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# In vivo function of Rnd2 in the development of neocortical pyramidal neurons

Kazuhiro Nakamura [a,b,1,*], Yoko Yamashita [b,1], Nobuaki Tamamaki [a,2], Hironori Katoh [b], Takeshi Kaneko [a,c], Manabu Negishi [b]

[a] Department of Morphological Brain Science, Graduate School of Medicine, Kyoto University, Sakyo-ku, Kyoto 606-8501, Japan
[b] Laboratory of Molecular Neurobiology, Graduate School of Biostudies, Kyoto University, Sakyo-ku, Kyoto 606-8501, Japan
[c] Core Research for Evolution Science and Technology, Japan Science and Technology, Japan

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**Abstract**

The present study examined the in vivo role of Rnd2, a Rho family small GTPase, in brain development. Rnd2 was expressed by radially migrating cells, which primarily develop to pyramidal neurons, during their stay in the subventricular zone of embryonic cerebral cortex and hippocampus. Exogenous expression of wild-type and a constitutively active Rnd2, but not a negative mutant of Rnd2, in radially migrating cells by in utero electroporation disturbed their morphology and migration to upper layers. These results indicate that Rnd2 functions in vivo as a regulator of the migration and morphological changes associated with the development of pyramidal neurons.


*Keywords:* Ganglionic eminence; Immunohistochemistry; In situ hybridization; In vivo electroporation; Neocortical development; Radial migration; Subventricular zone; Ventricular zone

It is an ultimate goal of developmental neurobiology to understand how the fine laminar structure and highly organized neuronal networks in the cerebral cortex is formed in the embryonic brain. Cortical pyramidal neurons are primarily divided from precursor cells of the germinal ventricular zone (VZ) of the dorsal telencephalon and migrate radially to their positions in the developing cortex in an ''inside-out'' manner. Most cortical interneurons are, on the other hand, generated in the VZ of the ganglionic eminence and migrate tangentially into the cortex (Nadarajah and Parnavelas, 2002). The neuronal development involves various cellular events, such as cell division, migration, axon and dendrite growth, and synapse formation, and these are associated with cellular morphological changes.

It is known that the Rho family of small GTPases act as key intracellular regulators in morphological changes of various cells by reorganizing cytoskeleton (Hall, 1998). Rho GTPases serve as molecular switches by cycling between GDP-bound inactive and GTP-bound active states, and once activated, they transduce signals to a variety of specific downstream effectors, leading to cytoskeletal reorganization. A number of in vitro studies have suggested that Rho GTPases are involved in the regulation of neuronal morphological changes (Luo, 2000). However, little is known about the role of Rho GTPases in vivo.

The Rnd subfamily composes a new branch of the Rho family of GTPases, and as members, Rnd1, Rnd2, and Rnd3/RhoE have been identified (Foster et al., 1996; Nobes et al., 1998). Although, of Rnd proteins, Rnd2 is predominantly expressed in the brain (Nobes et al., 1998; Nishi et al., 1999), the function of Rnd2 remains to be examined. In the present study, we determined the histochemical localization of Rnd2 in developing cerebral cortex and examined the role of this molecule in the development of cortical neurons in vivo by using an in utero electroporation technique.

* Corresponding author. Present address: Neurological Sciences Institute, Oregon Health & Science University, 505 N.W. 185th Av., Beaverton, OR 97006, USA. Tel.: +1 503 418 2672; fax: +1 503 418 2501.
*E-mail address:* nakamura@ohsu.edu (K. Nakamura).
[1] These authors equally contributed to this work.
[2] Present address: Department of Morphological Neural Science, Graduate School of Medical Sciences, Kumamoto University, Kumamoto 860-8556, Japan.

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Production of an antibody against Rnd2 protein and immunoblotting basically followed our previous method (Nakamura et al., 1999). Antisera were raised in rabbits against a GST protein fused with a ‘‘Rnd family insert region’’ of rat Rnd2 (GHRQLRRTDSRRGLQRSTQLPGRPDRGNEGEI), which is a portion unique to Rnd2 in the known Rho family members (Nobes et al., 1998). A polyclonal antibody was affinity-purified from the sera with a column conjugated with a synthetic peptide (CGRPDRGNEGEI) by eluting with 0.1 M triethylamine (pH 11.8).

In utero electroporation was performed according to the procedure by Saito and Nakatsuji (2001). Timed pregnant Wistar rats at E16 and C57/BL6 mice at E13 were anesthetized with pentobarbital (50 mg/kg, i.p.). The abdomen was opened and the uterus was taken out. DNA vectors (1.5 μg/μl, 1–2 μl) were injected into the lateral ventricle of embryos in utero through a glass micropipette and introduced into the VZ of the dorsal cortex by delivering electric pulses to the embryos through the uterus. The uterus was repositioned in the abdominal cavity, and the abdominal wall and skin were sewed up with surgical sutures. The embryos were perfused by postnatal day (P) 0.

