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Neurite Outgrowth in Retinal Ganglion Cell Culture

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Summary

Retinal ganglion cells (RGC) are the projection neurons of the eye. The RGC is the primary cell type injured in a variety of diseases of the optic nerve, including glaucoma and optic neuritis. The most well-established extrinsic signal of RGC survival and axonal outgrowth is the neurotrophin brain-derived neurotrophic factor. An immunopurification system has been adapted in order to filter large enough quantities of RGCs from the mixed population of retinal neurons in order to perform high-throughput screening in a 96-well format. Using this assay, the screening of a combinatorial chemical library for compounds with a similar effect to brain-derived neurotrophic factor may be preformed. Follow-up validation studies are performed by evaluating for a dose–response relationship.

Key Words: Axon; BDNF; calcein AM; cellomics; chembridge; immunomagnetic; neuron; optic nerve; retinal ganglion cells.

1. Introduction

Retinal ganglion cells (RGCs) are the projection neurons of the eye, their axons extending along the inner retina to the optic nerve, coursing through the chiasm and optic tract, and synapsing on lateral geniculate neurons, which project to the visual cortex. The human optic nerve is made up of slightly over one million RGC axons, which are bundled by a meshwork of connective tissue septae containing small arterioles, venules, and capillaries. Other cellular components include astrocytes, oligodendrocytes provide myelination to retinal ganglion cell axons.

RGC axons might be injured along their course by a number of disease processes, which result in axonal degeneration, apoptosis of RGCs, and irreversible vision loss. RGCs are well suited for the study of axonal injury because of their accessible location outside the brain. From a clinical point of view, many RGC diseases have their initial insult at the axon level, including glaucoma, optic neuritis, anterior ischemic optic neuropathy, traumatic optic neuropathy, and compressive optic neuropathy. As such, the adoption of this model for the general study of axonal injury and regeneration has led to many insights, yet there has been no definitive impact on the clinical practices, which diagnose and manage optic nerve diseases: neuro-ophthalmology and glaucoma.

When the optic nerve is injured at the orbital apex, the entire length of individual axons degenerate simultaneously as early as 3 wk and as late as 6 wk after injury *(1)* and death of RGCs ensues. The mechanism by which RGCs die is programmed cell death or apoptosis, which is observed in both clinical *(2)* and experimental *(2,3)* optic neuropathies. Why do RGCs die following injury? Although the decisive event triggering RGC apoptosis after axotomy is not known, the downstream molecular signaling pathways involved in RGC apoptosis have been an

From: *Methods in Molecular Biology, vol. 356:*
High Content Screening: A Powerful Approach to Systems Cell Biology and Drug Discovery
Edited by: D. L. Taylor, J. R. Haskins, and K. Giuliano © Humana Press, Inc., Totowa, NJ

area of considerable investigation. Once activated, the highly regulated cell death pathway is generally launched in an all or none fashion. In the final stages, a series of proteases of the caspase family (cysteine proteases that cut after an aspartate residue) undergo aggregation and activation in a self-amplifying cycle called the caspase cascade. Inhibition of the caspase cascade at the time of optic nerve transection is protective against RGC death *(4)*. The caspase cascade might be initiated by cell surface receptors, such as the Fas ligand binding to Fas receptor. Patients with Leber's hereditary optic neuropathy causing mitochondrial mutations might be particularly susceptible to Fas *(5)*. The caspase cascade might also be initiated internally, when mitochondria are induced to discharge the electron carrier protein cytochrome-*c* into the cytosol, where it binds and activates an adaptor protein called Apaf-1.

Central to the apoptosis pathway in general and to RGCs in particular are the Bcl-2 family of intracellular proteins. Bcl-2 and Bcl-xL are important antiapoptotic genes that function in part by blocking release of cytochrome-*c* from the mitochondria. Other members of the Bcl-2 family, including Bad, Bax, and Bak, are proapoptotic, either inactivating antiapoptotic factors or stimulating the release of cytochrome-*c* from mitochondria. In the retina, the antideath gene Bcl-xL is the predominant Bcl-2 family member, and its level decreases after optic nerve crush *(4,6)*. In retinal ganglion cell culture, expression of Bcl-2 reduces cell death *(7)*. With the ability to modulate the signaling pathways in apoptosis comes the ability to dissociate survival from axonal regeneration.

