Neuroscience 163 (2009) 586–593

RhoE IS SPATIOTEMPORALLY REGULATED IN THE POSTNATAL MOUSE CNS

B. BALLESTER-LURBE,[a] E. POCH,[b] E. MOCHOLÍ,[b] R. M. GUASCH,[c] I. PÉREZ-ROGER[b] AND J. TERRADO[a]*

[a]*Department of Animal Medicine and Surgery, University CEU-Cardenal Herrera, Avda Seminario sn, 46113 Moncada, Valencia, Spain*

[b]*Department of Chemistry, Biochemistry and Molecular Biology, University CEU-Cardenal Herrera, Avda Seminario sn, 46113 Moncada, Valencia, Spain*

[c]*Department of Cellular Pathology, Centro de Investigación Príncipe Felipe, Avda Autopista del Saler, 16, 46012, Valencia, Spain*

Abstract—Rnd proteins are a family of small GTPases that have been involved in axon path finding and CNS development by their control of actin cytoskeleton dynamics. Rnd proteins are constitutively activated and, subsequently, their functions determined by their localization and expression levels. In this work we have analyzed by Western blot and immunohistochemistry the levels and localization of Rnd3/RhoE during mouse postnatal development. CNS was found to be the main tissue for RhoE protein expression, which was detected in all regions of the adult brain and spinal cord, with the highest levels in the olfactory bulb and cortex. RhoE protein levels were considerably higher in all the regions of the CNS the first 2–3 weeks of postnatal development, undergoing later a decrease that led to low levels in the adult. Immunohistochemical detection of RhoE at postnatal day 21 showed an intense and widespread labelling throughout the CNS. RhoE immunoreactivity was detected in the granular and mitral cells and anterior olfactory nuclei of the olfactory bulb and in all cerebral layers. In the striatum, diencephalon, mesencephalon, pons, medulla oblongata and spinal cord, RhoE was widely distributed with higher intensity in the motoneurones and in some brainstem nuclei such as the red nucleus or the reticulotegmental nucleus. The pyramidal cells of CA1-3 and the polymorph layer, but not the granular cells of the dentate gyrus in the hippocampus were strongly labelled. At earlier stages the labelling was nearly similar; however, a prominent labelling was detected in the cells of the rostral migratory stream and in the external granule cells of the cerebellum. Our results suggest that RhoE can play important roles in the postnatal development and maturation of the CNS, especially in the migratory processes affecting the neurones.

Key words: Rnd, actin cytoskeleton, Rho GTPases.

Mammalian Rho GTPases comprise a family of 22 intracellular signalling molecules, best documented for their important roles in regulating the actin cytoskeleton. Most Rho GTPases switch between an active GTP-bound form and an inactive GDP-bound form (Heasman and Ridley, 2008). In the last years an emerging role for the Rho family of proteins has been demonstrated in the nervous system development and function (Govek et al., 2005; Linseman and Loucks, 2008). Because of their role in cytoskeleton regulation, they are involved in several aspects of neuronal migration, neurite formation and growth and in maintaining dendritic spines (Govek et al., 2005). Moreover, mutations of their regulators and effectors contribute to produce different neurological deficits. As a consequence, Rho proteins and/or their regulators have been involved in many pathologies of the nervous system, from cognitive deficits to nerve repair or motoneurone disease (Linseman and Loucks, 2008).

RhoE/Rnd3 (hereafter referred to as RhoE) is a member of the Rnd subfamily (Foster et al., 1996; Guasch et al., 1998), which is also composed of Rnd1 and Rnd2 (Chardin, 2006). These proteins lack GTPase activity and do not bind GDP, and are, therefore, in a constitutively active state. As a consequence, their physiological role essentially depends on their expression and localization (Chardin, 2006). RhoE binds and inhibits the RhoA effector ROCK I (Riento et al., 2003) and interacts with p190-Rho-GAP increasing the GTPase activity of RhoA and thus, inactivating it (Wennerberg et al., 2003). As a consequence, RhoE antagonises RhoA function altering the actin cytoskeleton organization and inducing cell motility (Guasch et al., 1998). In addition to this role in actin dynamics, RhoE is also involved in the control of cell cycle and survival in some cell lines (Villalonga et al., 2004; Bektic et al., 2005; Poch et al., 2007).

