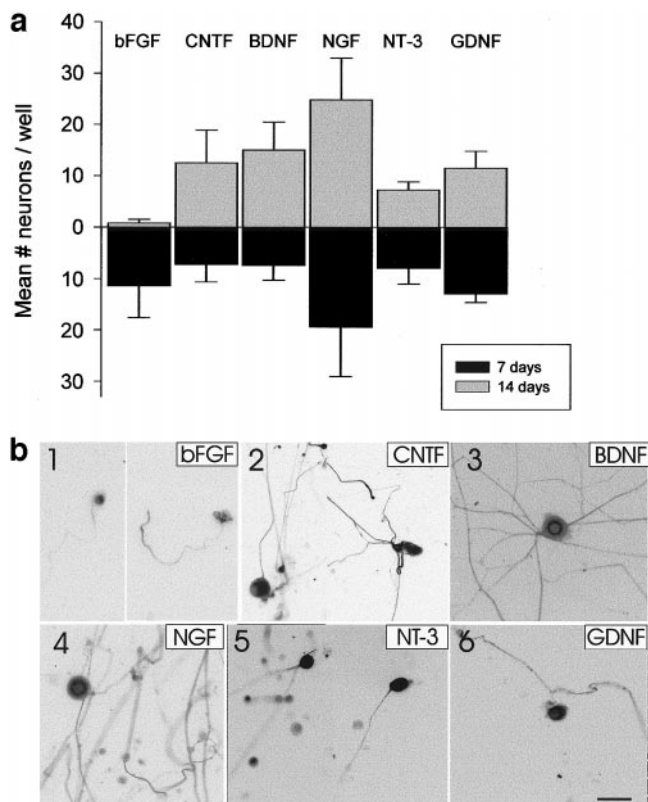


FIG. 2. Evidence that bFGF supports the survival and/or proliferation of neuronal precursors that then differentiate into neurons following addition of trophic factors. Comparison of effects of trophic factors on bFGF-sustained precursors from isolated DRG coimmunolabeled with MAP-2/NF-160. DRG cells were cultured in serum-free medium enriched with bFGF for 7 or 14 days followed by removal of bFGF and addition of a trophic factor to the medium for 4 days. Neurons with morphology characteristic of DRG neurons appeared. (a) While many neurons were still evident after 7 days in bFGF, only a few neurons survive culture in serum-free medium with bFGF after 14 days. However, subsequent addition of various trophic factors results in increased numbers of neurons ($P < 0.05$ except for CNTF, for which $P = 0.057$; Student's t test or rank sum test). (b) Examples of morphological phenotypes observed after 7 days in bFGF (b1) or after 14 days in bFGF followed by addition of trophic factors as shown for CNTF (b2), BDNF (b3), NGF (b4), NT-3 (b5), and GDNF (b6). Photographed images in b were converted to negative gray-scale bitmaps using CorelDRAW version 9. Scale bar, 50 μm .

ing. Then, bFGF was removed and NGF added for 4 days to induce differentiation. Cells displaying morphology typical of DRG neurons were targeted (Fig. 3a). All 17 cells recorded were spherical and possessed fine processes leaving the soma. The mean soma diameter of this group of neurons was $29.6 \pm 4.0 \mu\text{m}$, and mean resting membrane potential was -55 mV . In response to depolarizing current steps, 12 cells fired action potentials; 4 displayed repetitive firing (Fig. 3b). In response to depolarizing voltage steps, 16/17 cells displayed inward currents while all 17 generated outward currents. Addition of TTX at high concentrations

(5–7 μM) completely blocked the inward currents in only 1 of the 2 cells tested (Fig. 3c).

We next studied whether there was evidence of neurogenesis in a serum- and NGF-containing culture medium. This was undertaken for two reasons. First, *in vivo*, DRG are exposed to blood-borne elements (see 10) and also experience increases in NGF following axotomy (35). Second, NGF- and serum-containing media are commonly used for DRG culture (7), and we sought to replicate these conditions. BrdU was also added to the culture medium to identify proliferating cells. In these experiments, primary DRG cultures were divided into several groups and grown at a fixed density (15,000 cells/dish) on collagen-coated 35-mm dishes for 2, 7, 14, 21, and 28 days in a DRG culture medium containing serum and NGF with and without bFGF. Cells colabeled for BrdU and neuron-specific markers (NF-160/BrdU⁺ or MAP-2/BrdU⁺) were observed at all time periods sampled, suggesting that neurons were being generated in a “standard” DRG medium (Fig. 4a), as well as a group that also contained bFGF (Fig. 4b). Figure 4a depicts an example of a double-labeled cell (NF-160/BrdU⁺) that was cultured for 4 weeks in standard DRG medium. Morphologically, newly generated neurons were indistinguishable from preexisting, BrdU⁻ neurons.

