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 remodelling 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 post-synaptic 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.

Keywords: actin cytoskeleton; BDNF; dendritic spine; mRNA localization; Rho GTPase; synaptic plasticity; synaptosome

Introduction

Dendritic spines are highly dynamic protrusions that cover the dendritic surface of the neuron. They represent the main post-synaptic 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 like 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 has 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).

Synaptoneurosome are subcellular neuronal membrane fractions containing sealed pre-synaptic elements attached to their corresponding post-synaptic 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 synaptoneurosomes 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 synaptoneurosomes enrichment obtained. Furthermore, since characterization has been primarily focused on the pre-synaptic 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 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 synaptoneurosomes 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 immunoblotting. Two pre-synaptic markers, CSP α and synapsin, and three post-synaptic 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 pre- and post-synaptic 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. S1A). Nevertheless,

GFAP protein was increased in neocortical 1P2, whereas it was notably reduced in the 1P4 fraction (Supplemental Fig. S1A). Remarkably, the O2-derived 2P4 fraction showed no enrichment in synaptic markers (Supplemental Fig. S1A). 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. S1B). 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 synaptoneuroosomes by EM requires that both the pre- and the post-synaptic elements must be properly placed in the plane of sectioning. In spite of this probabilistic restriction, synaptoneuroosomes (among other vesicular structures) were frequently observed in electron micrographs at low magnification (Fig. 3A). Synaptoneuroosomes were easily identified by the presence of: i) synaptic vesicles of about 45 nm in the pre-synaptic element, ii) a synaptic cleft of about 20 nm, and iii) an electron-dense membrane in the post-synaptic element (post-synaptic density) (Fig. 3B). Thus, the presence in the 1P4 fraction of abundant *bona fide* synaptoneuroosomes was confirmed.

As mentioned above, transport and local translation of dendritic mRNAs in response to neuronal activity is a relevant aspect of post-synaptic 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).

As shown in Fig. 3C, α -*CaMKII* mRNA was enriched in 1P4 about 10-fold compared to P1. In the case of *HPRT* a modest 2.5-fold enrichment was detected in 1P4, probably due to some

somatic contamination. Thus, although slightly contaminated by non-dendritic mRNA, fraction 1P4 showed the mRNA profile expected for synaptoneurosomes. Moreover, α -*CaMKII* mRNA was nearly undetectable in the other O1-derived Percoll fractions (Fig. 3C). To check if 1P4 synaptoneurosomes were metabolically active, radiolabeling of proteins with a mix of ^{35}S -Met and ^{35}S -Cys was carried out in the presence of chloramphenicol to inhibit mitochondrial protein synthesis (Polosa and Attardi, 1991). Similarly to previous reports (Villanueva and Steward, 2001; Bagni et al., 2000), about thirty major bands with variable intensities corresponding to newly synthesized proteins were evident after 30 min of incubation (Fig. 3D), demonstrating the competence of these preparations for mRNA translation.

***RhoA* mRNA is locally translated in response to BDNF**

Altogether, the results so far here described let us conclude that the 1P4 fraction constituted a highly enriched synaptoneurosome preparation suitable for biochemical and functional studies. Accordingly, we used this preparation to assess the possible dendritic localization and translation of *RhoA* mRNA.

Segregation of RhoA protein to dendrites has been described *ex vivo*, in mature cultured hippocampal neurons (Da Silva et al., 2004). Synaptoneurosome preparations should reflect the *in vivo* localization of synaptic proteins in adult brain. To test if RhoA protein was enriched in 1P4 neocortical synaptoneurosomes, quantitative immunoblot analysis was performed. We found that RhoA was strongly enriched in 1P4 synaptoneurosomes (Fig. 4A) compared to P1 (7-fold), to O1 (4-fold) or to other tested subcellular fractions (1P1, 1P2, 1P3 and O2; not shown). Thus, the enrichment of RhoA GTPase in this synaptoneurosome preparation most probably reflects its accumulation at synapses of adult mouse neocortex.

Local translation of dendritic mRNAs represents an effective means to rapidly synthesize new proteins in response to neuronal activity (Bramham and Wells, 2007). An attractive hypothesis is that at least some of the synaptic RhoA protein may be originated from dendritic mRNA. As a first step to determine if *RhoA* is a local translated gene, we checked the presence of the corresponding mRNA in the 1P4 fraction by real time RT-PCR quantification. This analysis showed that *RhoA* mRNA was 10-fold enriched in these synaptoneurosomes, compared to P1 and using *HPRT* to normalize mRNA levels. This is very high considering that α -*CaMKII* mRNA, a well-established dendritic mRNA, showed a 4.4-fold increase (Fig. 4B). These results strongly suggest the dendritic localization of *RhoA* mRNA in adult mouse brain.