Following axotomy, RGC axons fail to regenerate past the injury site *(8)*. This is thought to be because of a developmental loss of the intrinsic capability of RGCs to regenerate and a prohibitive environment of the glial scar and myelin. The extent to which the signals are controlled in the axonal outgrowth during development and axonal regeneration in adulthood overlap is not yet known. The loss of the intrinsic ability of RGCs to grow neurites occurs early in postnatal development is irreversible, and extrinsically signaled by amacrine cells *(9)*. Another important developmental change is the switch from axonal extension to the elaboration of the dendritic arbor *(10)*. This event is mediated by a calcium responsive transactivator called CREST (for calcium-responsive transactivator) *(11)* and NeuroD *(12)*.

In another development, it was reported that inhibition of the ubiquitin ligase anaphase-promoting complex (APC) in primary neurons specifically enhanced axonal growth in the developing rat cerebellum *(13)*. The APC is essential to the coordination of cell cycle transitions, particularly exit from the cell cycle, and is highly expressed in postmitotic neurons. In this study, APC was inhibited by means of small hairpin RNA knockdown Cdh-1, a gene that activates APC. This suggests a mechanism by which axonal outgrowth might be under the developmental influence of cell cycle exit.

The most well-established extrinsic signal of RGC axonal outgrow is the neurotrophin brain-derived neurotrophic factor (BDNF). BDNF interacts with the Trk B receptor and signals through the ras-raf-MAP kinase and the phosphatidylinositol-3-kinase pathways *(14–16)*. With the development of the ability to dissociate cell survival from neurite outgrowth signaling by introducing Bcl-2 into RGCs, it was shown that BDNF, as well as electrical activity, specifically promote axonal outgrowth *(7)*. How well will a small molecule modulate RGC survival? BDNF and forskolin synergistically promote RGCs in culture. Forskolin increases intracellular cAMP by stimulating adenylate cyclase and subsequent activation of protein kinase A. The role of cAMP in synergistically modulating response to BDNF signaling has been shown to occur by translocation of TrkB receptors from the intracellular compartment to the membrane surface, thus making cells more responsive to BDNF *(17)*. The use of a high-throughput screen to identify potentially therapeutic compounds has been successfully used in neuronal cell cultures, and more specifically in retinal cell cultures. In a search for molecules that might be effective in the treatment of Huntington's disease, high-throughput screen of a small molecule library successfully identified a novel molecule that supported Huntington's disease neurons in cell culture, inhibits polyglutamine aggregation, and suppress neurodegeneration in vivo *(18)*.

In retinal cell culture, Leveillard et al. ***(19)*** demonstrated the power of this approach, when they screened a retinal expression library in chicken retinal cultures for a cone survival factor that might be effective in the treatment of retinal degeneration. For this study, an expression library from wild-type mouse neural retina was constructed in pcDNA-3. The library was collected into pools containing approx 100 clones each and introduced into COS-1 cells for a total of 2100 pools of 100 clones each. After successfully screening those 2100 pools for the cone survival phenotype, positive pools were diluted into subpools of 10 clones and finally tested as individual clones. The novel survival factor was named rod-dependant cone viability factor-1 (RdCVF1). RdCVF1 rescues cones in chicken cultures as well as cones from a mouse with retinitis pigmentosa (rd1 mouse). Expression of RdCVF1 is restricted to the retina. It is expressed by rods and its expression is markedly reduced following retinal degeneration. Restoration of RdCVF1 following rod degeneration might provide a novel strategy for the treatment of retinitis pigmentosa and/or other retinal degenerations. Because of that time, the library has been sequenced in collaboration with Novartis and is presently being organized into a set of individual clones that should make it more versatile.