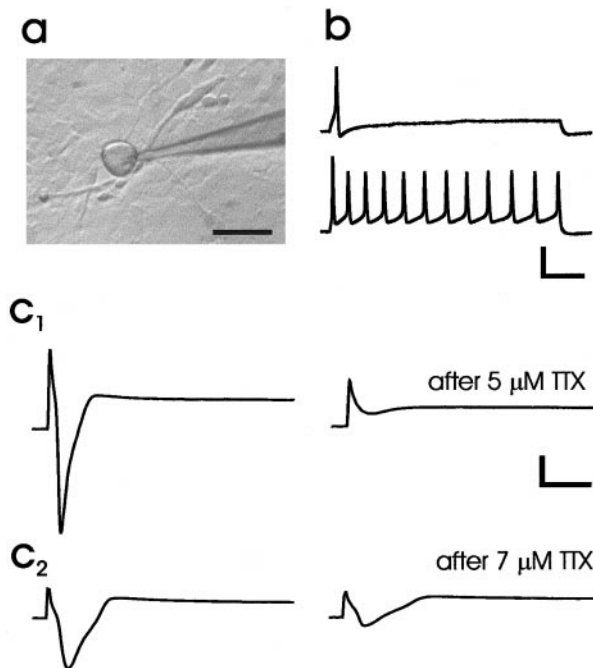


FIG. 3. bFGF-proliferated spherical cells with DRG neuron morphology are electroresponsive. (a) Example of spherical cell soma targeted for whole-cell recordings. A microelectrode is seen approaching the cell from the right. (b) In response to depolarizing current steps most neurons fired a single spike (top) but repetitive firing was also observed (bottom). (c) In voltage clamp, the inward currents were TTX-sensitive, confirming that voltage-gated Na⁺ channels contributed to the generation of action potentials. Scale bars: a, 50 μm ; b, 50 mV, 50 ms; c, 2 nA, 5 ms.

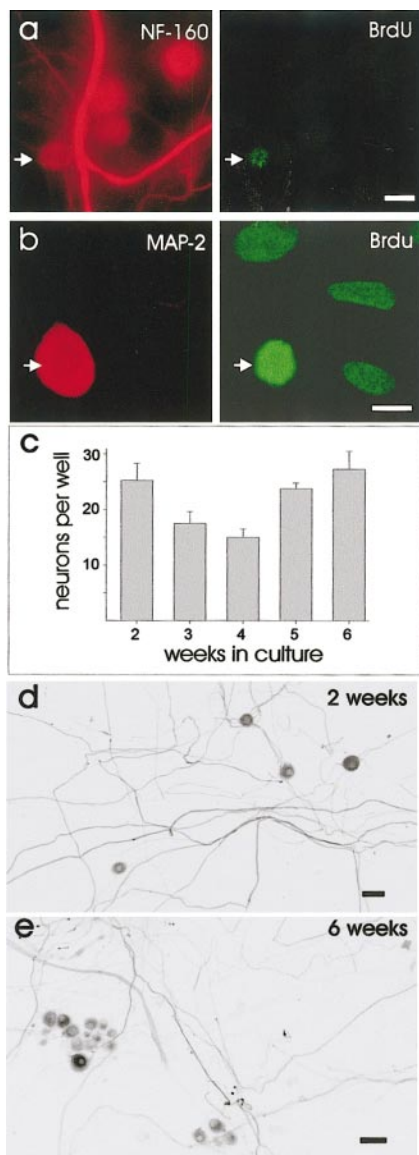


FIG. 4. Neurogenesis is observed in a standard culture medium that contains NGF. (a) A group of NF-160⁺ DRG neurons after 4 weeks in culture medium containing horse serum and NGF. Only one of these NF-160⁺ cells (left) is BrdU⁺ (right), identifying it as a newly generated neuron. Arrows denote the same cell. A short and thin neurite emanates from the cell soma but is not visible at this magnification. Note that several other nonneuronal cells with oval nuclei are also BrdU⁺. (b) MAP-2⁺/BrdU⁺ cell cultured for 1 week as above but also enriched with bFGF. Arrows denote same cell. A short and thin neurite emanates from the cell soma but is not visible at this magnification. Note that several other nonneuronal cells with oval nuclei are also BrdU⁺. (c) Histogram of the number of NF-160/MAP-2⁺ cells over time in NGF- and serum-containing medium. Each value represents the mean number of neurons \pm SE from four wells examined (d and e) Examples of NF-160/MAP-2⁺ neuronal staining following 2 (d) or 6 weeks (e) in culture. Photographed images in d and e were converted to negative gray-scale bitmaps using CorelDRAW version 9. Scale bars: a and b, 10 μ m; d and e, 50 μ m.

In order to quantify this apparent neurogenic effect, a similar procedure was undertaken on collagen-coated, eight-well plates in two separate experiments. Figure 4c presents the mean values (\pm SE). In the

standard DRG medium containing serum and NGF, \sim 25 neurons were observed per well 2 weeks after being plated, and neuron number decreased for the next 2 weeks in culture, after which neuron number increased in weeks 5 and 6 ($P < 0.01$; Student–Newman–Keuls multiple pairwise comparison; Fig. 4c). At all time periods, neurons displayed characteristic DRG morphology with extensive process formation (Figs. 4d and 4e).