To determine if dendritic *RhoA* mRNA could be locally translated, radioactive metabolic labeling of synaptoneurosomes followed by RhoA immunoprecipitation was carried out. As BDNF has been shown to induce local translation of dendritic mRNAs in the context of memory consolidation (reviewed by Bramham, 2008), promoting long-lasting structural changes at dendritic spines (Tanaka et al., 2008), the effect of this neurotrophic factor was also tested. In these experiments, a radioactive-labeled protein of about 25 kDa corresponding to RhoA (as confirmed by Western blot, not shown) was immunoprecipitated (Fig. 4C). Remarkably, the intensity of the RhoA-radioactive band was enhanced approximately two times in BDNF-treated synaptoneurosomes as determined densitometrically.

Together, these results strongly support the dendritic localization and local translation of *RhoA* mRNA, and its enhancement by BDNF.

Discussion

In spite of the high amount of information available on the role of Rho GTPases in neurons, few studies have addressed their subcellular localization. Interestingly, in cultured rat hippocampal neurons, RhoA shifts from a uniform distribution to a dendritic localization as neurons mature and become fully polarized (Da Silva et al., 2004). It has been also reported that *RhoA* mRNA is localized in developing axons and growth cones of embryonic rat dorsal root ganglia explants, where it is locally translated for regulating Sema3A-mediated growth cone collapse (Wu et al., 2005). These data suggest that local enrichment of RhoA could play key regulatory roles in neuronal functions.

In this work we have investigated the dendritic localization and local translation of *RhoA* in adult mouse forebrain.

As explained before, synaptoneurosomes have been used for the analysis of dendritic local translation on the basis that, in the adult CNS, axons do not contain mRNAs. However, these synaptoneurosomes preparations are not fully characterized lacking in most cases a quantitative biochemical characterization. For instance, Bagni et al. (2000) described a method that has been extensively used for the isolation of dendritic mRNAs (Bagni et al., 2000; Matsumoto et al., 2007; Di Nardo et al., 2007). However, enrichment of synaptic proteins as deduced from Western blot analysis is not evident. More recently, Villasana et al. (2006) described a new method for rapid isolation of synaptoneurosomes, but they reported a rather limited (about 1.7-fold) enrichment of synaptic proteins.

To overcome these problems we developed and described here an improved method for the preparation of mouse brain synaptoneurosomes which fulfills biochemical, morphological, and functional criteria. First, both pre- and post-synaptic proteins were highly enriched (Fig. 2 and Supplemental Fig. S1). Second, EM revealed the presence of sealed pre- and post-synaptic joint membrane elements which were morphologically identified as

synaptoneurosomes (Fig. 3A, B). Third, dendritic mRNA was highly enriched in this fraction (Fig. 3C) and was capable to synthesize proteins (Fig. 3D).

We have taken advantage of our method to demonstrate that both RhoA protein and mRNA are highly enriched in neocortical synaptoneurosomes (Fig. 4A, B). Furthermore, we have here shown that *RhoA* mRNA is indeed locally translated and BDNF enhances this local translation (Fig. 4C). Thus, our results provide the first evidence for dendritic local translation of *RhoA* in adult brain and its stimulation by a neurotrophic factor.

Although further studies will be required to analyze the involvement of local translated RhoA in synapse function, the well known role of RhoA as a regulator of actin cytoskeleton (Luo, 2000) and spine morphology (Nakayama et al., 2000; Tashiro et al., 2000) make tempting to hypothesize about a possible role of local translated RhoA in synapse remodeling. Several lines of evidence point in this direction.

Thus, activation of NMDA and AMPA receptors induces a general activation of RhoA in neurons, but a local synaptic inactivation which results in spine collapse (Schubert et al., 2006). These data could be explained by differential properties of somatic and dendritic RhoA proteins. In this sense, it has been recently proposed that local translated proteins might have differential functions because of lacking some post-translational modifications (Lin and Holt, 2008). Very interestingly, post-translational modifications (ie., prenylation) are required for RhoA activation (Roberts et al., 2008). As a consequence, dendritic-translated RhoA could, for example, interact with a different set of downstream partners. In support of this idea, it has been reported that serine phosphorylation of RhoA in response to NGF differentially affects its binding capacity to downstream effectors (Nusser et al., 2006). Another non-excluding possibility is that local translated proteins might need to be synthesized in close proximity to their binding or signaling partners (Lin and Holt, 2008). Regarding that, RhoA protein has been shown to interact with some post-synaptic proteins (Camera et al., 2003; Schubert et al.,

2006). Thus, translation of RhoA in dendrites might constitute a strategy not only to rapidly synthesize the protein in the right place and time in response to activity, but also to ensure subsequent interactions with the adequate partner(s) in order to couple synaptic activity to spine morphology modification.

