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RESEARCH ARTICLE

Co-culture of oligodendrocytes and neurons can be used to assess drugs for axon regeneration in the central nervous system

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Abstract

We present a novel *in vitro* model in which to investigate the efficacy of experimental drugs for the promotion of axon regeneration in the central nervous system. We co-cultured rat hippocampal neurons and cerebral cortical oligodendrocytes, and tested the co-culture system using a Nogo-66 receptor antagonist peptide (NEP1–40), which promotes axonal growth. Primary cultured oligodendrocytes suppressed axonal growth in the rat hippocampus, but NEP1–40 stimulated axonal growth in the co-culture system. Our results confirm the validity of the neuron-oligodendrocyte co-culture system as an assay for the evaluation of drugs for axon regeneration in the central nervous system.

Key Words: *nerve regeneration; experimental models; NEP1–40; oligodendrocytes; neurons; axon regeneration; Nogo; PC12 cells; neural regeneration*

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Introduction

Impaired axon regeneration always accompanies traumatic injury to the spinal cord or brain, hypoxic-ischemic brain damage, and demyelinating degenerative disease (Li et al., 2008; Harris et al., 2010; Ni et al., 2015). Therefore, axonal regeneration and remodeling play an important role in central nervous system recovery after damage. Indeed, the promotion of axon regeneration is gaining increasing attention among researchers. To date, the evaluation of drugs for axon regeneration has mainly involved experiments in animals and immortalized neuron-like cells. However, animal models have a number of limitations, such as the length of time required to perform each experiment, high cost, and complex surgery, which preclude convenient assessment of drug effects. *In vitro* cellular assays have become a common method of drug evaluation, because of their comparative rapidity, simplicity and accuracy. The commonly used cell line PC12 is derived from rat pheochromocytomas, and is a non-neuronal immortalized cell line (Genchi et al., 2015). However, immortalized cells differ structurally and functionally from normal cells, so the use of this cell line to evaluate drugs that promote axonal growth cannot truly reflect a drug's effects in real neurons.

It therefore remains necessary to establish an effective and convenient method of determining axon regeneration in primary neurons, in the investigation of the restorative action of drugs after neuronal injury. Previously, Schwann cells co-cultured with neurons have been used to observe axon regeneration in the peripheral nerve system (Beaudoin et al., 2012; Xu et al., 2012). However, the mechanisms underlying axon regeneration in the central nervous system are different from those in the peripheral nervous system (Niu et al., 2012; Rodriguez et al., 2014), meaning that methods used to study primary cells in the periphery are not suitable for studying the central nervous system. Oligodendrocytes are the main factor affecting axonal regeneration

in the central nervous system (Watabe et al., 2014). Here, we attempted the direct co-culture of oligodendrocytes and neurons to simulate axonal growth restriction after central nervous system injury, and observed the effects of the Nogo-66 receptor antagonist peptide, NEP1–40, which promotes axonal growth, in this co-culture system (Wang et al., 2002; Huebner and Strittmatter, 2009; Gou et al., 2010; Hong et al., 2013; Fujita and Yamashita, 2014; Suehiro et al., 2014). Our results confirm the efficacy of the oligodendrocyte-neuron co-culture system, and present further ideas for the investigation of drugs for axon regeneration in the central nervous system.

Materials and Methods

Ethics statement

Experiments were approved by the Animal Ethics Committee of the Affiliated Hospital of Logistics University of Chinese People's Armed Police Force. All procedures were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Precautions were taken to minimize suffering and the number of animals used in each experiment.

