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In vitro growth conditions and development affect differential distributions of RNA in axonal growth cones and shafts of cultured rat hippocampal neurons

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ABSTRACT

Local synthesis of proteins in the axons participates in axonogenesis and axon guidance to establish appropriate synaptic connections and confer plasticity. To study the transcripts present in the growth cones and axonal shafts of cultured rat hippocampal neurons, two chip devices, differing in their abilities to support axonal growth and branching, are designed and employed here to isolate large quantities of axonal materials. Cone-, shaft- and axon-residing transcripts with amounts higher than that of a somatodendritic transcript, *Actg1* (γ-actin), are selected and classified. Since the chips are optically transparent, distribution of transcripts over axons can be studied by fluorescence in situ hybridization. Three transcripts, *Cadm1* (cell adhesion molecule 1), *Nefl* (neurofilament light polypeptide), and *Cfl1* (non-muscle cofilin) are confirmed to be preferentially localized to the growth cones, while *Pfn2* (profilin2) is preferentially localized to the shafts of those axons growing on the chip that restricts axonal growth. The different growing conditions of axons on chips and on conventional coverslips do not affect the cone-preferred localization of *Cadm1* and shaft-preferred localization of *Pfn2*, but affect the distributions of *Nefl* and *Cfl1* over the axons at 14th day in vitro. Furthermore, the distributions of *Cadm1* and *Nefl* over the axons growing on conventional coverslips undergo changes during in vitro development. Our results suggest a dynamic nature of the mechanisms regulating the distributions of transcripts in axonal substructures in a manner dependent upon both growth conditions and neuronal maturation.

Introduction

A typical neuron consists of three morphologically distinct compartments: soma (cell body), dendrites and axon. Axons serve as cables for propagation of action potentials and as conduits for transportation of various metabolites, bio-molecules and organelles between the somata and axonal termini. During early phases of axonal development, the growth cone, a highly dynamic structure at the tip of an axon, actively explores its surrounding and guides the growth of axon toward target area under the influences of various cues (Gordon-Weeks, 2000; Vitriol and Zheng, 2012). At later phases of axonal development, axons form contacts and subsequently synapses with the dendrites and somata in their target area (McAllister, 2007). Finally, neuronal activities further strengthen, attenuate or even eliminate synapses between neurons (Cohen-Cory, 2002; Okabe, 2002). Developments of molecular, cellular and biochemical methods have greatly advanced our understanding of the mechanisms underlying axonogenesis and axon guidance. In addition, novel culturing techniques are continuously developed to aid the study of axonogenesis.

Proteins underlying the various functions and development of axons are synthesized in the somata and subsequently transported to the axons and/or translated locally from the mRNAs residing in axons. Although the requirement of local protein synthesis for basal axonal functions is still controversial (Blackmore and Letourneau, 2007; Campbell and Holt, 2001; Eng et al., 1999; Kar et al., 2013), recent studies have indicated that local protein synthesis may participate in the turning behavior, regeneration and collateral branching of axons (Gumy et al., 2011; Jung et al., 2012; Spillane et al., 2012; Taylor et al., 2009; Vogelaar et al., 2009; Willis et al., 2005, 2007; Wu et al., 2005; Zivraj et al., 2010).

Identification of the RNA species present in axons is the first step toward understanding the roles played by local protein synthesis in

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various axonal functions and is greatly aided by the development of techniques in isolating axons and the substructures. Axons have been isolated from various neurons cultured on filter membrane, Campenot chambers, microfluidic devices and other apparatuses (Campenot, 1977, 1994; Taylor et al., 2005; Wu et al., 2010; Zheng et al., 2001). Axonal substructures, i.e., growth cones and axonal shafts, have also been microdissected from FM1-43-labeled and paraformaldehyde-fixed retinal ganglion cells (Zivraj et al., 2010). Analyses of the RNA prepared from these samples have shown that a large variety of RNA species, coding for proteins participating in protein synthesis, metabolism, signaling and the construction and regulation of cytoskeleton systems of neurons, are present in axons and growth cones (Gumy et al., 2011; Jung et al., 2012; Taylor et al., 2009; Vogelaar et al., 2009; Willis et al., 2005; Zivraj et al., 2010). Studies have also indicated that the RNA compositions of axons undergo changes after exposures to exogenous stimulations (Willis et al., 2007) as well as during development and regeneration processes (Gumy et al., 2011; Taylor et al., 2009; Vogelaar et al., 2009; Zivraj et al., 2010).

Here, a chip device for the isolation of large quantities of axonal shafts and growth cones is developed. We report the results of semi-quantitative RT-PCR surveys of RNA isolated from axons, growth cones and axonal shafts of rat hippocampal neurons cultured on this newly developed and a previously reported chip devices (Wu et al., 2010). Thirty three transcripts, encoding the building blocks and regulatory proteins of cytoskeleton, cell adhesion molecules and proteins participating in protein synthesis and in establishing structural polarity, present in axons and axonal substructures are selected and classified by their relative abundances. Comparisons of transcripts found in axons, growth cones and axonal shafts suggest that a majority of these transcripts may have an uneven distribution over axons of hippocampal neurons. The cone-preferred localizations of *Cadm1* (cell adhesion molecule 1), *Nefl* (neurofilament light polypeptide) and *Cfl1* (non-muscle cofilin) as well as the shaft-preferred localization of *Pfn2* (profilin 2) in the distal 50 μm of axons grown on the chip used to collect growth cones were confirmed by fluorescence in situ hybridization (FISH). Further FISH analysis of axons grown on conventional coverslips shows that the cone-preferred localizations of *Cadm1*, *Nefl* and *Cfl1* in distal axons are affected by the growth conditions and by in vitro development.