The observation that neurogenesis occurred in the presence of NGF-containing serum in the absence of bFGF was unexpected (e.g., Fig. 4a). Surprisingly, we observed evidence of mitosis in putative neurons in NGF- and serum-containing medium, as demonstrated in Fig. 5. The neuron double labeled for MAP-2/BrdU in Fig. 5a appeared to be in metaphase. A clear example of a neuron with two BrdU⁺ nuclei is shown in Fig. 5b. Figures 5c1 and 5c2 depict two sets of BrdU⁺ neurons that appear to have just completed cytokinesis, and the neurons in Fig. 5c2 appear to be siblings migrating along axon tracts (cf. 40). Neurons having clear processes and two nuclei accounted for 3% of the total sample. The mean soma diameter of binucleate neurons was smaller ($28.4 \pm 1.4 \mu$ m) than that of neurons with only one nucleus ($37.8 \pm 1.4 \mu$ m; $P < 0.001$, Mann–Whitney rank sum test) consistent with the notion that NGF supports not only survival (8, 54) but also mitosis of small-diameter neurons, presumably via trkA receptor activation (6).

If NGF is acting as a mitogen and promoting neurogenesis, then neural proliferation should be inducible in a serum-free medium containing NGF. Thus, we compared the actions of NGF and other putative mitogens in serum-free medium. DRG were cultured for 6 weeks in flasks (120,000–135,000 cells/flask) containing 4 ml of serum-free medium alone or with NGF, sonic hedgehog (Shh), bFGF, or EGF, all at 10 ng/ml. The cell-containing medium was then transferred to collagen-coated eight-well plates for 4 days in the presence of various trophic factors (as in Fig. 2a). In two separate experiments after 6 weeks, NGF (Fig. 6a), Shh (Fig. 6b), bFGF (Fig. 6c), and EGF (Fig. 6d), but not serum-free medium alone, produced spherical cell clusters containing MAP-2/NF-160⁺ cells bearing processes in all trophic factors tested. Thus, many factors appear to proliferate neural precursors that can then differentiate into cells with a DRG neuronal phenotype.

DISCUSSION

Recent *in vivo* experiments have shown that following axotomy, mRNA for the mitogen bFGF is upregulated in DRG neurons (28), and axotomy also increases DRG neuron number in postmetamorphic bullfrog (18) and possibly adult rat (37). In our experiments in DRG cell culture, bFGF supports cell proliferation. Spheres

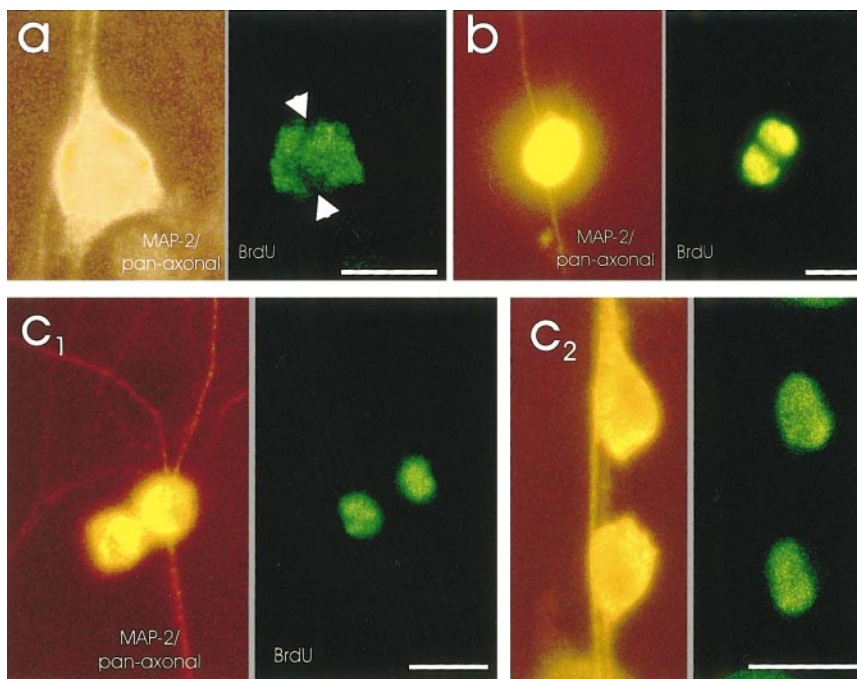


FIG. 5. Evidence that differentiated DRG neurons can undergo mitosis. (a) While DRG neurons are usually spherical, this neuron is spindle-shaped and its DNA, while blurred, appears to be in metaphase (arrowheads denote furrow). (b) Further example of a neuron displaying axons and containing two nuclei presumably in the telophase stage of cell division. (c1 and c2) Examples of pairs of neurons in close proximity that stain for BrdU. It is likely that these cells recently completed cytokinesis. The antibodies used are indicated. Scale bars, 10 μm .

of cells form in the presence of bFGF, and similar spheres can contain differentiating neurons in the presence of trophic factors. BFGF-derived progenitors can also be induced to differentiate into neurons with characteristic DRG neuron morphology and electrophysiological properties. These observations support the existence of neural precursors in postnatal DRG.