In vitro culture of oligodendrocytes

Six newborn (≤ 48 hours) specific-pathogen-free Sprague-Dawley rats were provided by the Animal Center of the Academy of Military Science of the Chinese PLA (animal licence No. SCXK (Army) 2012-0004). Animals were anesthetized by ether inhalation for 4–5 minutes, then sacrificed by immersion in 75% ethanol for 5 minutes. As described previously (Zhu et al., 2007; Franco et al., 2015), the cerebral cortex was cut, triturated, digested, centrifuged, and incubated in high-glucose Dulbecco's modified Eagle's medium (DMEM; HyClone, Logan, UT, USA) containing 15% fetal bovine serum (HyClone) at 37°C and 5% CO_2. The medium was replaced every 3 days. After 3–4 days in culture, the flask was blocked with sealing films, shaken on a swing bed for 2 hours at 150 r/min and at 37°C, and purified after removal of microglia (GrandPre et al., 2002). The suspension was discarded. Samples were then incubated

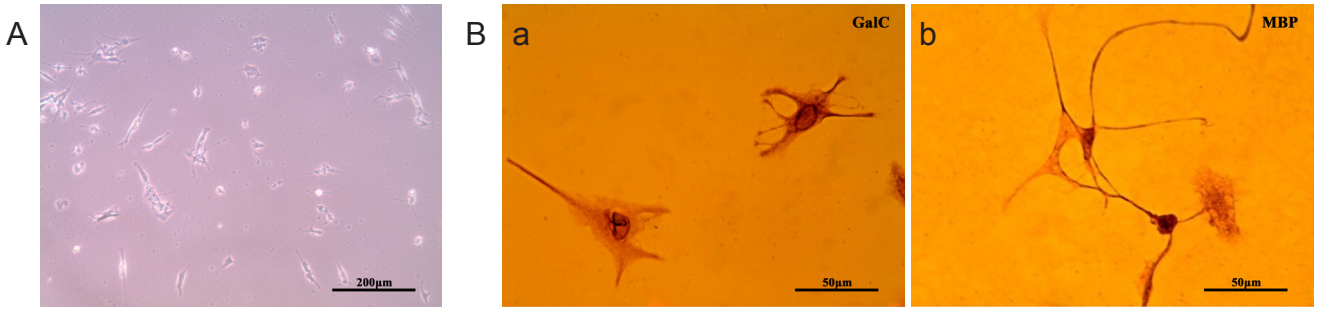

Figure 1 Oligodendrocytes of the rat cerebral cortex cultured *in vitro* (immunocytochemistry, inverted fluorescence microscope).
(A) Morphology of oligodendrocyte precursor cells after 3 days of culture. (B) Immunocytochemistry confirmed that oligodendrocyte precursor cells had differentiated into oligodendrocytes; (a) GalC staining revealed small cell bodies with a few processes and a dark cell membrane; (b) brown myelin sheaths were visualized using MBP staining. Scale bars: A, 200 μm; B, 50 μm. GalC: Galactosylceramidase; MBP: myelin basic protein.