Following identification of a drug that promotes growth of RGCs in culture, several hypothesis driven questions follow. One might hypothesize that the factor influences the competence of the cells to respond to neurite promoting cues or alternatively stimulate the cell directly, either by influencing known signaling pathways or electrical activity. This might be examined by culturing cells from animals at different ages in the presence of the factor and determining the percent of outgrowth neurons (*see* **Note 1**). One might hypothesize that it influences survival to a greater degree neurite extension and examine whether the factor promotes outgrowth in RGCs that are overexpressing Bcl-2, which allows cells to survive in culture without promoting axonal outgrowth ***(7)***. One might hypothesize that the factor might differentially modulate axonal vs dendrite promoting factor and assess this with immunostaining. One might hypothesize that the factor modulates a known signaling pathway, particularly if it is synergistic with known RGC growth factors. This can be tested in vitro with known inhibitors of MAPK kinase, phosphoinositide 3-kinase, or RhoA. In order to identify the drug target, one might biotinylate the compound followed by hybridization it to a proteome array ***(20)***. These are some of the lines of inquiry that might be pursued following drug identification.

A convergence of technologies in robotics, cell culture techniques, use of fluorescence in cell culture, digital imaging, image analysis, and combinatorial chemistry has made the outlined strategy possible. As with many of the recent technology driven advances in science, which have allowed broader questions to be asked, this represents a development of scale and proportion rather than a completely novel paradigm. That is to say, the underlying principle of identifying specific molecules in cell culture that promote cell survival that led to the discovery of BDNF and ciliary neurotrophic factor (CNTF) is no different from the strategy being employed in this proposal.

The screening of a combinatorial chemical library is highlighted by the NIH Roadmap and offers a distinct translational benefit. The development of such libraries is fundamental to the emerging field of chemical genomics. Chemical compounds used by biological systems represent a exceedingly small fraction of the total possible number of small carbon-based compounds with molecular masses in the same range as those of living systems ***(21)***. In terms of numbers of compounds, "biologically relevant chemical space" is only a tiny fraction of complete "chemical space" ***(21)***. The goal of chemical genomics is to identify small molecules that might be used as analogs of genetic mutations for studying mammalian systems.

Depending on the goals of the study one might wish to screen target oriented vs diversity oriented libraries. The diversity of a library is a quantitative description of how different compounds are from each other. A target-oriented library is made up of a collection of small molecules that has a specific attribute thought to be of biological significance often targeting a specific protein. Screening a library of FDA approved drugs led the observation that β-lactams promote expression

of a specific target, the glutamate transporter, and were neuroprotective in an animal model of amyotrophic lateral sclerosis *(22)*. This has prompted the development of a collection of small molecules based on the β-lactam structure, that might have more potent activity. Diversity-oriented libraries are not targeted to any specific protein class and are often used in broad screens in which the target proteins are not known. Such libraries are made up of an assorted collection of small molecules based on a computational approaches used to sift through much larger numbers of more varied compounds thought to be biologically active. Although employing the Chembridge Diverset, a diversity oriented compound library, this assay can be applied to nonsmall molecule libraries. The retinal expression library described in the preceding section is of particular interest for vision science. Another library of significant interest is lentivirus-based RNA interference (RNAi) library that is being developed by the Viral shRNA Library Consortium, a joint effort of several research groups at the Whitehead Institute, the Broad Institute, the Dana Farber Cancer Institute, and the Harvard Institute of Proteomics *(23)*.