We demonstrated that following 2-week incubation in serum-free bFGF-containing medium few neurons survive. However, it is possible that many more neurons survive in an atrophic state but are MAP-2/NF160⁻ and hence undetectable and that they then reexpress neuronal markers following reintroduction of trophic support. If this were true, then perhaps there are no neural precursors. However, this is unlikely, for the following reasons: (i) After 1 week in bFGF-containing serum-free culture many DRG-like neurons are detected in an atrophied state (Fig. 2b1), whereas after 2 weeks virtually no MAP-2/NF160⁺ DRG-like neurons are seen. (ii) BFGF is responsible for the survival and proliferation of neuronal precursors, not the survival of neurons (31). (iii) During the embryonic period, bFGF does not promote and is not responsible for the survival of sensory neurons. BFGF is, however, responsible for survival of nonneural cells (30). (iv) BFGF is mitogenic with maximal effects at 10 ng/ml but must be removed before differentiation can take place (45). However, in the adult, bFGF may afford some protection against

axotomy-induced cell death following sciatic nerve lesion (48).

During development, the DRG are derived from precursors in the neural crest (24, 43) and neurogenesis ends several days before birth in both mouse and rat DRG (20, 32). In mouse DRG, Berg and Farel (3) reported that postnatal day 4 animals have more neurons than those observed at embryonic day 18. However, in this study it was impossible to determine whether continued neurogenesis occurred postnatally or, consistent with the other studies cited above, during late embryogenesis. Our experiments suggest that early postnatal DRG continue to house a population of neuronal precursors that retain their capacity for neurogenesis beyond embryological development. As there is suggestive evidence that mature DRG house neural precursors (5, 18, 50, 57), our findings also support the possibility that adult animals retain the capacity for neurogenesis.

Our results suggest that neuronal precursors can be instructed to differentiate into neurons following culture of mammalian postnatal DRG. The culturing of DRG neurons mimics phenotypic changes observed following axotomy *in vivo* (39). For example, substance P and CGRP levels are reduced *in vivo* following axotomy and following cell culture *in vitro* (27). As we demonstrate that the cell culture procedure can lead to the generation of new neurons, it is possible that neuro-

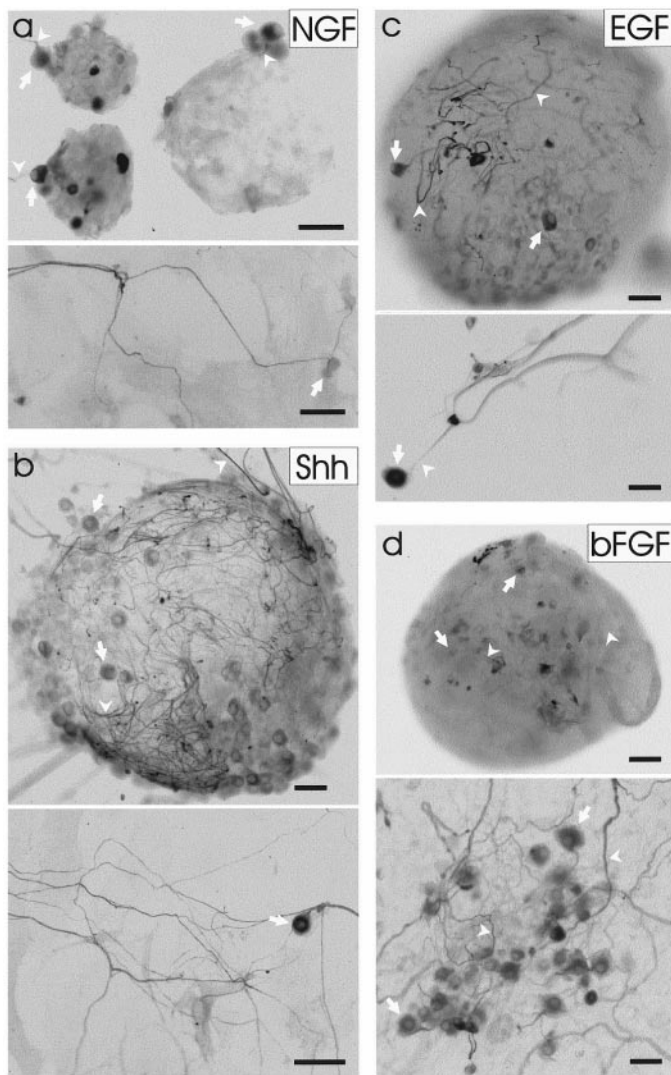


FIG. 6. Evidence of precursor proliferation and neurogenesis after long-term culture in serum-free medium. After 6 weeks culture in serum-free medium enriched with NGF (a) or Shh (b), bFGF (c) or EGF (d), the cell-containing medium was transferred to collagen-coated eight-well plates for 4 days in various trophic factors (cf. Fig. 2). Spherical cell aggregates were observed (top) to contain NF-160/MAP-2⁺ cells (arrows), some of which extended axon-like processes (arrowheads). In addition DRG-like neurons could be observed outside the spherical cell aggregates that adhered to the collagen-coated substrate and extended long axon-like processes (bottom). All photographed images are presented as negative gray-scale bitmaps. Scale bars, 50 μm .

genesis also occurs *in vivo* following nerve injuries that functionally axotomize DRG neurons. For example, in juvenile bullfrog, DRG house a population of immature, incompletely differentiated neurons that can be induced to differentiate into a mature form in response to injury (18).

