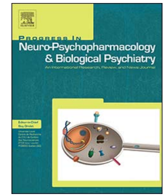
Progress in Neuropsychopharmacology & Biological Psychiatry xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

Progress in Neuropsychopharmacology & Biological Psychiatry

journal homepage: www.elsevier.com/locate/pnp



Characterisation of spinophilin immunoreactivity in postmortem human brain homogenates

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ARTICLE INFO

Keywords:
Spinophilin
Human brain
Postmortem
Western blot

ABSTRACT

Spinophilin is a multifunctional scaffold protein that regulates the formation and function of dendritic spines and plays a role in neuronal migration. The distinct roles of spinophilin depend on its localization and the direct interaction with other proteins, which may target spinophilin to specific locations within the cell. Several studies suggest a role of spinophilin in the pathophysiology of neurological or psychiatric diseases. However, the majority have been performed in animals or cultured cells. Thus, the aim of the present study was to characterise the regional and subcellular expression of spinophilin immunoreactivity by western blot in postmortem human brain. Two specific immunoreactive bands for spinophilin were observed: an intense band migrating at around 120 kDa, which seems to correspond to the apparent molecular weight of spinophilin described by other authors, and a less intense band of around 95 kDa. This second form seems to be a proteolysis or cleavage product of the ~120 kDa spinophilin. Interestingly, the subcellular distribution of both bands was different. In membrane fraction, the ~120 kDa spinophilin band was the most abundant, whereas in cytosol it was the ~95 kDa form. Furthermore, a different regional distribution for ~120 kDa spinophilin band was observed, with the highest expression in prefrontal cortex, followed by hippocampus and cerebellum, and the lowest in caudate nucleus. Altogether, these results constitute a useful reference for future studies of spinophilin in pathological and non-pathological human brain tissues.

1. Introduction

Spinophilin, also called neurabin II, is a multifunctional scaffold protein, initially described as a direct binding partner of protein phosphatase 1 (PP1) (Allen et al., 1997) and F-actin (Satoh et al., 1998). Since, much more partner proteins of spinophilin have been discovered, including cytoskeletal and cell adhesion molecules, enzymes, membrane receptors, ion channels, guanine nucleotide exchange factors, regulator of G-protein signaling proteins and tumour suppressors (Sarrouilhe et al., 2006).

The functions of spinophilin have been mainly studied in the central nervous system, where it is highly enriched in dendritic spines (Allen et al., 1997; Muly et al., 2004; Ouimet et al., 2004; Satoh et al., 1998). Studies with spinophilin knock out mice have revealed that this protein regulates the formation and function of dendritic spines (Feng et al., 2000). It has been shown that it stabilises actin-based cytoskeleton in dendritic spines and filopodia (Satoh et al., 1998; Yan et al., 1999). Spinophilin also regulates the activity of PP1, an enzyme implicated in postynaptic signal integration, by targeting the enzyme in a close vicinity to its substrate and allowing its dephosphorylating activity (Sarrouilhe et al., 2006; Satoh et al., 1998; Yan et al., 1999). Among the substrates of PP1 are the enzyme calcium/calmoduling-dependent protein kinase II (Yoshimura et al., 1999) and neurotransmitter receptors, such as the ionotropic AMPA glutamate channel (Yan et al., 1999). Spinophilin directly binds different membrane receptors for neurotransmitters too: dopaminergic D2 (Smith et al., 1999), α-adrenergic (Richman et al., 2001; Wang et al., 2004; Wang and Limbird, 2002), opioid (Fourla et al., 2012) and glutamatergic metabotropic (Di Sebastiano et al., 2016) and ionotropic AMPA receptors (Yan et al., 1999). It seems that spinophilin acts as a scaffold protein that brings intracellular signaling proteins into close proximity of cell membrane receptors, and might serve as a link between synaptic transmission and changes in spine morphology and density. In addition, spinophilin has been proposed to play a role in neuronal migration, by its interaction

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http://dx.doi.org/10.1016/j.pnpbp.2017.09.019
Received 19 April 2017; Received in revised form 14 September 2017; Accepted 19 September 2017
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with doublecortin, a microtubule-associated protein required for this process (Tsukada et al., 2003).

The distinct roles of spinophilin depend on its localization and the direct interaction with other proteins, which may target spinophilin to specific locations within the cell. Spinophilin is present in brain regions such as prefrontal cortex, hippocampus, caudate/putamen and cerebellum (Ouimet et al., 2004). Immunolabeling studies have determined that it is highly expressed in dendritic spines and postsynaptic density enriched forms, but it can also be found in preterminal axons and glia (Muly et al., 2004; Ouimet et al., 2004).

It is of great interest to study the role of spinophilin in the pathophysiology of different neurological and psychiatric diseases. Several studies have focused on the implication of spinophilin in schizophrenia (Baracskay et al., 2006; Clinton et al., 2005; Sweet et al., 2009), Alzheimer's (Akram et al., 2008; Palavicini et al., 2013) and Parkinson's diseases (Brown et al., 2008). However, the majority of the research studying the physiological or phathophysiological roles of spinophilin has been performed in animals or cultured cells. In this context, the use of postmortem human brain provides a unique opportunity to advance research in this area (McCullumsmith et al., 2014). In the present study, a characterisation of regional and subcellular expression of spinophilin immunoreactivity was performed by western blot assays in several postmortem human brain regions.

