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A reliable *in vitro* model for studying peripheral nerve myelination in mouse

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HIGHLIGHTS

- The study presents a reliable protocol for BL/6 mouse dorsal-root cultures to study myelination.
- The study evaluates analytic myelin read-outs, also for studying myelination in vital cultures.
- We show an optimised rat DRG protocols with improved myelin ratio and its comparison, respectively.
- The study presents an effective method to study demyelination in the mouse model.

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ABSTRACT

The rat dorsal root ganglia (DRG) model is a long-standing *in vitro* model for analysis of myelination in the peripheral nervous system. For performing systematic, high throughput analysis with transgenic animals, a simplified BL6 mouse protocol is indispensable.

Here we present a stable and reliable protocol for myelinating co-cultures producing a high myelin ratio using cells from C57BL/6 mice. As an easy accessible and operable method, Sudan staining proved to be efficient in myelin detection for fixed cultures. Green fatty acid stain turned out to be highly reliable for analysis of the dynamic biological processes of myelination in vital cultures. Once myelinated we were able to induce demyelination by the addition of forskolin into the model system. In addition, we provide an optimised rat DRG protocol with significantly improved myelin ratio and a comparison of the protocols presented.

Our results strengthen the value of *ex vivo* myelination models in neurobiology.

1. Introduction

The rat dorsal root ganglia (DRG) model is a well established *in vitro* model used for studying mechanisms of myelination in the peripheral nervous system (PNS). DRG contain neurons as well as Schwann cells (SCs), which differentiate into their various subtypes, e.g. myelinating and non-myelinating SCs attached to axons, satellite cells ensheathing the cell bodies of sensory neurons (Hanani, 2005) and perisynaptic SCs of the neuromuscular junction (Corfas et al., 2004). Using appropriate experimental DRG protocols, SCs arrange around the axons and form myelinated internodes (Mary et al., 2001).

Nowadays work with knock-out animals or spontaneous mutants has become a standard tool in basic science (LeDoux, 2005). Therefore reliable and reproducible DRG C57BL/6 (BL6) mouse models which are applicable for high throughput analysis are indispensable.

We present a protocol including a feasible culture surface coating for mouse myelination cultures based on a previously published intricate mouse co-culture model (Paivalainen et al., 2008). This protocol was used to analyse basic questions in myelin research (He et al., 2010). Others performed rat/mouse mix-cultures using rat SCs and mouse DRG neurons to conduct successful myelination experiments (Syed et al., 2010; Taveggia et al., 2008). In various publications the description for performing mDRG co-cultures is vague; here we evaluated and simplified the present protocols. Our model system was further explored by implementing a strategy for demyelination by adding forskolin (FKL). There is evidence that FKL elevates intracellular cAMP and furthermore increases the expression of myelin-related genes in SCs (Kioussi et al., 1995;

Abbreviations: AA, L-ascorbic acid; BL6, C57BL/6 mouse strain; DRG, dorsal root ganglia; FCS, fetal calf serum; FFA, fluorescent fatty acids; FKL, forskolin; Gluc, glucose; Glut, L-glutamine; HS, horse serum; MBP, myelin basic protein; MEM, minimal essential media; N2, N2-supplement; NGF, nerve growth factor; NF, neurofilament; P0, myelin protein zero; PEB, pituitary extract bovine; PFA, paraformaldehyde; P/S, penicillin/streptomycin; SC, schwann cell.

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Lemke and Chao, 1988; Morgan et al., 1991). It is assumed that myelination exclusively occurs in a non-proliferative condition of SCs (Morgan et al., 1991). Interestingly, it is also well known that FKL promotes SC proliferation at low concentrations (Porter et al., 1986; Raff et al., 1978; Rahmatullah et al., 1998; Sobue et al., 1986). Conceivable molecular mechanisms for these effects are the initiation of p27Kip degradation and thereby the sustained ERK activity, the promotion of G1 progression and furthermore the decreases of Krox-20 (Iacovelli et al., 2007). FKL may also lead to an acceleration of S-phase initiation, to a transcriptional activation of cyclin D3 expression, sustaining MEK/ERK activation, augmenting the Akt-signalling pathway (Fuentealba et al., 2004; Monje et al., 2006; Rahmatullah et al., 1998).