BDNF is emerging as an activity-dependent regulator of excitatory transmission and synaptic plasticity in the adult brain (Bramham, 2008). Interestingly, it has been recently demonstrated that BDNF is necessary and sufficient to induce long-lasting structural changes at dendritic spines in a protein synthesis-dependent manner (Tanaka et al., 2008). Thus, the enhancement of the local translation of *RhoA* by BDNF could represent a way to modulate the actin cytoskeleton in such a scenario.

Experimental Methods

Animals

Mice were maintained, handled and sacrificed in accordance with international laws. All protocols were approved by the University of Seville Animal Care and Use Committee.

Synaptoneurosome isolation

Membrane fractions enriched in synaptoneurosome were prepared from neocortex and hippocampus of mice by sequential centrifugation through Optiprep and Percoll gradients (Fig. 1). Three (for neocortex) or eight (for hippocampus) young adult male mice (2-4 months) were used per experiment. Tissue homogenization was made in 12 ml of homogenization solution (HS: 10 mM HEPES, pH 7.4; 320 mM sucrose; 1 mM MgSO₄; protease inhibitors leupeptin (10 µM), aprotinin (2 µg/ml) and pepstatin (1 µM)) using a 15-ml glass-teflon Dounce-type homogenizer at 650 rpm. The homogenate was centrifuged at 1,000xg for 10 min at 4°C, and the resulting pellet (P1) was resuspended in 1 volume of HS

and adjusted to 40% Optiprep (Sigma-Aldrich) with Optiprep working solution (OWS: 50% Optiprep; 10 mM HEPES, pH 7.4; 65 mM sucrose; 1 mM MgSO₄ and protease inhibitors; final pH adjusted to 7.4 with NaOH). This typically resulted in a 8-10 ml of 40% Optiprep-P1 pellet solution, which was distributed in four 12-ml polycarbonate tubes to make four Optiprep gradients. Each gradient consisted in 2-ml steps of 35%, 25%, 15%, 12.5% and 9% Optiprep, prepared by diluting the corresponding volume of OWS in Optiprep gradient buffer (OGB: 10 mM HEPES, pH 7.4; 300 mM sucrose; 1 mM MgSO₄; protease inhibitors). Gradients were centrifuged in a SW28.1 Beckman rotor at 16,500xg for 30 min at 4°C. Fractions located at interfaces 9%-12.5% (O1) and 12.5%-15% (O2) were removed with a Pasteur pipette, washed with 2 volumes of OGB and pelleted by centrifugation at 20,000xg for 10 min at 4°C in JA25.50 Beckman rotor. Pellets O1 and O2 were independently resuspended in 800 µl of HS, manually dispersed by using a 2-ml glass-glass Dounce tissue grinder, and loaded on separate Percoll (Sigma-Aldrich) gradients. These gradients consisted in: 25% Percoll (1-ml step), 20%, 16%, and 14% Percoll (2.5-ml steps) and 10% Percoll (2.15-ml step). Percoll solutions were made by diluting with HS a 30% Percoll stock (30% Percoll; 320 mM sucrose; 10 mM HEPES, pH 7.4; 1 mM MgSO₄; protease inhibitors). Gradients were centrifuged in a SW41 Ti Beckman rotor at 32,400xg for 20 min at 4°C. Fractions were removed with a Pasteur pipette, diluted with HS and pelleted by centrifugation at 20,000xg for 10 min at 4°C in 1.5-ml Eppendorf tubes.

For a detailed lab protocol of the described method see Supplementary material file S2.

Quantitative immunoblot analysis

Subcellular fractions were subjected to SDS-PAGE, and gels were either stained with Coomassie blue and scanned for relative quantification of protein, or transferred to polyvinylidene difluoride (PVDF) membranes for Western blot analysis. Antibodies against

the following proteins were used: Glutamate AMPA receptor 2 (GluR2) (1:2,000; NeuroMab clone L21/32); NMDA receptor 1 (NR1) (1:4,000; Cl54.1 antibody); Cysteine string protein α (CSP α) (1:4,000; R807 antibody); Synapsins (1:4,000; E028 antibody, which recognizes synapsin Ia, Ib, IIa and IIb); Glial fibrillary acidic protein (GFAP) (1:10,000; Sigma clone G-A-5); Post-synaptic density 95 (PSD95) protein (1:10,000; AbCam); RhoA (1:1,000; Santa Cruz Biotechnology clone 26C4). Activity of horseradish peroxidase-conjugated secondary antibodies (Promega) was revealed with ECL or ECL plus (GE Healthcare Life Sciences), depending on the required sensitivity. Chemiluminescence from blots was directly measured using a ChemiDoc XRS apparatus (BioRad). Linearity of chemiluminescent signals was determined in pilot experiments for each antibody, using 3-fold dilutions of P1 sample.