RhoE, as well as the other Rnd proteins, is expressed in the brain, and functional studies suggest that Rnd proteins are involved in axon path finding in neurones and may have important roles in CNS development (Chardin, 2006). Recent works have shown the role of Rnd2 in brain development (Heng et al., 2008) and the expression of Rnd1 in the brain (Nobes et al., 1998). However, little is known regarding the role of RhoE in the nervous system, although it contributes to the inflammatory response induced by ethanol in astrocytes (Guasch et al., 2007). Nevertheless, the role and regulation of RhoE proteins in the CNS remain to be elucidated.

The aim of our work is to study the regulation and distribution of RhoE expression during the postnatal CNS development. Although the levels of RhoE in the adult brain are considered low, higher levels of this protein during development would suggest a role in CNS formation as it has been shown for other proteins of the same family.

*Corresponding author. Tel: +34-961-369-000; fax: +34-961-395-272.
E-mail address: jterrado@uch.ceu.es (J. Terrado).
Abbreviations: EGL, external granule cell layer; IGL, internal granule cell layer; IR, immunoreactivity; RMS, rostral migratory stream.

0306-4522/09 $ - see front matter
doi:10.1016/j.neuroscience.2009.06.062

EXPERIMENTAL PROCEDURES

Animals

C57/BL6/129 mice from Harlan (Barcelona, Spain) were housed, bred and sacrificed according to the European Council legislation 86/609/EEC concerning experimental animal protection. Breeding pairs of mice were monitored daily for litters, and the day of birth was taken as day=0. All animals were maintained on a 12-h light/dark cycle, at constant room temperature (22 °C), and with free access to water and standard mouse fodder. The mice were sacrificed by decapitation or by an overdose of pentobarbital. The brains and spinal cords were obtained at postnatal (P) days 0, 7, 14 and 21 and 3 months (considered as adults). The kidneys, spleen, intestine, lung, liver, testis and thymus were also obtained from the adult mice. All experimental protocols were approved by the Ethical Committee of the CEU-Cardenal Herrera University. All efforts were made to minimize the number of animals used and their suffering.

Western blot

The brains were dissected separating the olfactory bulb, brainstem (pons and medulla oblongata), cerebellum, mesencephalon, cerebral cortex, hippocampus, striatum and diencephalon. The spinal cords were separated in their cervical, thoracic and lumbar segments. Samples from the different tissues were homogenised in lysis buffer (150 mM NaCl, 1% Triton X-100, 1 mM DTT, 50 mM Tris, pH 8.0, 10 mM NaF, 1 mM Na_3VO_4) and a cocktail of protease inhibitors (Complete Mini, Roche, Mannheim, Germany) and centrifuged at 20,800×*g* during 20 min, and the supernatant was collected and quantified by the Bradford assay (Bio-Rad, Hercules, CA, USA). Fifty microgram samples were resolved on 11% gels by SDS-PAGE and transferred to PVDF membranes (Millipore, Billerica, MA, USA). Then they were blocked with 5% nonfat milk for 20 min at room temperature and incubated at 4 °C overnight with the appropriate antibody, a monoclonal mouse anti-RhoE from Upstate (Lake Placid, NY, USA) at 1:500, or mouse anti-actin bound to peroxidase from Sigma-Aldrich (St. Louis, MO, USA) at 1:20,000. Then the membranes were washed three times with 0.1 M phosphate buffer containing 0.1% Tween 20 and incubated with a peroxidase-conjugated secondary antibody. Finally, the blots were developed using an enhanced chemiluminescence (ECL) Western blotting detection kit from Amersham Pharmacia Biotech (Little Chalfont, UK). The specificity of the anti-RhoE antibody was assessed by pre-incubating the antibody with an excess of *in vitro* produced full-length RhoE protein, which was obtained by a transcription–translation-coupled reaction. Briefly, 2 μg of pcDNA Flag RhoE was incubated with TNT T7 Quick Coupled Transcription/Translation System (Promega, Madison, WI, USA) for 2 h at 30 °C. Then, the reaction product was pre-incubated with the RhoE antibody during 2 h at 4 °C, before the incubation of the blot.