Rat embryos were electroporated with an expression vector that contains a gene encoding enhanced green fluorescent protein tagged with the N-terminal palmitoylation signal sequence of growth-associated protein-43 (GGFP; Moriyoshi et al., 1996) under a ubiquitous CAG promoter (Niwa et al., 1991). GGFP enables us to visualize the morphology of the expressing cells in a Golgi stain-like manner (Tamamaki et al., 2000). Mouse embryos were introduced with a vector enabling dual expression of Rnd2 and GGFP; Rnd2 gene was driven by the CAG promoter and the GGFP gene was under the control of internal ribosomal entry sites (IRES) (pCAG-Rnd2-IRES-GGFP). For expression of Rnd2 mutant genes, the Rnd2 gene was replaced with a $Rnd2^{V16}$ or $Rnd2^{N21}$ gene. For control expression, pCAG-IRES-GGFP was used.

Rat embryos later than E15 and neonatal mice were perfused transcardially with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4), and the brains were post-fixed in the same fixative at 4 °C for 8 h. Rat embryos at E11 and 13 were fixed by overnight immersion in the fixative. The tissues were cryoprotected in 25% sucrose solution at 4 °C for 48 h, and frontal sections (40 μm thick) were prepared with a cryostat and mounted onto gelatin-coated glass slides.

Immunohistochemical staining was based on our previous procedure (Nakamura et al., 2000, 2001). The sections were incubated with 2 μg/ml anti-Rnd2 antibody or 0.1 μg/ml anti-enhanced green fluorescent protein rabbit antibody (Tamamaki et al., 2000), and then with 10 μg/ml biotinylated donkey antibody to rabbit IgG (Chemicon, CA, USA). The sections were further incubated with avidin-biotinylated peroxidase complex (1:50; Vector, CA, USA), and bound peroxidase was finally visualized by a diaminobenzidine method. For immunofluorescence staining, sections were incubated with the anti-Rnd2 antibody, with the biotinylated antibody, and then with 2 μg/ml Alexa594-conjugated streptavidin (Molecular Probes, OR, USA).

Nonradioactive in situ hybridization followed our previous method (Hioki et al., 2004). The total coding region of rat Rnd2 cDNA was selected for the antisense and sense probes. Hybridization using the sense probe showed no signals. Although the coding region of human Rnd2 shows 61% and 62% homology to those of human Rnd1 and Rnd3, respectively, significant hybridization signals from the Rnd2 antisense probe were not detected in rat cortical plate (see Fig. 1i), where signals for Rnd1 mRNA were found in our previous observation (unpublished

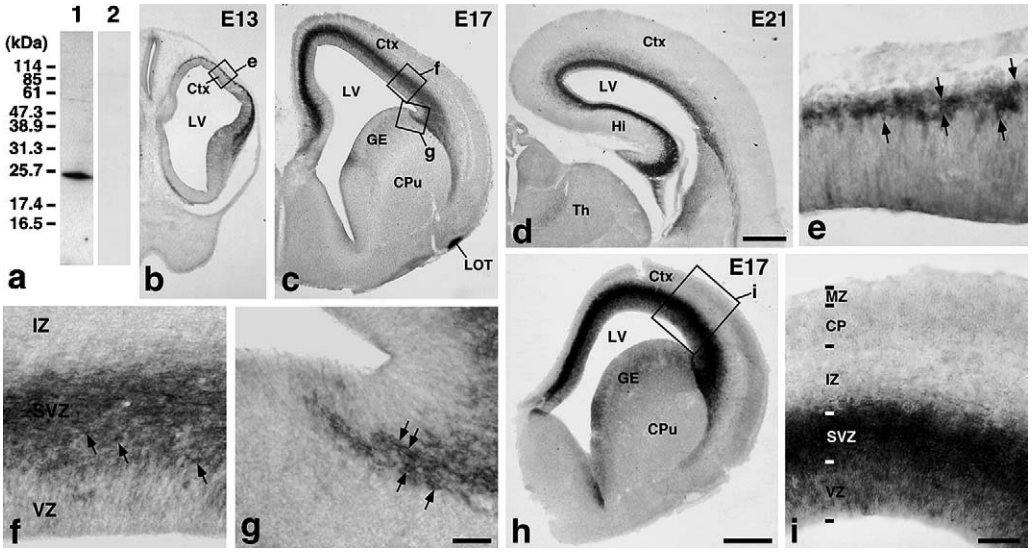


Fig. 1. (a) Immunoblotting of endogenous Rnd2 protein in rat embryonic brain at E19. A single band observed around 25 kDa (lane 1) was absorbed by preincubation of the primary antibody with the antigenic protein (lane 2). (b–g) Immunohistochemistry for Rnd2 in developing rat forebrain. Highly magnified images of the regions indicated by squares in (b) and (c) are shown in (e)–(g). Arrows indicate Rnd2-immunoreactive cell bodies. (h and i) In situ hybridization for Rnd2 mRNA in rat forebrain at E17. A highly magnified image of the squared region in (h) is shown in (i). CP, cortical plate; CPu, caudate putamen; Ctx, cerebral cortex; GE, ganglionic eminence; Hi, hippocampus; IZ, intermediate zone; LOT, lateral olfactory tract; LV, lateral ventricle; MZ, marginal zone; Th, thalamus. Scale bars: (b–d and h) 0.5 mm; (e–g) 50 μm; (i) 100 μm.