The observation of BrdU incorporation in neurons from a NGF-containing DRG culture medium demonstrated that neurogenesis occurs in a commonly used

DRG culture environment, as it is highly unlikely that the BrdU labeling observed was due to DNA repair mechanisms (49). Moreover the apparent observation of mitotically active neurons in this environment supports the intriguing possibility that some neurons retain the capacity for mitosis. The ability of neurons to undergo cell division and proliferate in culture is not a novel concept (4). Because NGF supports the survival of nociceptors (8), and NGF activation of the *trkA* receptor can induce a mitogenic response (6), it is possible that it is the NGF in the culture medium that induces neurogenesis of these cells. This is supported by the observed formation of “neurospheres” after 6 weeks in NGF-containing serum-free medium. Moreover, Ljunberg *et al.* (37) reported unexplainable increases in DRG neuron numbers following spinal nerve transection with immediate application of NGF to the proximal stump. While NGF levels in DRG return to normal values within a few days following spinal nerve ligation (35), NGF mRNA increases fourfold after spinal nerve ligation, peaking after 1 day and lasting for at least 3 weeks (55). One source of NGF may be satellite cells that surround DRG neurons (35) since NGF is upregulated in DRG satellite cells following peripheral nerve injury (64), implicating this neurotrophin in a paracrine neurogenic response. It is also conceivable that upregulated neurotrophins work together with increased bFGF expression following axotomy (28) to proliferate DRG neurons.

Neurotrophic factors are essential for the survival and phenotypic differentiation of several subtypes of sensory neurons (1, 8, 16, 17, 19, 36, 42, 58). Hence, it was not surprising that they were also able to support the differentiation of cells into DRG-like neurons. Neurotrophic factors have also been shown to support the development of diverse sensory axon morphologies. For example, NGF increases axon length with minimal branching while NT-3- or BDNF-responsive neurons produce extensive branching (36). While not quantified, mitogen-proliferated neurons may respond to the addition of trophic factors in a similar manner.

C fibers derive from small-diameter sensory neurons that signal tissue damage from the periphery. These nociceptors possess features suggestive of a developmentally immature phenotype. For example polymodal nociceptors are unmyelinated, terminate in unspecialized free nerve endings, and retain long-duration action potentials (12, 22). Moreover, similarly small-diameter neurons continue to express bFGF mRNA in control adults (28) and we observed that that smaller diameter “neurons” were binucleate and appearing to undergo mitosis. For these reasons, C fibers are good candidate neurons to retain an ability to divide postnatally. The observation that differentiated mammalian DRG neurons appear to retain the capacity to divide is consistent with recent *in vitro* observations of bFGF-induced mitosis in hip-

pocampal neurons (4) as well as *in vivo* observations in the anterior subventricular zone of mitotic migrating TuJ1⁺ “neurons” destined to become olfactory bulb interneurons (41).

Our data also suggest that, in the presence of mitogens, postnatal DRG in culture are capable of proliferating neuronal precursors that can then be induced to differentiate into neurons. BFGF, EGF, NGF, and Shh supported the proliferation of cell clusters that could then produce cells that stain for neuron-specific markers with characteristic DRG morphology. The findings are consistent with findings in the adult mammalian CNS of mitogen-induced proliferation of neural progenitors that form cell clusters in culture that can then be induced to generate neurons (52, 60).

In conclusion, these findings may provide a greater range of plasticity available to somatosensory systems experientially and following growth, injury, and repair. Further studies are required to elucidate the cellular mechanisms that activate neurogenesis in postnatal DRG and to explore possible neurogenic repair responses of potential clinical importance.

ACKNOWLEDGMENTS

We are indebted to Eleanor Ling and Carolyn Gibbs for expert technical assistance. We thank Charles Buck, Tim Cope, Sandra Garraway, David Machacek, and K. W. Cheng for their comments on an earlier version of the manuscript. We also thank *Regeneron* for supplying NT-3, BDNF, and CNTF; *Amgen* for supplying GDNF; and *Ontogeny* for supplying Shh. This work was supported by University of Manitoba Research Development Fund and Medical Research Council of Canada grants to S.H.

