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Axonal mRNAs: Characterisation and role in the growth and regeneration of dorsal root ganglion axons and growth cones

Christina F. Vogelaar [a,1,2], Noreen M. Gervasi [a,2], Laura F. Gumy [a], David J. Story [a], Ruma Raha-Chowdhury [a], Kin-Mei Leung [b], Christine E. Holt [b], James W. Fawcett [a,*]

[a] Cambridge University Centre for Brain Repair, Department of Clinical Neurosciences, University of Cambridge, Robinson Way, Cambridge CB2 0PY, UK
[b] Department of Physiology Development and Neuroscience, University of Cambridge, Cambridge CB2 3DY, UK

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ABSTRACT

We have developed a compartmentalised culture model for the purification of axonal mRNA from embryonic, neonatal and adult rat dorsal root ganglia. This mRNA was used un-amplified for RT-qPCR. We assayed for the presence of axonal mRNAs encoding molecules known to be involved in axon growth and guidance. mRNAs for *β-actin*, *β-tubulin*, and several molecules involved in the control of actin dynamics and signalling during axon growth were found, but mRNAs for microtubule-associated proteins, integrins and cell surface adhesion molecules were absent. Quantification of *β-actin* mRNA by means of qPCR showed that the transcript is present at the same level in embryonic, newborn and adult axons. Using the photoconvertible reporter Kaede we showed that there is local translation of *β-actin* in axons, the rate being increased by axotomy. Knock down of *β-actin* mRNA by RNAi inhibited the regeneration of new axon growth cones after *in vitro* axotomy, indicating that local translation of actin-related molecules is important for successful axon regeneration.

Introduction

Over the last two decades it has become clear that dendrites and also certain types of axons contain mRNA (Piper and Holt, 2004; Twiss and van Minnen, 2006, Giuditta et al., 2002; Kiebler and Bassell, 2006; Vogelaar and Fawcett, 2008a,b). This was first shown in invertebrate axons from squid and snails which contain numerous mRNAs, mainly encoding cytoskeletal and metabolic proteins, and proteins involved in local translation, as well as neuropeptides (VanMinnen et al., 1997; Gioio et al., 2001; Giuditta et al., 2002; Gioio et al., 2004; van Kesteren et al., 2006). There have been various studies on vertebrate neurites from developing neurons, axons from adult dorsal root ganglia (DRGs) that received a conditioning lesion, and also from cytoplasm squeezed out from adult nerves (axoplasm). These have identified localised cytoskeletal mRNAs, such as *β-actin*, *β-tubulin*, *neurofilament*, *vimentin*, mRNAs encoding small GTPases and CREB (Bassell et al., 1998; Eng et al., 1999; Lee and Hollenbeck, 2003; Sotelo-Silveira et al., 2006; Yao et al., 2006; Leung et al., 2006, Giuditta et al., 2002; Perlson et al., 2005; Wu et al., 2005; Cox et al., 2008). A study of pre-conditioned adult DRG neurons identified 27 mRNAs in these axons (Willis et al., 2005). While several mRNAs have been identified in immature mammalian axons, and in axons from developing *Xenopus* and goldfish, mRNAs appear to be present in only a few types of axons in the adult mammalian CNS. Studies on adult rat olfactory and hypothalamic axons demonstrated the presence of mRNA (Vassar et al., 1994; Wensley et al., 1995; Mohr and Richter, 2000; Nedelec et al., 2005). However, adult retinal axons do not contain detectable amounts of ribosomal protein, and in the spinal cord the only axons in which ribosomal protein is detectable are the central branches of DRG axons, suggesting that most adult CNS axons are not capable of local translation (Verma et al., 2005, Verma and Fawcett, unpublished results).

Several functions of local translation of axonal mRNA have been established. In *Xenopus* retinal axons it was shown that asymmetrical translation of *β-actin* mRNA is essential for growth cone turning (Yao et al., 2006; Leung et al., 2006). Knock-out mice for the RNA binding protein SMN1 show decreased axonal *β-actin* mRNA and protein in motor neuron axons *in vitro*, causing decreased axon growth and a reduction in growth cone size (Rossoll et al., 2003). siRNA directed against axonal *rhoA* abolished Sema3A-induced growth cone collapse of embryonic DRG axons *in vitro* (Wu et al., 2005), and local translation of CREB is involved in NGF signalling (Cox et al., 2008). Many types of axon are able to regenerate after axotomy, but their

* Corresponding author. Fax: +441223 331174.
E-mail address: jf108@cam.ac.uk (J.W. Fawcett).
[1] Present address: Molecular Neurobiology Laboratory, Department of Neurology, Heinrich-Heine University, Düsseldorf, Germany.
[2] These authors contributed equally to this work.

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regenerative ability varies greatly, with PNS axons showing a strong regenerative response and many CNS axons showing little regeneration, even when presented with a permissive environment. The different regenerative ability of CNS and PNS axons can be modelled *in vitro* where DRG axons of all developmental stages are usually capable of regenerating a new growth cone after transection but adult retinal axons often fail to regenerate (Chierzi et al., 2005). The regenerative ability of DRG axons is much reduced by protein synthesis inhibitors and these axons contain ribosomal proteins and translation elongation factor at all developmental stages (Verma et al., 2005). However, the poorly regenerating adult retinal axons do not contain ribosomal protein, and their limited regeneration is not reduced by protein synthesis inhibitors (Verma et al., 2005). Local axonal translation of vimentin and importins play an important part in retrograde signalling from damaged sensory axons to the cell body (Hanz et al., 2003; Perlson et al., 2006).