2. Materials

1. DNAse (0.4%; 12,400 U/mL).
2. Poly-D-lysine (dilute 1/100 leads to a concentration of 0.01 mg/mL) (Sigma, cat. no. P-6407).
3. L-cysteine.
4. Papain (Worthington Biochemical Corporation, Lakewood, NJ).
5. Ovomucoid solution (10X; 40 mL).
   a. To 40 mL DPBS, add 600 mg of BSA (Sigma, St. Louis, MO; cat. no. A8806), 600 mg of Trypsin.
   b. Inhibitor (Roche Basil, Switzerland; cat. no. 109878), and adjust pH to 7.4 (add approx 400 µL of *N* NaOH).
6. Laminin (Sigma, cat. no. L-2020, maintain initially at –80°C).
7. Tris-buffer, 50 m*M*, (Sigma, cat. no. T1503).
8. Rat Serum: (Jackson Immunoresearch West Grove, PA; cat. no. 012000-120).
9. Mouse antirat Thy1 (Chemicon International, Temecula, CA; cat. no. MAB1406).
10. Mouse antirat cd11 b/c (BD Biosciences, Pharmingen, Bedford, MA; cat. no. 554859).
11. Calcein AM (Molecular Probes, Eugene, OR).
12. Hocest (Molecular Probes).
13. BDNF.
14. Forskolin.
15. Neurobasal medium.
16. Glutamine.
17. Penicillin/streptomycin.
18. Dynal microbeads.

3. Methods

3.1. Methods Overview

Our culture system, based on the work of Barres et al. *(24)* employs an efficient two-step immunomagnetic purification of the mixed cell population of cells from the postnatal rat retina to yield a highly enriched population of RGCs for culture. The first step consists of immunomagnetic depletion of Thy1 bearing non-RCGS using a cd11b/c antibody followed by a second step consisting of immunomagnetic selection of the RGCs with an antibody that binds Thy1 on the RGC cell surface. In the final step, the cells are released from the magnetic beads by digesting the DNA linker with a low concentration of DNAase. Cells are grown on poly-D-lysine/laminin coated plastic plates in a basic medium consisting of neurobasal media, B27 supplement, glutamine, and penicillin/streptomycin.

These cultures are highly enriched for RGCs, are morphologically typical of RGCs in cell culture, and reproducibly respond to BDNF and forskolin as expected for RGCs. To assess the efficiency of our selection technique, RGCs were retrograde labeled by bilateral transcranial injection of DiI into the superior colliculus 3 d before sacrifice. With this technique, 94.8 ± 1.3%

of the cells in culture were labeled. After 5 d in culture, the cells have developed a robust arbor of neurites typical of what has been reported for RGCs. Finally, the addition of BDNF and forskolin, factors known to promote RGC survival, to our basic culture medium results in increased overall cell survival and more significantly in the survival of neurite bearing cells.

We typically harvest between 18 and 20 retinas per experiment in a procedure which takes less than 5 h. Based on an estimate of 100,000 RGCs per retina, our yield is approx 50%. Thus, we typically harvest 1 million cells per experiment. We estimate our initial postseeding viability by staining with Hoechst (a blue fluorescent nuclear dye taken up by all cells) and TOTO3 (a far red fluorescent nuclear dye retained by dead cells). Our viability ranges from 90 to 95% using this technique.

3.2. Cell Source and Purification

Retinas are dissected from postnatal d 3–5 Sprague Dawley rats, digested with activated papain (Worthington) and DNase (Sigma) before dissociation by trituration. Immunomagnetic depletion of macrophages is performed by incubating the retinal cell suspension with magnetic microbeads (Dynal Biotech) conjugated to mouse antirat cd11 b/c antibody (BD Biosciences, Pharmingen) and removal by magnetic separation. This is followed by immunomagnetic selection of Thy1 antigen bearing RGCs by incubating the cell suspension with magnetic microbeads conjugated to mouse antirat Thy1 antibodies (Chemicon) and selection by magnetic separation. In a final step, the magnetic beads are cleaved from the cells by incubation in DNAse, which cuts the DNA linker between the magnetic bead and the antibody.