REFERENCES

- Acheson, A., J. C. Conover, J. P. Fandl, T. M. Dechiara, M. Russell, A. Thadani, S. P. Squinto, G. D. Yancopoulos, and R. M. Lindsay. 1995. A BDNF autocrine loop in adult sensory neurons prevents cell death. *Nature* **374**: 450–453.
- Bennett, G. J. 1994. Neuropathic pain. Pages 201–224 in P. D. Wall and R. Melzack, Eds., *Textbook of Pain*. Churchill Livingstone, Edinburgh.
- Berg, J. S., and P. B. Farel. 2000. Developmental regulation of sensory neuron number and limb innervation in the mouse. *Brain Res. Dev. Brain Res.* **125**: 21–30.
- Brewer, G. J. 1999. Regeneration and proliferation of embryonic and adult rat hippocampal neurons in culture. *Exp. Neurol.* **159**: 237–247.
- Ciaroni, S., T. Cecchini, R. Cuppini, P. Ferri, P. Ambrogini, C. Bruno, and P. Del Grande. 2000. Are there proliferating neuronal precursors in adult rat dorsal root ganglia? *Neurosci. Lett.* **281**: 69–71.
- Cordon-Cardo, C., P. Tapley, S. Q. Jing, V. Nanduri, E. O'Rourke, F. Lamballe, K. Kovary, R. Klein, K. R. Jones, and L. F. Reichardt. 1991. The trk tyrosine protein kinase mediates the mitogenic properties of nerve growth factor and neurotrophin-3. *Cell* **66**: 173–183.
- Crawford, J. H., J. F. Wootton, G. R. Seabrook, and R. H. Scott. 1997. Activation of Ca²⁺-dependent currents in dorsal root ganglion neurons by metabotropic glutamate receptors and cyclic ADP-ribose precursors. *J. Neurophysiol.* **77**: 2573–2584.
- Crowley, C., S. D. Spencer, M. C. Nishimura, K. S. Chen, S. Pitts-Meek, M. P. Armanini, L. H. Ling, S. B. MacMahon, D. L. Shelton, and A. D. Levinson. 1994. Mice lacking nerve growth factor display perinatal loss of sensory and sympathetic neurons yet develop basal forebrain cholinergic neurons. *Cell* **76**: 1001–1011.
- Devor, M. 1996. Pain mechanisms. *Neuroscientist* **2**: 233–244.
- Devor, M. 1999. Unexplained peculiarities of the dorsal root ganglion. *Pain Suppl.* **6**: S27–S35.
- Devor, M., and R. Govrin-Lippmann. 1985. Neurogenesis in adult rat dorsal root ganglia. *Neurosci. Lett.* **61**: 189–194.
- Djohri, L., L. Bleazard, and S. N. Lawson. 1998. Association of somatic action potential shape with sensory receptive properties in guinea-pig dorsal root ganglion neurones. *J. Physiol. (London)* **513**: 857–872.
- Doetsch, F., I. Caille, D. A. Lim, J. M. Garcia-Verdugo, and A. Alvarez-Buylla. 1999. Subventricular zone astrocytes are neural stem cells in the adult mammalian brain. *Cell* **97**: 703–716.
- Elsdale, T., and J. Bard. 1972. Collagen substrata for studies on cell behaviour. *J. Cell Biol.* **54**: 626–637.
- Eriksson, P. S., E. Perfilieva, T. Björk-Eriksson, A. M. Alborn, C. Nordborg, D. A. Peterson, and F. H. Gage. 1998. Neurogenesis in the adult human hippocampus. *Nature Med.* **4**: 1313–1317.
- Ernfors, P., J. Kucera, K. F. Lee, J. Loring, and R. Jaenisch. 1995. Studies on the physiological role of brain-derived neurotrophic factor and neurotrophin-3 in knockout mice. *Int. J. Dev. Biol.* **39**: 799–807.
- Ernfors, P., K. F. Lee, J. Kucera, and R. Jaenisch. 1994. Lack of neurotrophin-3 leads to deficiencies in the peripheral nervous system and loss of limb proprioceptive afferents. *Cell* **77**: 503–512.
- Farel, P. B., and A. Boyer. 1999. Transient effects of nerve injury on estimates of sensory neuron number in juvenile bullfrog. *J. Comp. Neurol.* **410**: 171–177.
- Farinas, I., K. R. Jones, C. Backus, X. Y. Wang, and L. F. Reichardt. 1994. Severe sensory and sympathetic deficits in mice lacking neurotrophin-3. *Nature* **369**: 658–661.
- Fariñas, I., C. K. Yoshida, C. Backus, and L. F. Reichardt. 1996. Lack of neurotrophin-3 results in death of spinal sensory neurons and premature differentiation of their precursors. *Neuron* **17**: 1065–1078.
- Gage, F. H., J. Ray, and L. J. Fisher. 1995. Isolation, characterization, and use of stem cells from the CNS. *Annu. Rev. Neurosci.* **18**: 159–192.
- Gee, M. D., B. Lynn, S. Basile, F. K. Pierau, and B. Cotsell. 1999. The relationship between axonal spike shape and functional modality in cutaneous C-fibres in the pig and rat. *Neuroscience* **90**: 509–518.
- Gould, E., A. J. Reeves, M. S. Graziano, and C. G. Gross. 1999. Neurogenesis in the neocortex of adult primates. *Science* **286**: 548–552.
- Greenwood, A. L., E. E. Turner, and D. J. Anderson. 1999. Identification of dividing, determined sensory neuron precursors in the mammalian neural crest. *Development* **126**: 3545–3559.
- Hernandez, M. A., J. Avila, F. Moya, and C. Alberto. 1989. Rearrangement of microtubule associated protein parallels the morphological transformation of neurons from dorsal root ganglion. *Neuroscience* **29**: 471–477.