PNS axotomy affects axonal transport, including probably that of mRNA (Willis et al., 2007). Currently, no localised mRNA identification data exist on axons from adult mammalian non-conditioned DRGs. In the present study we describe a new compartmented system for obtaining pure axonal material from DRG explants. We have used it to look for the presence of candidate mRNAs encoding proteins involved in the cytoskeleton, cytoskeletal control, signalling pathways and cell surface molecules. We have investigated axonal translation of *β-actin* mRNA and demonstrated its importance for successful axonal regeneration.

Results

A new culture method for isolation of axon-only RNA

Obtaining sufficient axonal mRNA for quantitative studies, free of glial and neuronal cell body contamination, is challenging. We have developed a new compartmented culture system for extracting axonal material from adult, neonatal and embryonic rat DRG explants. In Fig. 1, we compare our method with the currently used compartmented culture systems. Because the compartment divider is not placed in the culture dish until robust axon growth has begun, the method overcomes the inability of some axons to grow through silicone grease barriers and the need of DRGs to be held down by surface tension until adherent. It also allows for the culture of 20 or more DRG explants, and therefore the collection of significant quantities of axonal material. In the method DRG explants are placed in a line next to parallel scratches to direct axon growth and allowed to adhere and begin axon growth for one or two days. After this, triangular barriers cut from silicon elastomer with walls 1 mm thick are placed next to the DRGs without the application of silicon grease. Axons from embryonic, newborn and adult DRGs grow under the barrier for around 1 cm, but almost all fibroblasts and Schwann cells are excluded (Fig. 2). Fresh silicon elastomer is slightly sticky, and forms an effective seal between the compartments as long as fluid levels in the barrier-enclosed compartment and the rest of the dish are kept equal. To demonstrate the separation of compartments, ink was placed in the central compartment and showed almost no diffusion underneath the barrier over 12 h (Fig. 2b). After mitomycin-C treatment to the outer compartment to kill any Schwann cells or fibroblasts, mRNA could be extracted by tilting the dishes and scraping off the axons into extraction solutions. This provided sufficient RNA to detect *β-actin* mRNA in axons of all developmental stages without pooling or amplification. From dishes containing around 20 DRGs, between 100 ng and 150 ng of total RNA were isolated from the each culture dish, measured by nanodrop.

Axon-only preparations were devoid of cell contamination

The silicon inserts prevented almost all Schwann cell and fibroblast migration into the axon compartment. Mitomycin C was used to eliminate any cells that had got under the barrier. After RNA extraction we tested all preparations for the presence of *P0* and *DNA polymerase* mRNA by qPCR to ensure there was no cell contamination. The sensitivity of the *P0* qPCR for detecting contaminating Schwann cells was examined using a dilution series of 100 ng to 1 pg of Schwann cell RNA, corresponding to 10^4 to 10^{-2} cells (10^6 cells yielded 10 μg of total RNA). We were able to detect *P0* mRNA in cDNAs corresponding to as little as 10^{-1} cells per preparation (data not shown). This indicates that our method was sensitive enough to detect contamination of less than a single cell. Around 80% of all axon-only preparations were negative for both *P0* and *DNA polymerase*, the remaining 20% being rejected.

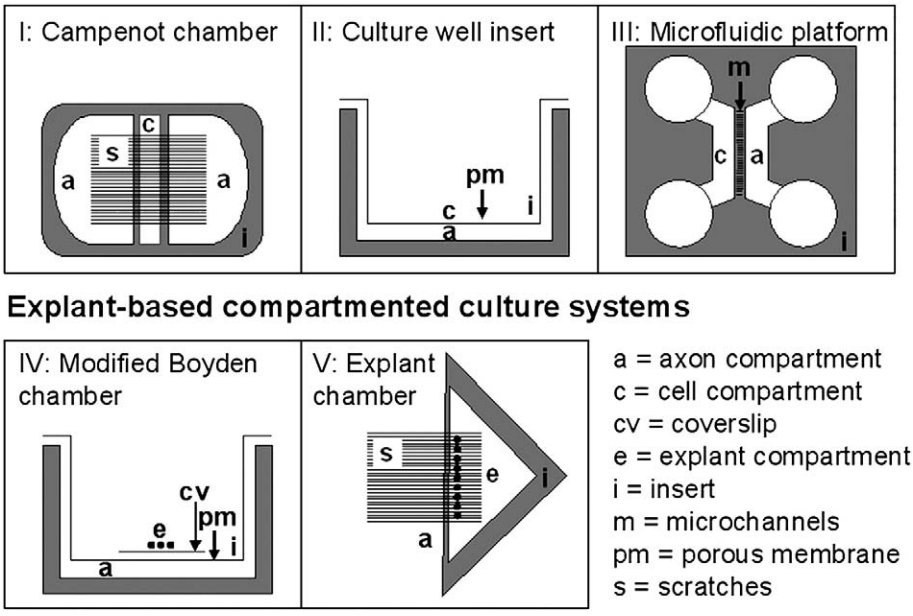

Fig. 1. Schematic representation of current compartment culture models I–IV (Eng et al., 1999; Zheng et al., 2001; Wu et al., 2005; Taylor et al., 2006). Our newly developed "explant chamber" is shown in (V).