In high concentrations, FKL causes demyelination and a reduction of myelin protein zero (P0) expression in the rat model. Subsequent removal of FKL leads to remyelination. In ultrastructural analysis, demyelinated areas exhibit myeloid bodies and peeling of myelin lamellae, which would be analogous to different pathological conditions tested on SCs (Kamakura et al., 2005; Lehmann et al., 2007; Paivalainen et al., 2008; Zhu and Glaser, 2008). This body of evidence suggests FKL as a potent agent for standardised demyelination in the present mouse DRG model, evaluated in the present study.

The Wistar rat DRG (rDRG) co-culture model is the established *in vitro* myelination model (Bunge, 1983; Lehmann et al., 2009).

However, various immunological questions *in vivo* are addressed in the Lewis rat, such as experimental autoimmune neuritis (EAN), a widely accepted model for acute inflammatory neuropathy (Jung et al., 1995; Meyer zu Horste et al., 2007). As such, the rDRG co-culture model still remains essential for translating immunological questions from the animal model to the culture dish, e.g. the effect of sera or purified immune cells on rDRG co-cultures.

Thus, *ex vivo* translation requires optimised Lewis DRG protocols (Bruck et al., 1994). Accordingly, we furthermore modified the standard protocol used in rats.

2. Materials and methods

2.1. Preparation of dorsal root ganglia

DRG of rats (rDRG) were prepared from embryonic Wistar or Lewis rat (E17) as described earlier (Eldridge et al., 1989; Lehmann et al., 2009; Podratz et al., 2001). DRG from C57BL/6 mice (BL6) and CD1 mice (E15) were prepared by opening the cutis and subcutis along the spine and removing the spinal cord not yet completely enclosed by the vertebral column. Detached DRG as well as DRG in the nerve root sheath were collected, centrifuged and resuspended for further treatment (Paivalainen et al., 2008).

For undissociated cultures, entire ganglia, containing anchorage dependent cells, were plated on collagen-coated 35 mm plastic dishes or in 6 (alternatively 24) well plates (Greiner Bio-One AG, Frickenhausen, Germany). A coating solution containing collagen type I (Becton Dickinson AG, New Jersey, USA) and 0.02 M acetic acid (1:7) was surfaced and dyed two times. For some cultures, basement membrane matrix (BD, New Jersey, USA) was applied according to the manufacturer's manual.

DRG cultures were kept in neurobasal medium for at least 24 h, containing for rat cultures: DMEM (BioWhittaker, Lonza AG, Basel, Switzerland) 1% foetal calf serum (FCS, PAA Laboratories GmbH, Pasching, Austria), 50 U/mL Penicillin/Streptomycin (P/S, Invitrogen Corp., CA, USA), 100 ng/mL nerve growth factor (NGF, Sigma–Aldrich Corp., MO, USA), and 10 g/l D-(+)-glucose (Gluc, Sigma–Aldrich Corp., MO, USA). Mouse neurobasal medium contained: DMEM, 10% HS, 50 U/mL P/S, 100 ng/mL NGF, 4 g/l Gluc. Neurobasal medium was subsequently exchanged by myelination media; media compositions are listed in Fig. 3B for rat media (M1–M5). Mouse myelination media is in accordance with M5, listed in Fig. 3B, containing components (if not listed above): minimal essential media (MEM, Invitrogen Corp., Carlsbad, CA, USA), pituitary extract bovine (PEB, Merck Millipore, Merck KGaA Darmstadt, Germany), L-ascorbic acid (AA, Sigma–Aldrich Corp., Missouri, USA), forskolin (FKL, Sigma–Aldrich Corp., Missouri, USA), L-glutamine (Glut, Invitrogen Corp., CA, USA), horse serum (HS, Invitrogen, Corp., Carlsbad, CA, USA), and N2-supplement (N2, Invitrogen Corp., CA, USA). The culture myelination medium was renewed every 3–4 days and the degree of myelination was assessed after 4 weeks, if not indicated otherwise.