Electron microscopy

Material from 1P4 neocortical subcellular fraction was pelleted and fixed for electron microscopy with 2% glutaraldehyde in 0.1M cacodylate (pH 7.4) for 1 h at 4°C. Sample was then washed with cacodylate, secondary-fixed in 1% OsO₄, gradually dehydrated and embedded in Spurr resin. Sections were visualized in a Philips CM-10 transmission electron microscope equipped with a CDD camera.

RNA isolation and real time quantitative RT-PCR

RNA from subcellular fractions was isolated (PureLink Micro to Midi total RNA, Invitrogen) and treated on column with DNase (Sigma-Aldrich) during the isolation procedure. RNA was quantified with a NanoDrop apparatus (Thermo Scientific) and reverse transcribed with random hexamer primers (Transcriptor First Strand cDNA Synthesis Kit, Roche). cDNA samples (and negative controls in which retrotranscriptase was omitted) were subjected to quantitative real-time PCR (3-5 replicates) using Brilliant SYBR Green QPCR Master Mix

(Stratagene) in a Mx3000P apparatus (Stratagene). Primers sequences for α -Ca²⁺-calmodulin-dependent protein kinase II (α -CamKII), *RhoA* and hypoxanthine-guanine phosphoribosyltransferase (*HPRT*) amplification were:

α -CamKII-fw 5'-GGAGGAAACAAGAAGAACGATG

α -CamKII-rv 5'-TCAGCTGCTCTGTCACTTTGAT

RhoA-fw 5'-AAACTATGTGGCGGATATCGAG

RhoA-rv 5'-CGGTGTCTGGATAAGAGAGAGG

HPRT-fw 5'-GGACCTCTCGAAGTGTTGGATA

HPRT-rv 5'-ACGTGATTCAAATCCCTGAAGT

The efficacies of primers, calculated from standard curves using cDNA serial dilutions, were the following: 90.4% for α -CaMKII, 107.3% for *RhoA* and 85.5% for *HPRT*; r^2 values of standard curves were 0.991, 0.993 and 0.996, respectively. These figures were taken into account for calculations using the qBase software (Hellemans et al., 2007).

Metabolic labeling of synaptoneurosomes

Neocortical synaptoneurosomes were resuspended in synaptoneurosome buffer (SB), consisting in 10 mM HEPES, pH 7.4; 2.2 mM CaCl₂; 0.5 mM Na₂HPO₄; 0.4 mM KH₂PO₄; 4 mM NaHCO₃ and 80 mM NaCl, supplemented with 100 µg/ml chloramphenicol (Huang and Richter, 2007). After pre-incubation at 37°C for 3 min they were incubated for 20-30 min at 37°C in the presence of EasyTag Express Protein Labeling Mix ³⁵S (Perkin Elmer). For induction experiments, BDNF (Sigma-Aldrich) was also included (100 ng/ml).

Samples were pelleted, washed with SB, and subjected to SDS-PAGE (or to immunoprecipitation). Before drying and exposing gels, they were treated with Amplify following the manufacturer's instructions (GE Healthcare Life Sciences). Radioactive proteins were detected with a Cyclone Plus Phosphor Imager system (Perkin Elmer).

Immunoprecipitation

Synaptoneurosome pellets were resuspended in 100 μ l of lysis buffer containing 10 mM Tris-HCl, pH 8.0, 1% SDS, 1 mM Na_3VO_4 and protease inhibitors. After incubation at 68°C for 5 min, 900 μ l of immunoprecipitation buffer (10 mM Tris-HCl, pH 8.0; 100 mM NaCl; 2 mM EDTA, 1% Triton X-100, 1 mM Na_3VO_4 and protease inhibitors) supplemented with 2% BSA were added to the samples. After centrifugation for 10 min at 10,000xg, supernatants were subjected to RhoA immunoprecipitation (3 μ g antibody per tube) using Dynabeads Protein G (Invitrogen), following manufacturer's instructions. Immunoprecipitated proteins were subjected to SDS-PAGE and transferred to a PVDF membrane. Radioactive bands were detected by exposing the membrane on a BioMax MS film (Kodak), using a BioMax TranScreen-LE intensifying screen (Kodak).