26. Himes, B. T., and A. Tessler. 1989. Death of some dorsal root ganglion neurons and plasticity of others following sciatic nerve section in adult and neonatal rats. *J. Comp. Neurol.* **284**: 215–230.
27. Hokfelt, T., X. Zhang, and H. Z. Wiesenfeld. 1994. Messenger plasticity in primary sensory neurons following axotomy and its functional implications. *Trends Neurosci.* **17**: 22–30.
28. Ji, R.-R., Q. Zhang, X. Zhang, F. Piehl, T. Reilly, R. F. Pettersson, and T. Hokfelt. 1995. Prominent expression of bFGF and its subcellular distribution in dorsal root ganglia after axotomy. *Eur. J. Neurosci.* **7**: 2458–2468.
29. Johansson, C. B., S. Momma, D. L. Clarke, M. Risling, U. Lendahl, and J. Frisén. 1999. Identification of a neural stem cell in the adult mammalian central nervous system. *Cell* **96**: 25–34.
30. Kalcheim, C. 1989. Basic fibroblast growth factor stimulates survival of nonneuronal cells developing from trunk neural crest. *Dev. Biol.* **134**: 1–10.
31. Kalyani, A. J., T. Mujtaba, and M. S. Rao. 1999. Expression of EGF receptor and FGF receptor isoforms during neuroepithelial stem cell differentiation. *J. Neurobiol.* **38**: 207–224.
32. Kitao, Y., B. Robertson, M. Kudo, and G. Grant. 1996. Neurogenesis of subpopulations of rat lumbar dorsal root ganglion neurons including neurons projecting to the dorsal column nuclei. *J. Comp. Neurol.* **371**: 249–257.
33. Koerber, H. R., K. Mirnics, P. B. Brown, and L. M. Mendell. 1994. Central sprouting and functional plasticity of regenerated primary afferents. *J. Neurosci.* **14**: 3655–3671.
34. La Forte, R. A., S. Melville, K. Chung, and R. E. Coggeshall. 1991. Absence of neurogenesis of adult rat dorsal root ganglion cells. *Somatosens. Mot. Res.* **8**: 3–7.
35. Lee, S. E., H. Shen, G. Tagliabatella, J. M. Chung, and K. Chung. 1998. Expression of nerve growth factor in the dorsal root ganglion after peripheral nerve injury. *Brain Res.* **796**: 99–106.
36. Lentz, S. I., C. M. Knudson, S. J. Korsmeyer, and W. D. Snider. 1999. Neurotrophins support the development of diverse sensory axon morphologies. *J. Neurosci.* **19**: 1038–1048.
37. Ljungberg, C., L. Novikov, J. O. Kellerth, T. Ebendal, and M. Wiberg. 1999. The neurotrophins NGF and NT-3 reduce sensory neuronal loss in adult rat after peripheral nerve lesion. *Neurosci. Lett.* **262**: 29–32.
38. Mannion, R. J., T. P. Doubell, R. E. Coggeshall, and C. J. Woolf. 1996. Collateral sprouting of uninjured primary afferent A-fibers into the superficial dorsal horn of the adult rat spinal cord after topical capsaicin treatment to the sciatic nerve. *J. Neurosci.* **16**: 5189–5195.
39. McMahon, S. B., D. L. H. Bennett, G. J. Michael, and J. V. Priestley. 1997. Neurotrophic factors and pain. Pages 353–379 in T. S. Jensen, J. A. Turner, and Z. Wiesenfeld-Hallin, Eds., *Proceedings of the 8th World Congress on Pain*. IASP Press, Seattle.
40. Memberg, S. P., and A. K. Hall. 1995. Dividing neuron precursors express neuron-specific tubulin. *J. Neurobiol.* **27**: 26–43.
41. Menezes, J. R., C. M. Smith, K. C. Nelson, and M. B. Luskin. 1995. The division of neuronal progenitor cells during migration in the neonatal mammalian forebrain. *Mol. Cell. Neurosci.* **6**: 496–508.
42. Molliver, D. C., D. E. Wright, M. L. Leitner, A. S. Parsadanian, K. Doster, D. Wen, Q. Yan, and W. D. Snider. 1997. IB4-binding DRG neurons switch from NGF to GDNF dependence in early postnatal life. *Neuron* **19**: 849–861.
43. Morrison, S. J., P. M. White, C. Zock, and D. J. Anderson. 1999. Prospective identification, isolation by flow cytometry, and in vivo self-renewal of multipotent mammalian neural crest stem cells. *Cell* **96**: 737–749.
44. Muma, N. A., H. H. Slunt, and P. N. Hoffman. 1991. Postnatal increases in neurofilament gene expression correlate with the radial growth of axons. *J. Neurocytol.* **20**: 844–854.
45. Murphy, M., K. Reid, M. Ford, J. B. Furness, and P. F. Bartlett. 1994. FGF2 regulates proliferation of neural crest cells, with subsequent neuronal differentiation regulated by LIF or related factors. *Development* **120**: 3519–3528.
46. Namaka, M., K. W. Cheng, E. Ling, and S. Hochman. 1999. Postnatal dorsal root ganglia generate new neurons in culture. *Soc. Neurosci. Abstr.* **25**: 1771.
47. Oliveira, A. L., M. Risling, M. Deckner, T. Lindholm, F. Langone, and S. Cullheim. 1997. Neonatal sciatic nerve transection induces TUNEL labeling of neurons in the rat spinal cord and DRG. *NeuroReport* **8**: 2837–2840.
48. Otto, D., K. Unsicker, and C. Grothe. 1987. Pharmacological effects of nerve growth factor and fibroblast growth factor applied to the transected sciatic nerve on neuron death in adult rat dorsal root ganglia. *Neurosci. Lett.* **83**: 156–160.
49. Palmer, T. D., A. R. Willhoite, and F. H. Gage. 2000. Vascular niche for adult hippocampal neurogenesis. *J. Comp. Neurol.* **425**: 479–494.
50. Popken, G. J., and P. B. Farel. 1997. Sensory neuron number in neonatal and adult rats estimated by means of stereologic and profile-based methods. *J. Comp. Neurol.* **386**: 8–15.
51. Ray, J., T. D. Palmer, J. Suhonen, J. Takahashi, and F. H. Gage. 1997. Neurogenesis in the adult brain: Lessons learned from the studies of progenitor cells from the embryonic and adult central nervous systems. Pages 129–149 in F. H. Gage and Y. Christen, Eds., *Isolation, Characterization and Utilization of CNS Stem Cells*. Springer, Berlin.
52. Reynolds, B. A., and S. Weiss. 1992. Generation of neurons and astrocytes from isolated cells of the adult mammalian central nervous system. *Science* **255**: 1707–1710.
53. Riederer, B. M., and I. Barakat-Walter. 1992. Differential distribution of two microtubule-associated proteins, MAP2 and MAP5, during chick dorsal root ganglion development in situ and in culture. *Brain Res. Dev. Brain Res.* **68**: 111–123.
54. Ruit, K. G., J. L. Elliott, P. A. Osborne, Q. Yan, and W. D. Snider. 1992. Selective dependence of mammalian dorsal root ganglion neurons on nerve growth factor during embryonic development. *Neuron* **8**: 573–587.
55. Shen, H., J. M. Chung, and K. Chung. 1999. Expression of neurotrophin mRNAs in the dorsal root ganglion after spinal nerve injury. *Mol. Brain Res.* **64**: 186–192.
56. Shortland, P., and C. J. Woolf. 1993. Chronic peripheral nerve section results in a rearrangement of the central axonal arborizations of axotomized A beta primary afferent neurons in the rat spinal cord. *J. Comp. Neurol.* **330**: 65–82.
57. St. Wecker, P. G., and P. B. Farel. 1994. Hindlimb sensory neuron number increases with body size. *J. Comp. Neurol.* **342**: 430–438.
58. Stucky, C. L., L. G. Abrahams, and V. S. Seybold. 1998. Bradykinin increases the proportion of neonatal rat dorsal root ganglion neurons that respond to capsaicin and protons. *Neuroscience* **84**: 1257–1265.
59. Tamatani, R., Y. Taniguchi, and Y. Kawarai. 1995. Ultrastructural study of proliferating cells with an improved immunocytochemical detection of DNA-incorporated bromodeoxyuridine. *J. Histochem. Cytochem.* **43**: 21–29.

