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Journal of Neuroscience Methods 169 (2008) 34–42

JOURNAL OF NEUROSCIENCE METHODS

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A high-throughput screen to identify novel compounds to promote neurite outgrowth

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Received 26 September 2007; received in revised form 20 November 2007; accepted 20 November 2007

Abstract

Following spinal cord injury, a variety of inhibitory molecules hinder the success of axon regeneration. The motile tip of the axon, the growth cone, shares a similar cytoskeletal array as a migrating cell, and in general the cytoskeleton is regulated by a conserved set of signaling pathways that act downstream of guidance cue and growth factor receptors. We exploit these similarities by using migrating cells as a model system to screen for extracts that promote axon outgrowth. The screen is a high-throughput wound-healing assay performed by a 96-pin tool Biogrid robot where positive candidates are identified as extracts that stimulate complete wound healing. Testing of positive candidates on chick DRG explants has lead to the identification of extracts that promote neurite outgrowth on permissive and inhibitory substrates. Extracts can be fractionated to purity, identifying novel compounds that promote neurite outgrowth on inhibitory substrates.

Keywords: Growth cone motility; Cell migration; High-throughput assay

1. Introduction

Following spinal cord trauma numerous inhibitory molecules are expressed at the injury site and prevent axon regeneration. These inhibitors include chondroitin sulfate proteoglycan (CSPG) and myelin-associated inhibitors that inhibit axon regeneration by activating RhoA (Monnier et al., 2003; Niederost et al., 2002). Other inhibitors include a number of repulsive guidance molecules that are expressed in the glial scar (Silver and Miller, 2004; Yiu and He, 2006). Interventions to treat spinal cord injury include manipulations both extrinsically and intrinsically. Extrinsic manipulations include the removal of inhibitory molecules present in the glial scar using various enzymes and blocking peptides (Bradbury et al., 2002; Li et al., 2004; MacDermid et al., 2004; Yang et al., 2006). Intrinsic interventions involve the manipulation of signaling pathways in the neuronal growth cone. For example, neurotrophic factors are often used to activate Trk signaling pathways that promote neuronal survival and axon regeneration (Hiebert et al., 2002; Kobayashi et al., 1997; Ramer et al., 2002). Other more specific methods include blocking signaling pathways that act downstream of inhibitory molecules such as the RhoA kinase (Chan et al., 2005) and PKC pathway (Sivasankaran et al., 2004).

While most of the above interventions are focused on blocking signaling pathways that act downstream of inhibitory molecules, there are few interventions that aim to enhance axon outgrowth itself. Indeed, one of the major obstacles inhibiting axon regeneration is the reduced intrinsic capacity of mature axons to grow (Kobayashi et al., 1997). Treatments that enhance axon outgrowth then may be useful to improve the success of axon regeneration. To identify novel compounds that can stimulate axon outgrowth, we focused our screen specifically on the basic mechanism underlying axon outgrowth. Briefly, axonal growth is initiated by a specialized structure at the tip of growing axons known as the growth cone. The growth cone consists of three basic regions: peripheral (P), intermediate (I) and central (C) regions (Dent and Gertler, 2003). The P region is the leading edge of the growth cone consisting of dynamic meshworks and bundles of F-actin that support lamellipodial and filopodial protrusions. Towards the center (C region) of the growth cone,

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0165-0270/$ – see front matter © 2007 Published by Elsevier B.V.
doi:10.1016/j.jneumeth.2007.11.013

the dynamic F-actin structures give way to more stable structures supported mainly by microtubules. The transition between the P and C regions is the intermediate zone where F-actin and microtubules interact. As the P region advances through F-actin polymerization, microtubules invade into the intermediate zone where the disassembly of F-actin results in axon consolidation (Dickson, 2001). The balance of F-actin assembly and disassembly governs the rate of axon growth or retraction, and this balance is regulated by Rho family GTPases that act downstream of guidance cues (Dickson, 2001; Luo, 2002). For example, attractive guidance cues activate Rac and cdc42 to enhance actin assembly that results in growth cone advance and subsequently axon outgrowth (Dickson, 2001; Gallo and Letourneau, 2002), while repulsive guidance cues activate RhoA to stimulate actin disassembly that results in growth cone collapse and subsequent axon retraction (Dickson, 2001; Gallo and Letourneau, 2002).