Results

Isolation of axonal RNA

A prototype chip device for harvesting axons, which are composed of axonal shafts and a small portion of growth cones, of neurons has been reported by Wu et al. (2010). As depicted in Fig. 1A, rat hippocampal neurons were initially seeded on the cell region, and subsequently their axons grew into the axon region via the 800 μm-long poly-L-lysine (PLL)-coated lines at DIV14 (14th day in vitro). The axon region was separated from the cell region, containing somata, dendrites and axons of neurons, by cleaving along the groove 2 of the chip. Purity of the separated axons was studied by fluorescence immunocytochemistry preformed on the cleaved chip fragments (Fig. 1B). The axon region on the cleaved chip fragment was densely covered by processes, which were positively stained by antibody SMI312, recognizing the phosphorylated epitopes of neurofilaments in axons, but not by the antibody against MAP2 (microtubule associated protein 2), a dendritic marker. The immunocytochemistry study demonstrated that the axon region was occupied by axons and free of dendrites. Cellular and axonal RNA were respectively extracted from the separated cell and axon regions and analyzed by one-tube multiplex RT-PCR (Fig. 1C). Similar to the previous report (Wu et al., 2010), PCR amplicons of *Actb* (β-actin) mRNA and 18S ribosomal RNA (*18S rRNA*) were detected in the cellular and axonal RNA. Consistent to the RT-PCR analysis of cultured cortical neurons (Bassell et al., 1998; Mayford et al., 1996), the amplicon of a well-characterized somatodendritic transcript *Camk2a* (calcium/calmodulin-dependent protein kinase IIα) was only detected in the cellular RNA but not in the axonal RNA.

Isolation of axonal shaft and growth cone RNA

A modified chip was developed for the isolation of axonal shafts and growth cones (Fig. 2A). The modified chip lacked the PLL-coated axon region of the prototype chip shown in Fig. 1A. On the chip surface, somata of neurons were also restricted to the cell region, and the growths of axons were guided along arrays of PLL-coated lines (850 μm long) until reaching the ends of these lines. The processes residing on the PLL-coated lines were positively stained by the antibody against βIII-tubulin (a

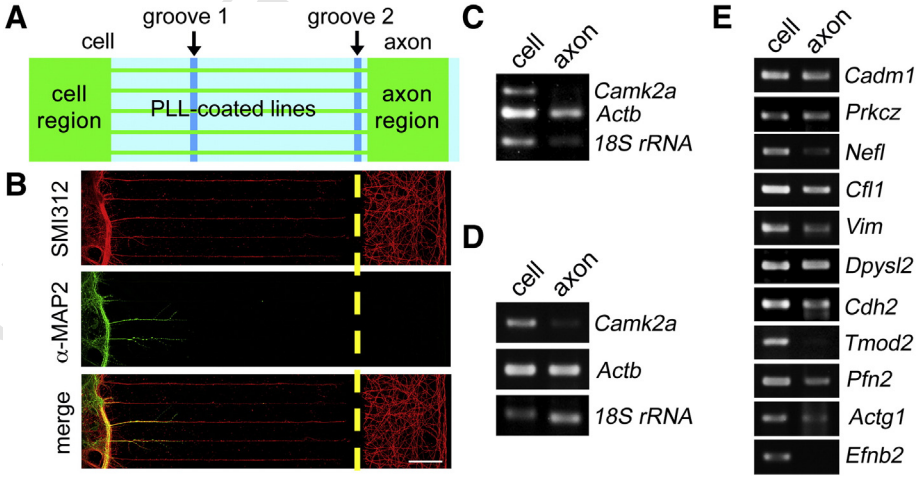

Fig. 1. *Isolation of axons of cultured hippocampal neurons for RNA survey.* (*A*) A schematic diagram of prototype chip (Wu et al., 2010) used for isolating axons. Green areas and horizontal lines depict the PLL-coated regions. The two vertical blue lines indicate grooves placed on the opposite surface. (*B*) Characterization of the neuronal structures residing on chip fragments after cleavage. The chip with DIV14 neurons grown on its surface was cleaved along the groove (yellow broken line) into two fragments and immunostained by SMI312 (red) and anti-MAP2 (green) antibodies. Scale bar: 100 μm. (*C*) Multiplex RT-PCR analysis of the total RNA prepared from the cleaved chip fragments. Cellular (cell) and axonal (axon) RNA were respectively isolated from the cell and axon regions shown in A. Three transcripts, *Camk2a*, *Actb* and *18S rRNA*, were simultaneously amplified by one tube RT-PCR. (*D*) PCR analysis of pre-amplified cDNA. *Camk2a*, *Actb* and *18S rRNA* were separately amplified after pre-amplifications of cDNA prepared from the corresponding total RNA samples analyzed in C. (*E*) Representative results of PCR analysis using pre-amplified cDNA prepared from the cellular and axonal RNA. Names of genes are indicated to the right of the ethidium bromide-stained agarose gel. Full names and accession numbers are listed in Table S1.

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