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Local translation of dendritic *RhoA* revealed by an improved synaptoneurosome preparation

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ABSTRACT

Changes in dendritic spine morphology, a hallmark of synaptic plasticity, involve remodeling of the actin cytoskeleton, a process that is regulated by Rho GTPases. RhoA, a member of this GTPase family, segregates to dendrites in differentiated neurons. Given the emerging role of dendritic mRNA local translation in synaptic plasticity, we have assessed the possible localization and translation of *RhoA* mRNA at dendrites. At this end, we have developed and describe here in detail an improved method for isolating hippocampal and neocortical mouse synaptoneurosomes. This synaptoneurosomal preparation is much more enriched in synaptic proteins than those obtained in former methods, exhibits *bona fide* electron microscopy pre- and postsynaptic morphologies, contains abundant dendritic mRNAs, and is competent for activity-regulated protein synthesis. Using this preparation, we have found that *RhoA* mRNA is dendritically localized and its local translation is enhanced by BDNF stimulation. These findings suggest that some of the known functions of RhoA on spine morphology may be mediated by regulating its local translation.

Introduction

Dendritic spines are highly dynamic protrusions that cover the dendritic surface of the neuron. They represent the main postsynaptic compartments for excitatory input in the mature mammalian brain and are involved in synaptic plasticity. Thus, changes in number, size, and shape of dendritic spines have been correlated with modulation of synaptic strength (reviewed by Tada and Sheng, 2006). Remarkably, abnormal spine morphology and density have been associated with mental retardation diseases such as Fragile X, Down, Rett, and Williams syndromes (Kaufmann and Moser, 2000) and other neurological disorders such as schizophrenia and epilepsy (Roberts et al., 1996; Swann et al., 2000).

Morphological changes in dendritic spines involve remodeling of the actin cytoskeleton. Rho GTPases, as RhoA and Rac1, are well-characterized regulators of actin polymerization (reviewed by Luo, 2000). For instance, it is well known that active RhoA reduces the number and length of dendritic spines, whereas Rac1 induces spine development (Nakayama et al., 2000; Tashiro et al., 2000). Very interestingly, while RhoA and Rac1 are uniformly distributed in immature neurons, they segregate to dendrites and axons, respectively, in fully polarized neurons, strongly suggesting that they play differential roles in those compartments (Da Silva et al., 2004). Although the underlying mechanisms responsible for spatial and functional segregation of RhoA to dendrites are unknown, transport and local translation of *RhoA* mRNA have been suggested (Da Silva et al., 2004) and constitutes an attractive possibility. mRNA localization and local translation are regulated by neurotrophins and synaptic activity and play key roles in synaptic plasticity (reviewed by Kindler et al., 2005; Schuman et al., 2006; Bramham and Wells, 2007).

Synaptoneurosomes are subcellular neuronal membrane fractions containing sealed presynaptic elements attached to their corresponding postsynaptic counterparts. They can be a very useful experimental system for the molecular analysis of diverse aspects of synapse physiology, including local translation of dendritic mRNAs (Bagni et al., 2000; Schratt et al., 2004; Di Nardo et al., 2007; Matsumoto et al., 2007). Nevertheless, since the original report by Hebb and Whittaker (1958), most synaptoneurosome preparation methods so far described lack a quantitative biochemical and/or ultrastructural characterization of the subcellular fractions obtained, rendering difficult to compare the degree of synaptoneurosome enrichment obtained. Furthermore, since characterization has been primarily focused on the presynaptic terminal, it is not always clear if functional dendritic elements are present in those preparations.

To overcome this problem, we have developed a protocol for the isolation of membrane subcellular fractions from mouse hippocampus or neocortex highly enriched in synaptoneurosomes that fulfills biochemical, morphological, and functional criteria. Taking advantage of this new synaptoneurosomal preparation, we demonstrate that *RhoA* mRNA is dendritically localized and locally translated. In

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addition, we show that *RhoA* local translation is activated in response to brain-derived neurotrophic factor (BDNF). We discuss the implications of these findings for the mechanisms underlying the role of RhoA in the regulation of spine morphology.

Results

An improved synaptoneurosome preparation from mouse forebrain

Based in a previously published method for purifying rat hippocampal CA3-dendritic spines with mossy fiber terminals (Kiebler et al., 1999), we developed a protocol for isolating mouse synaptoneurosomes.

In brief, after tissue homogenization, a pellet (P1) was obtained by low-speed centrifugation, resuspended and subjected to isopycnic centrifugation on a discontinuous Optiprep gradient. Four bands (O1 to O4, from top to bottom) corresponding to subcellular fractions with different densities were obtained (Fig. 1).

In contrast to the method described by Kiebler et al. (1999), in which material from bands O1 and O2 was mixed and subsequently fractionated in Percoll gradients, based on our Western blot analysis (see below), we decided to process the O1 and O2 fractions separately in two equal Percoll-sucrose gradients (Fig. 1). Secondly, the concentration of Percoll gradient steps and centrifugation time were optimized for having a better resolution in the density range in which synaptoneurosomes appeared. Finally, the iso-osmolarity of the gradient steps was precisely adjusted. Thus, five bands, which we named 1P1 to 1P5 (from O1 fraction) and 2P1 to 2P5 (from O2 fraction), were obtained in each case.

To identify the subcellular fractions enriched in synaptic markers, samples were analyzed by quantitative immunobloting. Two presynaptic markers, CSPα and synapsin, and three postsynaptic markers, PSD95, NR1, and GluR2 were used. In addition, to check the degree of contamination with glial membranes, GFAP was used as a marker (Bignami et al., 1972).