A conventional method for identifying novel compounds that can enhance axon outgrowth is to test the effects of these compounds on primary neuronal cultures. One of the most popular and well-characterized systems for axon outgrowth assays is the chick dorsal root ganglion (DRG) explant. Although this model is often feasible for testing a small sample of drugs, the low efficiency of this type of screen due to laborious preparation and low throughput, limits the likelihood of identifying a positive candidate. To increase the probability of finding axon outgrowth-promoting compounds, we devised a high-throughput screen using cell motility as a general model for axon outgrowth. The rationale of this approach is that many of the basic mechanisms underlying axon outgrowth are also conserved in the motile cell. Specifically, both systems share a dynamic leading edge consisting of filopodial and lamellipodial protrusions, and a trailing edge where F-actin meshworks and bundles are disassembled (Dickson, 2001). Furthermore, the balance of F-actin assembly and disassembly in both systems is regulated by Rho family GTPases (Dickson, 2001). With these similarities in mind, we designed a primary high-throughput wound healing assay and a secondary axon outgrowth assay to identify compounds that stimulate motility. Specifically, we used a 96-pin tool robot to perform a scratch assay in 96-well plates containing confluent HEK 293 cells and added extracts from marine sponge and microbial library. Extracts that promote complete reinvasion of cells into the scratch are considered positive candidates, and were further tested on dorsal root ganglion (DRG) explants cultured on various substrates. Using this two-part screen, we have demonstrated that positive candidate extracts identified in the high-throughput screen can promote axon outgrowth even on inhibitory substrates. Furthermore, purification of extracts can yield specific compounds that promote robust neurite outgrowth on permissive and inhibitory substrates.

2. Materials and methods

2.1. Cell cultures

HEK 293 cells were cultured to confluency in 100 mm cell-culture plates (Falcon) in DMEM supplemented with 5% FBS (Sigma). Once confluent, the cells were mechanically sheared off the plate and collected in fresh medium. The cells were then plated in 96-well plates (Falcon) at a density of 100,000 cells per well, and incubated at 37 °C in 5% CO_2 for 24 h to allow the cells to reach confluency.

2.2. High-throughput migration assay

The high-throughput migration assay was performed with a Biogrid robot, which consists of a 96-pin tool, and designated racks where 96-well plates containing the extracts (source) and the cells (destination) can be loaded. Confluent 96-well plates were first loaded into the 'destination' racks (Biogrid), and were subjected to automated scratching and extract transfer procedures. For the automated scratching procedure, the following settings were employed. Briefly, the 0.7 mm 96-pin tool is set to insert into the destination plate and to stop at the height of 0 mm where the pins touch the bottom of the plate. Upon contact with the plate, the tool wiggles for 5 s to make a scratch in the confluent layer of cells. Immediately following the scratch, 96-well plates containing an array of marine sponge extracts were loaded into the 'source' racks. To add extracts directly from the library (source) to the cells (destination), the 0.7 mm 96 pin tool picks up 30-μL droplets per pin of extract from each well of the source plate, and transfers the droplet into the corresponding wells in the destination plate. At the destination plate, the pin stops at the height of 3 mm and wiggles for 5 s to gently stir in the extracts into the cell-culture medium. Immediately after adding the extracts, the cells were incubated for 18 h at 37 °C in 5% CO_2 to allow cell reinvasion back into the scratch. The cells were then fixed in 3.7% formaldehyde and imaged using an inverted microscope.

The wells were screened manually to identify positive extracts that promote cell migration. In the future, this step could be automated and done with relative ease. Based on preliminary experiments, we found that HEK 293 cells fail to completely reinvade into the scratch area following a time period of 18 h. In addition, we found that cell proliferation had little impact within this short-time period. We identified positive extracts as those that can drive complete cell reinvasion into the scratch within the 18-h period. To ensure consistency in our results, the screens for each plate of extracts were repeated at least three times.

2.3. DRG explants

Dorsal root ganglia were isolated from embryonic days 8 and 14 chick embryos, and cultured in DMEM (Sigma–Aldrich; Oakville, ON) supplemented with 10% FBS and 50 ng/mL NGF (Invitrogen; Burlington, ON). The explants were cultured on glass coverslips coated with either poly-L-lysine (Sigma–Aldrich; Oakville, ON), CSPG (Chemicon; Temecula, CA) or myelin substrates. Specifically, E8 DRG explants were cultured on 100 μg/mL poly-L-lysine (PLL), or CSPG substrate consisting of a mixture of 4 μg/mL CSPG and 20 μg/mL PLL. Because neurons do not respond to myelin until embryonic day 13, we cultured E14 chick DRG explants on myelin substrates