3.3. Culture Conditions and Controls

The purified Thy-1 immunoreactive cells are resuspended in growth media (neurobasal media, B27 supplement, glutamine [2 m*M*], and penicillin/streptomycin [1 U/mL]) and quantified on a hematocytometer. Cells are seeded in 100 µL at a density of 4000 cells/well (150/mm^2) in 96-well culture dishes (Falcon BD Biosciences, Bedford, MA) that had been sequentially coated with poly-D-lysine (0.01 mg/mL) and laminin (0.01 µg/µL) (Sigma). Each well either contains 25 µL of media alone or 5X drug or 5X BDNF and forskolin. Final concentrations are: DMSO (1%), drug (20–30 µ*M*), BDNF (50 ng/mL) and forskolin (10 µ*M*). Culture dishes are placed in an incubator maintained at 37°C and 5% CO_2. One hour after seeding, a postseeding viability is determined.

The Diverset (Chembridge, Inc. San Diego, CA) is a diversity-oriented library of synthetic small molecules picked from the Chembridge collection of 300,000 chemicals. The organizing criteria for the collection are diversity, heterocyclic, predicted bioactivity, low molecular weight, production through multistep large-scale manual synthesis, and over 95% purity. The entire Diverset collection consists of five groups of 10,000. The compounds are diluted to 3 m*M* in DMSO and individually arrayed in columns 2–11 on a 96-well plate, leaving columns 1 and 12 for controls. Negative controls are placed in columns 1 and 2, wells A–E. Positive controls, which consist of media supplemented with BDNF and forskolin are placed in columns 1 and 2, wells F–H. The plates are stored at –20°C until ready for use. The final concentration of drug is approx 20–30 µ*M*.

3.4. Staining and Imaging

After 120 h of incubation, cells are stained with Hoechst (5 µ*M*, Molecular Probes) and Calcein AM (10 µ*M*) (live cell stain; Molecular Probes). Following 30 min of incubation, each well is rinsed three times with PBS and imaged. In earlier studies, a third dye was used to stain dead cell nuclei (TOTO3). It was observed that it was a rare cell that could not be distinguished as "live" without calcein alone; rare cells stain robustly with both Calcein AM and TOTO3. Thus, in order to reduce scan time and simplify our protocol, TOTO3 was eliminated. However, TOTO3 is still used to determine viability because of low background without rinsing.

In a standardized fashion that is identical for each well, twenty stereotypic fields are autofocus imaged on the Cellomics KineticScan High Content Scan Reader using epifluorescence

and filter sets corresponding to each of the two dyes. Scanning the entire plate takes just over 3 h and collects 3840 images.

3.5. Image Analysis

Images are processed using the Cellomics, Inc., Extended Neurite Outgrowth program. In the initial image processing, cells are identified on the Hoechst channel and selected based on size such that clusters of cells might be excluded from analysis. In addition, each nuclei on the Hoechst channel is matched to a calcein stained cell and counted as a "valid neuron," which might be regarded as a live cell. Neurons can be further selected based on size in the calcein channel. The Extended Neurite Outgrowth program is used to trace neurites extending from these selected neurons. The mean neurite length, mean neurite count, mean number of branch points, and mean number of crosspoints are determined. In addition, the percent of cells with neurite features (i.e., neurite length, neurite count) surpassing a threshold of one standard deviation more than the mean neurite characteristic of a set of reference wells containing growth medium without additional growth factors is determined. The "neurite outgrowth" is based on the combined mean neurite length and mean neurite count. Cells surpassing a threshold of one standard deviation more than the mean neurite outgrowth index for control wells are regarded as "outgrowth neurons." The number of outgrowth neurons is the most reliable indicator of the presence of growth factors in our system and the primary end point of this assay, yet several other parameters are assessed as well.

3.6. Statistical Analysis

The outgrowth count for each well is normalized to counts from all wells except for positive controls. As studies are done in quadruplicate, a mean, and standard deviation is calculated for each compound and a z-statistic as an indicator or reproducibility calculated:

$$z = [1 - (3 \times \text{standard deviation of controls}) + (3 \times \text{standard deviation of drug})]/ (\text{mean of drug} - \text{mean of controls}).$$

Those drugs with the highest z-scores are regarded as the most reproducible.