Immunohistochemistry

The animals were perfused with 0.1 M PBS and fixed in 4% paraformaldehyde. The brains were extracted and postfixed overnight in the same fixative solution, dehydrated in increasing concentrations of ethanol, embedded in paraffin, serially sectioned (5 μm) in an HM 310 Microm microtome (Walldorf, Germany) and collected on polylysine-coated slides. Antigen retrieval of deparaffined and rehydrated sections was performed by heating at 100 °C in a water-bath for 15 min in citrate buffer (10 mM pH 8). Then, the sections were washed three times in 0.1 M phosphate buffer with 0.2% Triton X-100 (PBST) and incubated with 3% H_2O_2 in methanol for 40 min to quench endogenous peroxidase activity. Nonspecific binding was blocked with 10% normal goat serum in 3% BSA. Immunohistochemistry for RhoE was performed using the immunoperoxidase procedure of Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA, USA). Briefly, the sections were incubated with the primary antibody overnight at 4 °C in a humidified chamber. Then they were incubated 1 h with a biotinylated secondary anti-mouse antibody, amplified with the avidin–peroxidase complex and finally revealed by diaminobenzidine tetrahydrochloride stain.

RESULTS

In the adult mice, RhoE is preferentially expressed in the brain

We first compared by Western blot the levels of RhoE protein in different organs in the adult mice. Two bands were detected for RhoE with slight variations in the apparent MW in different tissues, which may be due to post-translational modifications, as previously reported (Riento et al., 2005a; Fortier et al., 2008; Klein et al., 2008). Expression levels in the adult tissues were, in general, low, but the highest levels of RhoE were detected in the brain (Fig. 1A). It was also detected in the testis, liver and lung, whereas lower levels were observed in the protein extracts of spleen and intestine. The kidney and thymus were almost devoid of RhoE (Fig. 1A). To investigate in more detail the levels of RhoE protein in the different regions of the nervous system, adult brains were dissected separating the olfactory bulbs, striatum, cerebral cortex, hippocampus, diencephalon, mesencephalon, cerebellum and brainstem (comprising pons plus medulla oblongata) and the RhoE protein levels were analyzed by Western blot. RhoE was detected in all encephalic regions, as shown in Fig. 1B. The highest levels of RhoE were detected in the olfactory bulb and cortex. Intermediate levels were appreciated in the diencephalon, mesencephalon and striatum, whereas the hippocampus, cerebellum and brainstem showed the lowest levels. The cervical, thoracic and lumbar segments of the spinal cord also expressed RhoE at low levels. Preincubation of the antibody with an excess of RhoE protein confirmed the specificity of the antibody (Fig. 1B).

RhoE protein levels are progressively reduced in the postnatal mouse brain

To study the progression of RhoE expression, the CNS of mice from P0, P7, P14, P21 and from adult mice (about 3 months) was dissected and the levels of RhoE were analyzed by Western blot (Fig. 2). The highest levels of RhoE expression were detected, in most of the CNS regions, during the first postnatal weeks. In the spinal cord, brainstem, cerebellum, mesencephalon, striatum and diencephalon the expression of RhoE was higher at postnatal days P0, P7 and P14, and then it decreased into the adulthood, when it reached its final levels. On the other hand, the levels of Rhoe remained high in the olfactory bulb and in the cerebral cortex throughout the postnatal development, and only slightly decreased in the adult. Strikingly, the levels in the hippocampus peaked at P14, being lower before and after this age.

RhoE is widely expressed within the CNS

To establish the localization of RhoE in the CNS, brains and spinal cords at postnatal day 21 were analyzed by