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Trophic factor and hormonal regulation of neurite outgrowth in sensory neuron-like 50B11 cells

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HIGHLIGHTS

- 50B11 cells share features with nociceptor neurons including neurite formation.
- We show that PGP9.5 (but not other markers) accurately defines their axon arbors.
- All differentiated cells co-express receptors for both NGF and GDNF.
- NGF and GDNF increase neurite outgrowth, as do angiotensin II and estrogen.
- 50B11 cells provide a potential tool for high throughput analysis of axonogenesis.

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ABSTRACT

Sensory axon integrity and regenerative capacity are important considerations in understanding neuropathological conditions characterized by hyper- or insensitivity. However, our knowledge of mechanisms regulating axon outgrowth are limited by an absence of suitable high-throughput assay systems. The 50B11 cell line generated from rat embryonic dorsal root ganglion neurons offers a promising model for screening assays. Prior characterization shows that these cells express cytoskeletal proteins and genes encoding ion channels and neurotrophin receptors in common with sensory nociceptor neurons. In the present study we further characterized 50B11 cells in regard to their phenotypes and responsiveness to neurotrophic and hormonal factors. 50B11 cells express neuronal cytoplasmic proteins including beta-3 tubulin, peripherin (a marker of unmyelinated neurons), and the pan-neuronal ubiquitin hydrolase, PGP9.5. Only PGP9.5 immunoreactivity was uniformly distributed throughout soma and axons, and therefore presents the best means for visualizing the entire axon arbor. All cells co-express both NGF and GDNF receptors and addition of ligands increased neurite length. 50B11 cells also showed immunoreactivity for the estrogen receptor-α and the angiotensin receptor type II, and both 17-β estradiol and angiotensin II increased outgrowth by differentiated cells. 50B11 cells therefore show features reported previously for primary unmyelinated nociceptor neurons, including responsiveness to classical neurotrophins and hormonal modulators. Coupled with their ease of culture and predictable differentiation, 50B11 cells represent a promising cell line on which to base assays that more clearly reveal mechanisms regulating axon outgrowth and integrity.

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1. Introduction

Peripheral sensory innervation is critical in transducing environmental information necessary for awareness and protection of the organism. Pathological loss of peripheral axons of sensory dorsal root ganglion (DRG) neurons underlies common forms of neuropathy affecting many patients including diabetics and cancer survivors receiving chemotherapy [20,40]. Conversely, abnormal proliferation of DRG axons occurs in inflammatory pain syndromes [8,35]. Thus, appropriate structural geometry of peripheral axons appears integral in ensuring optimal sensory function.

Abbreviations: ANGII, angiotensin II; AT2, angiotensin II receptor type 2; DRG, dorsal root ganglion; E2, estradiol or estrogen; ER, estrogen receptor; GDNF, glial cell line derived neurotrophic factor; NGF, nerve growth factor; PGP9.5, protein gene product 9.5; ir, immunoreactivity.

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Factors regulating DRG target innervation are incompletely understood, in part because we lack robust assay systems. Primary DRG cultures provide the principal means for assessing factors regulating axon outgrowth *in vitro*. However, these are limited by low throughput, cellular heterogeneity, and tedious preparation protocols. Attempts to simulate sensory outgrowth *in vitro* have included PC12 cells and neuroblastomas [30,31], but with limited success. Immortalized cell lines from rat, mouse and human DRGs include the F11 cell line which fused mouse hybridoma with rat embryonic DRG neurons [27,28], ND lines generated by fusing neonatal mouse DRG with neuroblastoma cells [39] and HD10.6 lines derived by incorporating a tetracycline-inducible *v-myc* oncogene into human embryonic DRG neurons [32]. While these lines are useful for electrophysiological, cell signaling and biochemical studies [12,13,39], none display axonal morphologies similar to primary cultures, thus limiting their use in studying axonogenesis.

Recently, Hoke and colleagues created the 50B11 cell line by electroporating E14.5 rat primary DRG neurons to incorporate the SV40 large T-antigen and human telomerase reverse transcriptase. These cells remain largely undifferentiated under standard culture conditions, but in the presence of forskolin assume neuronal properties [9]. They express features in common with small diameter, nociceptor neurons including axonogenesis and gene expression for some neurotrophin receptors and voltage-gated ion channels [9]. Accordingly, 50B11 cells hold promise as a model for studying axon growth. However, DRG axonogenesis involves interactions among trophic and modulatory factors acting on multiple receptors regulating cytoskeletal proteins, and it remains unclear how closely 50B11 cells replicate outgrowth in primary neurons. We show here that differentiated 50B11 cells display some phenotypic properties and responses to growth factors that are highly similar to DRG neurons.