60. Weiss, S., C. Dunne, J. Hewson, C. Wohl, M. Wheatley, A. C. Peterson, and B. A. Reynolds. 1996. Multipotent CNS stem cells are present in the adult mammalian spinal cord and ventricular neuroaxis. *J. Neurosci.* **16**: 7599–7609.
61. Woolf, C. J., and T. P. Doubell. 1994. The pathophysiology of chronic pain—Increased sensitivity to low threshold A β -fibre inputs. *Curr. Opin. Neurobiol.* **4**: 525–534.
62. Woolf, C. J., P. Shortland, and R. E. Coggeshall. 1992. Peripheral nerve injury triggers central sprouting of myelinated afferents. *Nature* **355**: 75–78.
63. Woolf, C. J., P. Shortland, M. Reynolds, J. Ridings, T. Doubell, and R. E. Coggeshall. 1995. Reorganization of central terminals of myelinated primary afferents in the rat dorsal horn following peripheral axotomy. *J. Comp. Neurol.* **360**: 121–134.
64. Zhou, X. F., Y. S. Deng, E. Chie, Q. Xue, J. H. Zhong, E. M. McLachlan, R. A. Rush, and C. J. Xian. 1999. Satellite-cell-derived nerve growth factor and neurotrophin-3 are involved in noradrenergic sprouting in the dorsal root ganglia following peripheral nerve injury in the rat. *Eur. J. Neurosci.* **11**: 1711–1722.