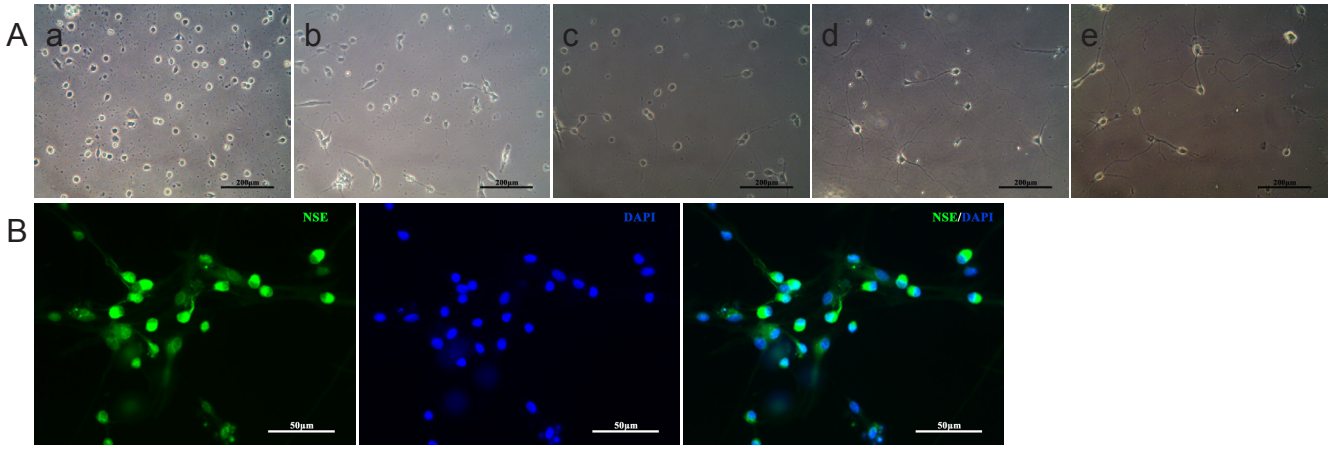

Figure 2 Hippocampal neurons cultured *in vitro* (immunofluorescence staining, inverted fluorescence microscope).
(A) Neuronal morphology at 24 hours (a), 48 hours (b), 72 hours (c), 5 days (d) and 8 days (e). (B) NSE immunofluorescence (green) showed neurons, polygonal or fusiform bodies, and weakly stained nuclei; DAPI counterstaining (blue) revealed consistently-sized, spindle- or oval-shaped nuclei. Neurons were identified as NSE and DAPI double-labelled cells. Scale bars: A-a–e, 200 μm; B, 50 μm. NSE: Neuron specific enolase; DAPI: 4′,6-diamidino-2-phenylindole.

Table 1 Effect of NEP1–40 on mean axon length (μm) in neurons co-cultured with oligodendrocytes

	Hours in culture		
Group	24	48	72
Control	61.4±3.3	70.5±3.1†	84.7±4.6†
Co-culture	54.2±2.7*	62.8±4.3*†	70.4±3.4*†
NEP1–40	59.3±3.7$^{\#}$	65.1±4.5$^{*\#\dagger}$	80.6±4.1$^{*\#\dagger}$

Data are expressed as the mean ± SD. Means were compared using two-way repeated-measures analysis of variance. Experiments were performed in triplicate. *P < 0.05, *vs.* control group; #P < 0.05, *vs.* co-culture group; †P < 0.05, *vs.* previous time point.

for 3 days with conditioned medium A (Wang et al., 2013), containing DMEM/F12 (HyClone) supplemented with 2.5 ng/mL platelet-derived growth factor AA (Peprotech, Rocky Hill, NJ, USA) and 2.5 ng/mL fibroblast growth factor (Peprotech). Afterwards, the samples were incubated with conditioned medium B, containing DMEM/F12 supplemented with 0.8 μg/mL sodium selenite, 50 mg/L human transferrin, 0.4 μg/L tri-iodothyronine, 5 mg/L insulin, 2.2 g/L sodium bicarbonate and 16.1 mg/L putrescine (Sigma-Aldrich, San Francisco, CA, USA), for 8–9 days. The medium was replaced every 3 days. Purified oligodendrocytes were collected and identified by galactosylceramidase (GalC) and myelin basic protein (MBP) staining. Oligodendrocytes were fixed with 4% paraformaldehyde for 30 minutes, washed three times with phosphate buffered saline (PBS) for 2 minutes each time, permeabilized with 0.5% Triton X-100 in PBS for 20 minutes, and washed in PBS as before. They were then incubated with 3% H_2O_2 for 10 minutes, washed again in PBS for 3 × 2 minutes, and blocked with 5% bovine serum albumin for 20 minutes. Rabbit anti-rat GalC monoclonal antibody (1:150; Abcam, Cambridgeshire, UK) or rabbit anti-rat MBP monoclonal antibody (1:150; Abcam) was placed on the coverslip and incubated in a wet box at 4°C overnight. In the next day, oligodendrocytes were washed with PBS for 3 × 2 minutes and incubated with goat anti-rabbit IgG-horseradish peroxidase polymer (1:100; Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd., Beijing, China) in a wet box at 37°C for 30 minutes, washed three times with PBS for 5 minutes each time, and visualized by reacting with with 3,3′-diaminobenzidine for 5 minutes, terminating the reaction with distilled water. The samples were air dried, mounted with neutral resin, and observed under an inverted fluorescence microscope (Leica, Danaher, Nußloch, Germany).