2. Materials and methods

2.1. Cell culture, differentiation and treatments

50B11 cells, a gift from Dr. Ahmet Hoke, were plated in 6 or 24 well plastic tissue culture plates in Neurobasal medium (Life Technologies, Gibco) supplemented with FBS (Sigma–Aldrich), B27 (Life Technologies), glucose (Fisher) and glutamine (Sigma–Aldrich) [9]. Cells were plated at different densities including low densities optimal for visualizing individual neurite arbors. 24 h after plating cells were differentiated by adding forskolin (Sigma–Aldrich, 75 μM) to the medium. Based on observations by Chen et al. [9] and our preliminary studies, neuronal phenotype was most stable between about 20 and 36 h post-forskolin, and treatment protocols were designed to be completed within this time frame.

Seventeen hours after initiating forskolin-induced differentiation, cells were treated with nerve growth factor (NGF, 50 ng/ml recombinant mNGF, Peprotech), glial cell line-derived neurotrophic factor (GDNF, 50 ng/ml recombinant hGDNF, Peprotech), estrogen (17β-estradiol, 20 nM, Sigma–Aldrich) or angiotensin II (ANGII, 100 nM, Sigma–Aldrich). Cells were maintained for 20 h and fixed with 4% paraformaldehyde.

2.2. Immunostaining

Fixed cells were washed and incubated in blocking solution containing 1% BSA (Sigma–Aldrich) and 5% normal donkey serum (Millipore) in phosphate buffered saline (Sigma–Aldrich) containing 0.3% Triton X-100 (Sigma–Aldrich) for 1 h at room temperature, and immunostained for PGP9.5 (1:700, rabbit antiserum, Serotec), βIII-tubulin (1:400, mouse antiserum, Millipore), peripherin (1:200, chicken antiserum Millipore), TrkA (1:200, rabbit antisera, Millipore), GFRα1 (1:200, goat antiserum, R&D Systems), GFRα2 (1:200, goat antiserum, R&D Systems), estrogen receptor alpha (ERα) (1:200, rabbit antiserum, Santa Cruz Biotech), and ANGII receptor type 2 (AT2, 1:200, rabbit antiserum, Alamone Labs). Donkey IgG (1:200 to 1:400, Jackson Immunoresearch) tagged with Cy3 or Alexa 488 was directed against host primary antibodies. All antibodies were diluted in PBS containing 0.3% Triton X-100 and 5% normal donkey serum. Antibody specificities were confirmed by preabsorption and omission controls in our lab and others [2,3,7,8,35].

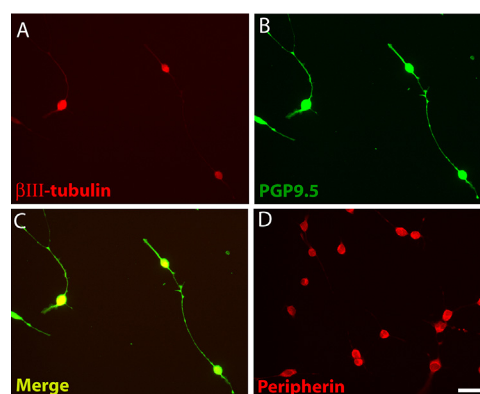

Fig. 1. Differentiated 50B11 cells show cytoplasmic proteins that may be useful in analyzing neurite outgrowth. (A) Staining for βIII-tubulin is prominent in the cell body but diminished in distal processes. (B) PGP9.5 immunostaining delineates soma and axons in good detail. (C) An overlay of A and B shows colocalization of these neuronal markers. (D) Peripherin immunohistochemistry shows strong expression in the soma but little in processes. Bar in D = 50 μm for all panels.

2.3. Quantitation of neurite outgrowth

For each treatment group in each experiment, 75–100 individual neurons were imaged. Neurons were counted from randomly collected images (about 15–20 per well). Single neurons with minimal or no overlapping of neurite arbors with adjacent cells were analyzed using NIH ImageJ software with the NeuronJ Plugin. Distances from soma perimeter to neurite tips were measured by tracing arbors, and expressed as the summed length of outgrowth and as length of the longest axon. All data are presented as mean ± the standard error of the mean, and treatment effects were compared by Mann–Whitney rank sum tests.

3. Results

3.1. Characterization of axonal markers in differentiated 50B11 cells

Differentiated 50B11 cells acquire many phenotypic features characteristic of DRG neurons including expression of the cytoskeletal proteins βIII-tubulin and hypophosphorylated neurofilament H [9]. We confirmed that 50B11 cells express βIII-tubulin, which was most intense in the cell body and fainter in the neurites (Fig. 1A). PGP9.5, a ubiquitin hydrolase expressed in all intact axons [22], has been used extensively for staining nerve fibers *in vitro* and *in vivo* [38]. PGP9.5-immunoreactivity (ir) was intense within cell bodies but also bright throughout the axons including finer processes (Fig. 1B). PGP9.5 showed consistent colocalization with βIII-tubulin within all differentiated cells (Fig. 1C), and lower expression in undifferentiated cells.

Peripherin is a type III intermediate filament protein that selectively marks unmyelinated axons *in vivo* and *in vitro* [15,16]. Expression in differentiated 50B11 cells was largely restricted to the soma (Fig. 1D) and weak in undifferentiated cells. When stained

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