3.7. Validation of Hits

Standard rationale for demonstrating a causal relationship between two factors is to modulate the level of function of one factor and correlate it with the desired outcome. The standard paradigm for studying gene functions is gain-of-function and loss-of-function manipulations. For small compound studies, the standard approach is a dose–response analysis. As such, one approach to the validation of hits is to demonstrate a dose–response relationship (*see* **Note 2**). This will demonstrate the underlying biological response. In addition, it will help to determine the potency of drugs so that the most potent drugs are identified. Although the developed technique for performing cell culture is effective and objective, it is desirable to assess ones outcome using a different technique. As such, the standard technique of culturing cells on poly-D-lysine and laminin-coated cover slips in 24-well culture dishes under various culture conditions followed by immunostaining with antineurofilament antibody might be performed followed by quantitative analysis.

As with genes in which precise functions can be further localized to specific domains, the essential bioactive element of small compounds can be determined. As with genes in which this involves site-directed mutagenesis and truncation, a combinatorial chemist can alter specific parts of a compound for testing in order to enhance the potency. Combinatorial chemistry is a group of methodologies developed by researchers in the pharmaceutical, agrochemical, and biotechnology fields to facilitate the production of effective new drugs. Combinatorial chemistry is used to create large populations of molecules that can be screened. By producing larger, more diverse compound libraries, the probability is increased that one might a find novel compounds of significant therapeutic value. The field represents a convergence of chemistry and biology, made feasible by fundamental advances in miniaturization, robotics, and receptor development.

For dose–response analysis, RGC harvest, and immunopurification is performed with the same technique as previously discussed. Drugs to be validated will be diluted to a final medium concentration of 3, 10, 15, 30, 60, 90, 120, and 150 μ*M* while controlling DMSO at 1% in all wells including media only and BDNF/Forskolin controls. Drugs and growth factor supplements are added, 25 μL to each well in a 5X concentration, before the seeding cells in a volume of 100 μL giving a final volume of 125 μL. Cells are incubated, stained, and imaged as stated earlier. Experiments are performed in duplicate. Following normalization to media only well, the dose–response is plotted using SPSS statistical software in order to perform curve fitting and analysis.

For each drug, resupply in quantities from 1 to 5 mg is available through online ordering from Chembridge, Inc. If larger quantities are needed, further drug can be synthesized. For validation studies, 1 mg of desiccated drug is resuspended in 22.2 μL of DMSO to a final stock solution of 150 m*M*. If the drug does not go into solution, the stock is further diluted until it is in solution. Afterwards, the drug is stored in the dark at –20°C until ready for use.

4. Notes

1. Phenotypic vs target end point. The use of a phenotypic end point, in this case "outgrowth neuron," as opposed to a target-based screening, in high-throughput analysis has the advantage of having a functional end point yet has the challenge of more difficult assay development and interpretation as well as downstream challenge of target identification.
2. What is a hit? The criteria of what constitutes a hit are important. The criteria should be broad enough to minimize type II error (failing to reject the null hypothesis when it is false), in which we might fail to select a particular compound for follow-up studies. Compounds selected for follow-up are those with an outgrowth count and *z*-score comparable to BDNF and forskolin as well as compounds that are the most robust in comparison with media alone. This might range from 0 to 3 per compound plate. Some of these are "false-positives" representing either an outlier or the tip in the distribution of outgrowth counts for a given plate. Others of course represent a "true-positives." Sorting out the false hits and the true hits are the reason for validation studies.

Acknowledgments

This work was supported by grants from the National Eye Institute, the Glaucoma Foundation, the Glaucoma Research Foundation, and the American Health Assistance Foundation, Research to Preven Blindness, Inc., the MICC, the Marriott Foundation, the William and Ella Owens Medical Research Foundation, and by a generous gift from Mr. and Mrs. Robert Smith.

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