2.2. Pure Schwann cell cultures

Preparation of rat SCs was done using a modified Brockes method (Brockes et al., 1979). Sciatic nerves were dissected from neonatal (P3) Wistar rats. Cells were plated in DMEM with 10% FCS, after digestion with 0.1% collagenase (Worthington, Lakewood, NJ, USA) and 0.25% trypsin. To reduce fibroblasts, cultures were treated with two cycles of 10 μM cytosine arabinoside, followed by complement lysis with anti-thymidine 1.1 antibody (AbD Serotec, Kidlington, UK) with a final purity between 90 and 98%, assessed using S100 staining in immunocytochemistry. Rat SCs were maintained in DMEM Gibco 3185 (Invitrogen, Carlsbad, CA, USA) with 10% FCS, 100 U/ml P/S, 2 mM Glut, and 1 μl/ml FKL.

For the preparation of mouse SCs, sciatic nerves were dissected of neonatal P3 BL6 mice. Nerves were shredded and digested, using 0.125% trypsin (Invitrogen, Carlsbad, CA, USA) and 0.05% collagenase (Sigma–Aldrich, St. Louis, MO, USA) in PBS for 30 min at 37 °C, and subsequently washed three times in DMEM containing 10% HS. SCs were then plated on PDL- (Sigma–Aldrich, St. Louis, MO, USA) coated dishes (Greiner Bio-One AG, Kremsmünster, Austria) using DMEM containing 10% HS, 100 U/ml P/S, 200 mM Glut, 2 ng/mL human heregulin (Cellsciences, Canten, MA, USA), and 0.5 μM FKL. After 5 days complement lysis was performed. An initial washing step with Hank's Balanced Salt Solution (Invitrogen Corp., Carlsbad, CA, USA) containing 20 mM HEPES (Invitrogen, Carlsbad, CA, USA), was followed by application of DMEM containing 20 mM HEPES, 10% HS, 4 mM Glut, 100 U/ml P/S. DMEM containing 4 μg/ml anti Thy Antibody 1.2 (AbD Serotec, Kidlington, UK), 20 mM Hepes, 10% HS, 4 mM Glut and 100 U/ml P/S was added. After 15 min at 37 °C, 400 μl rabbit complement (Cedarlane Laboratories Inc., Burlington, NC, USA) diluted in DEPC was added and incubated for 2 h at 37 °C. Cells were washed twice with HBSS containing 20 mM HEPES and cultured in DMEM containing 10% HS, 100 U/ml P/S, 200 mM Glut, 2 ng/mL human heregulin, 0.5 μM FKL, 10 ng/mL recombinant human Fibroblast Growth Factor (FGF-2, biomol GmbH, Hamburg, Germany), 20 μg/mL PEB (Merck Millipore, Merck KGaA, Darmstadt, Germany) and 1:4 mouse myelination DRG supernatants. If required, lysis was repeated up to four times. Cells were frozen in liquid nitrogen in 10% DMSO (Sigma–Aldrich), 45% HS and 45% DMEM in sufficient numbers.

2.3. Sudan staining

Sudan black staining was performed to assess *in vitro* myelination as described before (Stettner et al., 2011). After washing, cells were fixed for 1 h using 4% paraformaldehyde (PFA), washed three times with PBS and treated with 0.1% osmium tetroxide for 1 h. After sequential ethanol treatment (25%, 50%, 70% each for 5 min) for 1 h the 0.5% Sudan black solution (Sigma–Aldrich, St. Louis, MO, USA), dissolved in 70% ethanol, was added to the cultures, followed by descending ethanol treatment (70%, 50%, 25% each for 1 min).