2. Material and methods

2.1. Materials

The rabbit anti-human spinophilin polyclonal antiserum (LS-C162085) and the corresponding spinophilin antibody blocking peptide (LS-E4298) were obtained from LifeSpan BioSciences. Mouse anti-human spinophilin monoclonal antiserum (sc-373974) was purchased from Santa Cruz Biotechnology. Mouse anti-β-actin monoclonal antiserum (A1978) was purchased from Sigma-Aldrich. All other chemicals were obtained from Sigma-Aldrich, Panreac, MERCK, Bio-Rad, Carlo Erba reagents, National diagnostics and Quimipur.

2.2. Human samples

Human brain samples from subjects who died by sudden and violent causes were obtained at autopsy in the Basque Institute of Legal Medicine, Bilbao, Spain. All the subjects were determined to be free of psychiatric or neurological disorders based on medical histories and postmortem tissue examinations. Samples from the prefrontal cortex, caudate nucleus, hippocampus and cerebellum were dissected at the time of autopsy and immediately stored at − 70 °C until assay. The postmortem delay (PMD) of the samples ranged from 4 to 48 h. For all the experiments, with the exception of the correlations with different variables, a pool preparation containing four different samples was used. The study was developed in compliance with legal policy and ethical review boards for postmortem brain studies.

2.3. Preparation of membranes

Samples were prepared as previously published (Erdozain et al., 2015) with minor modifications. The frozen samples were cut and cleaned of meninges and white matter to the best extent possible. White matter was pooled and treated as another sample. Each tissue sample (1 g) was homogenized with an ultraturrax in 5 ml of homogenization buffer (0.32 M sucrose in 5 mM Tris-HCl pH 7.4), supplemented with protease and phosphatase inhibitors (50 μl/g of Sigma protease inhibitor Cocktail, 5 mM Na_3VO_4 and 10 mM NaF). In some degradation experiments, protease and phosphatase inhibitors were not added but when this is the case it is also mentioned in the Results section. The total homogenate was centrifuged 10 min (4 °C) at 1100 × *g* (Sorvall RC-5C centrifuge, SM-24 rotor) to remove the nuclear fraction. The supernatant was recentrifuged 10 min (4 °C) at 40000 × *g* (Sorvall RC-5C centrifuge, SM-24 rotor). The new supernatant corresponded to the cytosolic fraction. The pellet was resuspended in 5 mM Tris-HCl pH 7.4 buffer (supplemented with the above mentioned protease and phosphatase inhibitors) and recentrifuged again. This procedure was repeated twice to obtain the final pellet of membrane-enriched fraction (P_2 fraction). That was resuspended in 1.2 ml of the same buffer with protease and phosphatase inhibitors. The HT, Cyt and P2 fractions were stored at − 70 °C until assay. Protein content was measured by the Bradford's method (Bradford, 1976).

2.4. Western blot

Western blot assays were performed as previously described (Erdozain et al., 2015), with minor modifications. For general experiments, samples were prepared in the electrophoresis buffer in reducing and denaturing conditions (100 mM DTT, 2% SDS, 8% glycerol, 0.01% bromophenol blue, and heated at 95 °C for 5 min). In some experiments, samples were not heated 95 °C for 5 min (see Section 2.6) but when this is the case it is also mentioned in the Results section. Solubilized proteins (20 μg, except for the protein-dependent curves) were resolved on 10% SDS-PAGE gels and transferred to nitrocellulose membranes. After being blocked (5% non-fat dry milk in PBS with 1% Tween 20) for 1 h at room temperature, the membranes were incubated overnight at 4 °C with constant agitation with the primary antibodies against: spinophilin (LifeSpan BioSciences LS-C162085, 1:1000; Santa Cruz sc-373974, 1:250) or β-actin (Sigma A1978, 1:50000). For specificity assays with the blocking peptide, the antibody was previously incubated with the blocking peptide (1:500) for 1 h at room temperature in the same blocking solution. Next morning, membranes were washed again with PBS and incubated for 90 min at room temperature with the fluorescent secondary antibodies (IRDye™ 800 or Alexa Fluor® 680 conjugated). The immunoreactive signal (integrated intensity values) was detected using the Odyssey infrared imaging system (LI-COR Biosciences) and quantified using Image Studio Lite 5.2 (LI-COR Biosciences). For the quantification analysis the immunoreactivity values were normalized for β-actin signal.

2.5. Immunoprecipitation assay

Total homogenate lysate from postmortem human prefrontal cortex was prepared in lysis buffer (10 mM Tris-HCl, 1% triton X-100, supplemented with protease and phosphatase inhibitors as in Section 2.3). Immunoprecipitations were performed using either polyclonal rabbit anti-spinophilin (LifeSpan BioSciences, LS-C162085) or monoclonal mouse anti-spinophilin (Santa Cruz, sc-373974) antibodies. Lysates were precleared 3 h at 4 °C with protein A/G agarose (Santa Cruz, sc-2003), then incubated with the appropriate antibody (5 μg for 500 μg of tissue) at 4 °C overnight. Protein A/G agarose beads were added and samples were incubated for additional 3 h at 4 °C, followed by three washes with lysis buffer. Elution was performed at 95 °C for 5 min in electrophoresis sample buffer (see Section 2.4) and samples were centrifuged to pellet the agarose beads. The supernatants were analysed by western blot.

2.6. Proteolysis study

For general proteolysis study, membrane-enriched fractions were prepared as previously explained in the presence or absence or any protease inhibitors, and incubated at 37 °C for a range of time (0–180 min). Then samples were prepared in the loading buffer (see Section 2.4) and heated at 95 °C for 5 min. For temperature-dependent proteolysis assay, samples were heated at different temperatures (4 °C–95 °C) for 15 min directly in the loading buffer, and were not heated anymore. To study the effect of freeze/thaw cycles, cortical membrane-enriched fractions were either subjected to five cycles of

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