In vitro culture of neurons

One pregnant (gestational day 15) specific-pathogen-free Sprague-Dawley rat was provided by the Animal Center of the Academy of Military Science of the Chinese PLA, and sacrificed by ether anesthesia. Using the method described by Beaudoin et al. (2012), bilateral hippocampi of fetal rats were cut, triturated, digested, centrifuged, and incubated with DMEM/F-12 medium containing 10% fetal bovine serum. Cells were counted using a counting plate. Cell suspension was seeded on coverslips in a 6-well plate coated with 0.025% polylysine (Sigma-Aldrich) in 37°C 5% incubator for 4 hours, and then the 6-well plate was shaken gently. The medium was discarded and replaced by serum-free Neurobasal medium containing 2% B27 (Gibco, Carlsbad, CA, USA) (Franco et al., 2015). The medium was replaced every 3 days.

An immunofluorescence assay was conducted. Samples were fixed in 4% paraformaldehyde for 30 minutes, washed three times with PBS for 2 minutes each time, permeabilized with 0.5% Triton X-100 in PBS for 20 minutes, washed in PBS for 3 × 2 minutes, and blocked with 5% bovine serum albumin for 20 minutes. They were then incubated with rabbit anti-rat neuron specific enolase monoclonal antibody (1:100; Abcam) at 4°C overnight. In the next day, samples in wet box were placed at room temperature for 40 minutes, washed in PBS (3 × 2 minutes), incubated with goat anti-rabbit IgG-FITC (1:100; EarthOx, San Francisco, CA, USA) at 37°C for 30 minutes, washed on a swing bed with PBS (3 × 5 minutes), visualized by incubating with 4′,6-diamidino-2-phenylindole (DAPI) for 10 minutes, and washed a final time in PBS (3 × 5 minutes) on the swing bed. The sampes were then air dried, mounted with glycerol, and observed under an inverted fluorescence microscope.

Experimental groups

Neurons were assigned to three groups. In the control group, neurons were cultured normally. In the co-culture group, oligodendrocytes were directly co-cultured with neurons. In the NEP1–40 group, NEP1–40 was added to co-cultured neurons.

Construction of neuron-oligodendrocyte co-culture

Mature oligodendrocytes were incubated with 4 mL of trypsin (Solarbio, Beijing, China) for 3–4 minutes, which was stopped by 1 mL of fetal bovine serum as soon as spherical cell clusters had formed in order to terminate the digestion. Oligodendrocytes were lightly treated again, collected, and centrifuged. The supernatant was discarded, and the oligodendrocytes were resuspended in 2 mL of conditioned medium B. The cell suspension was pipetted onto sterile coverslips in a 6-well plate, and placed in an incubator for 30 minutes. Conditioned medium B (2 mL) was added to each well for 24 hours. Coverslips coated with confluent cells were placed in the 6-well plate with neurons for 24 hours of incubation with neuronal medium, and the coverslips with the oligodendrocytes were reversed to allow direct contact with the neurons.

NEP1–40 intervention

After 24 hours of co-culture, 80 nM NEP1–40 (Bioss, Beijing, China) was added to the oligodendrocytes and neurons.

Measurement of axon length

At 24, 48 and 72 hours, 100 axons in five fields of vision at 100× magnification were randomly selected from each group under the light microscope (Leica). Axon length was measured using Image-Pro Plus 7.0 software (Media Cybernetics, Sarasota, FL, USA).

Statistical analysis

Data are expressed as the mean ± SD, and were analyzed using SPSS 17.0 software (SPSS, Chicago, IL, USA). Intergroup differences were compared using a two-way repeated-measures analysis of variance. A value of $P < 0.05$ was considered statistically significant.