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Figure Legends

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

Fig. 2. Western blot analysis of gradient fractions from mouse hippocampus. Immunodetection of post-synaptic (NR1, PSD95 and GluR2), pre-synaptic (CSP α and synapsins Ia, Ib, IIa, IIb) and glial (GFAP) proteins present in: (A) P1, O1 and O1-derived Percoll fractions. (B) P1, O2 and O2-derived Percoll fractions. A representative experiment is shown. Protein loaded per lane, normalized to P1 sample (containing 10 μ g of protein) is shown in arbitrary units. Fractions 1P3 and 2P3 were not analyzed due to the low amount of material therein found. (C) Quantification of immunodetected proteins showed in panel A. (D) Quantification of immunodetected proteins showed in panel B. Measurement of direct chemiluminescence was normalized to the relative quantity of protein present in the corresponding sample. Normalized chemiluminescence for P1 was arbitrarily designed as 1.

Fig. 3. Morphological and functional characterization of neocortical 1P4 synaptoneurosomes. (A) Low magnification electron micrograph showing synaptoneurosomes with pre-synaptic (pre) and post-synaptic (post) elements. Mitochondria (mit) were present in some pre-synaptic elements. Bar = 1.0 μ m. (B) Higher magnification of panel A micrograph showing a synaptoneurosome in which synaptic vesicles (asterisk), synaptic cleft (arrow) and post-synaptic densities (arrowheads) can be easily distinguished. Bar = 500 nm. (C) Real time RT-PCR quantification of a dendritic (α -CaMKII) and a somatic (HPRT) mRNA in neocortical P1, O1 and O1-derived Percoll fractions. Data were normalized

to P1 sample. Error bars: standard deviation (SD) from 2 independent PCR determinations.

(D) Metabolic labeling of proteins in 1P4 synaptoneurosomes: autoradiography of proteins newly synthesized (^{35}S protein) when incubated in the presence of ^{35}S -Met and ^{35}S -Cys for 30 min. For sake of comparison, a Coomassie blue-stained gel showing both newly synthesized and pre-existent proteins contained in the sample (total protein) is also shown.

Fig. 4. Localization and translation of *RhoA* mRNA in neocortical 1P4 synaptoneurosomes. (A) Distribution of RhoA protein in fractions P1, O1 and 1P4 detected by quantitative Western blot. (B) Real time RT-PCR quantification of *RhoA* mRNA in 1P4 synaptoneurosomes. *α -CaMKII* and *HPRT* were used as dendritic and somatic controls, respectively. Error bars: standard deviation (SD) from 3 to 5 independent PCR determinations. (C) Immunoprecipitation of newly synthesized RhoA protein from 1P4 synaptoneurosomes. Synaptoneurosomes were incubated with (BDNF) or without (control) 100 ng/ml BDNF for 20 min in the presence of ^{35}S -Met and ^{35}S -Cys. An autoradiography of immunoprecipitated RhoA protein after transferring it to a PVDF membrane is shown.

Table 1

Amount of total protein contained in the isolated hippocampal fractions

Fraction	Protein (μg)
O1	4,229
O2	268
1P1	176
1P2	63
1P4	525
1P5	83
2P1	60
2P2	38
2P4	92
2P5	32

Figure 1
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- tissue homogenization
- centrifuge 1,000 g 10 min
- pellet (P1)

Figure 2
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Figure 3 grayscale
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Figure 3 color
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Figure 4
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Fig. S1. Quantification by immunoblot of synaptic proteins and glial contamination in gradient fractions from mouse neocortex. (A) Measurement of direct chemiluminescence from immunodetected proteins in P1, O1, O2 and the corresponding Percoll-derived fractions was normalized to the relative quantity of protein present in the sample. Normalized chemiluminescence for P1 was arbitrarily set to 1. A representative experiment is shown. Fractions 1P5, 2P1 and 2P5 were not fully characterized, due to the variable amount of material present in those fractions from experiment to experiment, but at least in the case of 2P1 and 2P5, no enrichment for NR1 and GluR2 proteins was observed (not shown). GFAP levels for fractions 2P2, 2P3 and 2P4 were not determined. (B) Comparison of synaptic protein enrichment and glial contamination level obtained when combining the O1 and O2 fractions versus processing the O1 fraction separately. Levels of GluR2, CSP α and GFAP proteins were determined and normalized as stated in panel A; data are shown as the percentage of enrichment of the corresponding marker found in Percoll fraction 4 (P4 when processing O1+O2 together), compared to the 1P4 fraction (100%).