The pattern of this protein repertoire was compared in two reference tissues, hippocampus and neocortex. For the hippocampus, enrichment in both presynaptic and postsynaptic proteins was evident in subcellular fractions 1P4 and 2P4 related to the starting material P1 (Fig. 2). Thus, an average 6-fold enrichment of synaptic markers was observed in both 1P4 and 2P4 fractions. Nevertheless, GFAP quantitative immunoblotting revealed a significant glial contamination in 2P4 in contrast to the 4-fold decrease found in 1P4, compared to P1 (Fig. 2). Furthermore, 1P4 contained six times more protein than 2P4 (Table 1). Thus, the contribution of fraction 2P4 to the final yield of synaptoneurosomes is too low, and therefore, 1P4 is the fraction of choice for the isolation of synaptoneurosomes from hippocampus.

For mouse neocortex, enrichment in synaptic proteins was found in fractions 1P2 and 1P4, with average values of 3- and 5-fold, respectively (Supplemental Fig. 1A). Nevertheless, GFAP protein was increased in neocortical 1P2, whereas it was notably reduced in the 1P4 fraction (Supplemental Fig. 1A). Remarkably, the O2-derived 2P4 fraction showed no enrichment in synaptic markers (Supplemental Fig. 1A). As a matter of fact, a higher enrichment of synaptic markers and a lower glial contamination (i.e., GFAP) was achieved when processing O1 fraction separately, instead of using O1 + O2 (Supplemental Fig. 1B).

Since both in hippocampus and neocortex the 1P4 fraction showed the higher degree of synaptic marker enrichment and the lowest level of glial contamination, we decided to further analyze it.

A morphological assessment of subcellular structures present in the 1P4 fraction was obtained by electron microscopy (EM). It must be taken into account that an accurate identification of synaptoneurosomes by EM requires that both the presynaptic and the postsynaptic elements must be properly placed in the plane of sectioning. Despite

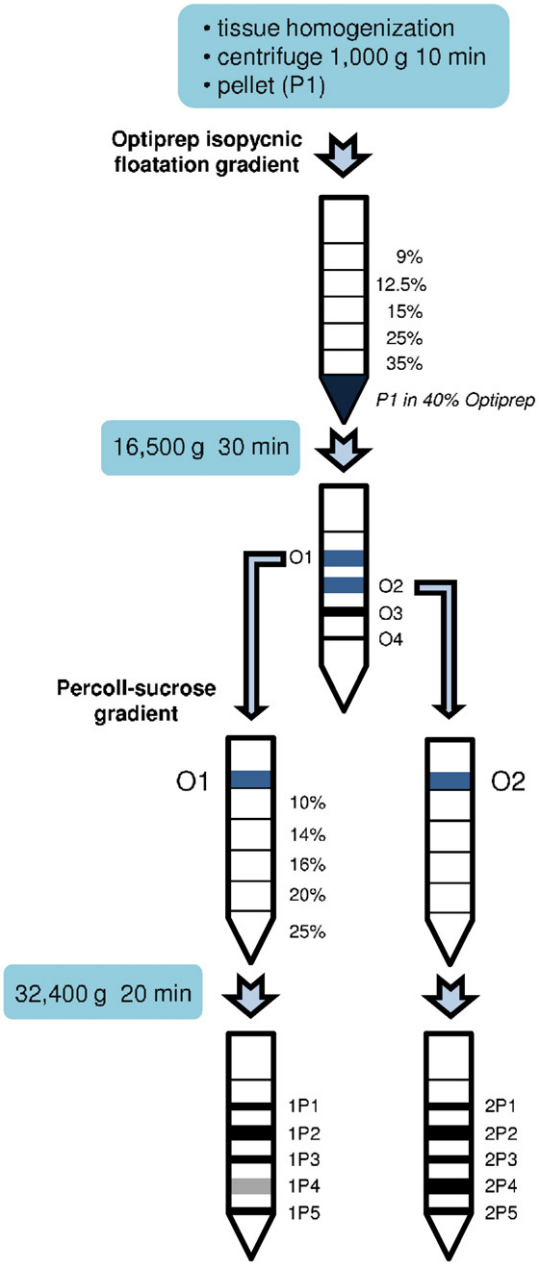

Fig. 1. Synaptoneurosome isolation. Schematic representation of main steps of the synaptoneurosome isolation procedure. Concentrations of Optiprep or Percoll gradient steps are indicated as percentage.

this probabilistic restriction, synaptoneurosomes (among other vesicular structures) were frequently observed in electron micrographs at low magnification (Fig. 3A). Synaptoneurosomes were easily identified by the presence of (i) synaptic vesicles of about 45 nm in the presynaptic element, (ii) a synaptic cleft of about 20 nm, and (iii) an electron-dense membrane in the postsynaptic element (postsynaptic density) (Fig. 3B). Thus, the presence in the 1P4 fraction of abundant *bona fide* synaptoneurosomes was confirmed.

As mentioned above, transport and local translation of dendritic mRNAs in response to neuronal activity is a relevant aspect of postsynaptic physiology. To determine if dendritic mRNA was present in the neocortical fraction 1P4, the level of the well-known dendritic α-*CaMKII* mRNA (Mayford et al., 1996) was quantified by real-time RT–PCR and compared to *HPRT*, a typical somatic mRNA (Di Nardo et al., 2007).