Results

Oligodendrocytes cultured *in vitro*

After 3–4 days of cortical cell culture, a large number of mixed glial cells were observed. Oligodendrocyte precursor cells were noted above astrocytes, and aggregated into spheres upon shaking and separating. Cell bodies were round, with unipolar or bipolar processes (**Figure 1A**). After 3 days of culture in conditioned medium A, oligodendrocyte precursor cells showed good survival and proliferation (Zhu et al., 2007), and differentiated into oligodendrocytes after culture in conditioned medium B. At 8–9 days, immunocytochemistry showed that the purity of oligodendrocytes was 95% (**Figure 1B**).

Neurons cultured *in vitro*

When cells were seeded, their bodies were round, lucent, uniform and floating. At 12–24 hours (**Figure 2A-a**), all cells were adherent and extended small processes. At 48 hours (**Figure 2A-b**), cells began to differentiate, cell bodies appeared conical or fusiform, and processes were apparent. Expanded cone-like structures were visible at the distal ends of the processes. A few glial cells were observed. At 72 hours (**Figure 2A-c**), cells exhibited typical features of neurons: large nuclei, fusiform or conical bodies, a few polygonal bodies, long processes, and growth cone-like structures. A few glial cells were evident. The purity of neurons was 80% (**Figure 2B**). At 4–7 days, neuronal cell bodies became enlarged, and the processes gradually formed fiber networks (**Figure 2A-d**). At 8 days, large neuronal cell bodies were observed, which formed masses and presented dense processes comprising one axon and multiple dendrites (**Figure 2A-e**).

Effects of NEP1–40 on axonal growth after co-culture of oligodendrocytes and neurons

As the culture progressed, axons grew significantly in length in each group ($P < 0.05$). Axons in the co-culture group were shorter than in the control group at each time point ($P < 0.05$), suggesting that oligodendrocytes suppressed axonal growth under the present experimental conditions. However, axons in the NEP1–40-treated co-cultured cells were longer than those in non-treated co-cultured cells, indicating that NEP1–40 promoted axonal growth in neurons co-cultured with oligodendrocytes (**Table 1**).

Discussion

It is important to establish an effective assay for the evaluation of experimental drugs for axon regeneration. At present, PC12 is the most commonly used cell line for the study of axon regeneration *in vitro*, and has been used to construct models of axon regeneration and determine the promoting effect of NEP1–40, protein kinase inhibitor sc82510, and nerve regeneration factor (Chiba et al., 2010; Hong et al., 2013; Marvaldi et al., 2014). PC12 cells can also be used to establish a model of oxygen-glucose deprivation to assess the effects of minocycline on axon regeneration (Tao et al., 2015). Although PC12 cells provide a simple method of culturing cells and observing axon regeneration, they derive from rat pheochromocytoma cells, not neurons. As such, PC12 cells cannot really simulate the physiological changes that occur in primary cells, which can impact later experiments and applications. In contrast, the direct effect of drugs can be assessed by *in vivo* studies, but these are complex, lengthy, and expensive. Chiba et al. (2010) observed the effects of fasudil combined with mesenchymal stem cells in rats with spinal cord injury. Their observations were performed 2–3 months after fasudil injection, illustrating the considerable limitations of *in vivo* studies in the development of new drugs for axon regeneration in the central nervous system. In the present study, we have shown that primary cultured oligodendrocytes inhibit axon regeneration in hippocampal neurons. We also showed that application of NEP1-40 to the co-culture system promoted axon regeneration. Drug testing *in vitro* was completed within 3 days, demonstrating the convenience of this technique.

In summary, the use of an *in vitro* co-culture of oligodendrocytes and neurons can considerably shorten the research period involved in the assessment of drugs for neuronal repair, while simulating true axon regeneration. In the present study, the promoting effect of NEP1–40 on axonal growth confirmed that our co-culture model has predictive validity, and will provide a useful new assay for future experimental drugs prior to i*n vivo* validation.

Author contributions: *LG was in charge of data collation and statistical analysis. YCY wrote the paper and participated in a part of cell experiment. YFL performed the experiment and provided data. YT conceived and desiged the study. XYC obtained the funding, provided data support, and oversaw the experiment. YPL and KY were responsible for cell culture and immunofluorescence assay. All authors approved the final version of the paper.*

Conflicts of interest: *None declared.*

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