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ORIGINAL ARTICLE

Hiroshi Isumi · Sachio Takashima · Kazuhiko Ikeda
Masashi Mizuguchi

Expression of a 45K subunit of platelet-activating factor acetylhydrolase in the developing mouse cerebellum

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Abstract The 45K subunit of platelet-activating factor acetylhydrolase (PAFAH-45K) is the product of a candidate gene for Miller-Dieker lissencephaly. We studied the expression of this protein in the developing mouse cerebellar cortex by immunochemical and immunohistochemical methods. Western blotting studies indicated that PAFAH-45K is more abundant in the fetal than the postnatal period. Immunohistochemical studies revealed developmental changes in the localization of PAFAH-45K-immunoreactivity, which shifted from the somata of Purkinje cells to the neuropil of the molecular layer. Our findings indicate that PAFAH expression is developmentally regulated and suggest its role in histogenetic processes in the cerebellar cortex other than neuronal migration.

Key words PAF acetylhydrolase · Cerebellum · Development

Introduction

Platelet-activating factor acetylhydrolase (PAFAH) inactivates platelet-activating factor (PAF) by removing the acetyl group at its *sn-2* position. The 45K-subunit of PAFAH (PAFAH-45K) purified from bovine brains is a homologue of human LIS-1, a candidate gene for a typical migration disorder called Miller-Dieker lissencephaly (Hattori et al. 1994). We recently examined the expression patterns of human PAFAH-45K in normal human subjects and patients with Miller-Dieker lissencephaly using two specific polyclonal antibodies raised against synthetic peptide fragments (N1 and I2) of this protein (Mizuguchi et al. 1995). PAFAH-45K is abundant in normally developing brains at an early fetal stage, when immature neurons are migrating, and a high level of expression remains at subsequent stages. By contrast, PAFAH-45K immunoreativity is markedly decreased in Miller-Dieker lissencephaly brains. These findings are compatible with the possibility that PAFAH plays a critical role in neuronal migration. We have also shown that both anti-N1 and anti-I2 antibodies recognize its homologues in cows, rats and mice, and that PAFAH-45K is also abundant in the cerebellum, a structure that develops later than the cerebrum (Mizuguchi et al. 1995). In this study, we immunochemically and imunohistochemically examined the developmental expression of PAFAH-45K in the mouse cerebellum in order to explore the role of this protein at early stages of brain histogenesis.

H. Isumi (✉) · S. Takashima
Department of Mental Retardation and Birth Defect Research,
National Institute of Neuroscience,
National Center of Neurology and Psychiatry,
4-1-1 Ogawahigashi, Kodaira, Tokyo 187, Japan,
Tel.: 81-0423-46-1713; Fax: 81-0423-46-1743

K. Ikeda
Department of Ultrastructure and Histochemistry,
Tokyo Institute of Psychiatry, Tokyo, Japan

M. Mizuguchi
Department of Pediatrics, Jichi Medical School, Tochigi, Japan

Materials and methods

Brains were taken from BALB/c mice at six developmental stages: embryonic days 14 (E14) and 17 (E17), and postnatal days 5 (P5), 10 (P10), 40 (P40), and 80 (P80), which had been anesthesized by inhalation of ether. For immunochemical analyses, tissues were kept frozen at –80°C until use. Proteins were extracted from tissue samples with TRIS/saline buffer containing 1% Triton X-100 (Mizuguchi et al. 1995). The subsequent sodium dodecyl sulfate-polyacrylamide gel electrophoresis, semi-dry electrophoretic transfer, antibody binding, and detection with horseradish peroxidase were performed as described previously (Mizuguchi et al. 1994). The amount of protein loaded on each lane was adjusted to 40 µg. The anti-LIS-1 antisera were used at a dilution of 1:750.

For immunohistochemistry, brains were cut coronally or sagittally, fixed for 7 days with 4% paraformaldehyde in 0.1 mol/L phosphate buffer, pH 7.4. They were embedded in paraffin, since the results of preliminary experiments were identical between frozen and paraffin-embedded sections. Subsequentry, 6 µm-thick sections were prepared and were immunostained for PAFAH-45 K by the biotin-streptavidin-peroxidase method, as described previously (Mizuguchi et al. 1995). In an attempt to identify immature Purkinje cells, some of the adjacent sections were stained with a mouse monoclonal antibody against calbindin D-28k (Sigma, St. Louis, Mo., USA; diluted 1:200), a immunohistochemical marker specific to Purkinje cells (Baimbridge and Miller 1982), followed by biotinylated anti-mouse IgG (Seikagaku, Tokyo, Japan).

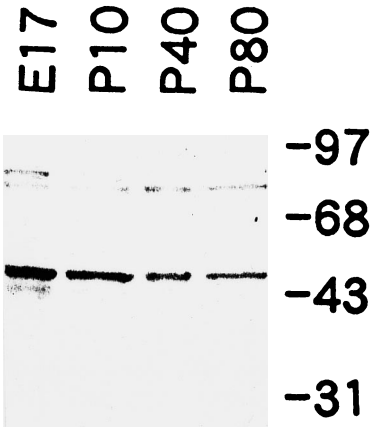


Fig. 1 Western blots of cerebellar homogenates of mice at E17, P10, P40 and P80, stained with the N1 antibody against the 45K subunit of platelet-activating factor acetylhydrolase (PAFAH-45K)

Results

In Western blots of cerebellar tissues at all ages, PAFAH-45K was detected with both anti-N1 and anti-I2 antibodies as a band of approximately 45 kd (Fig. 1). The amount of the protein was highest at E17 and then decreased with increasing age.

The results of immunostaining were identical with the two antibodies. At E14–17, staining was most intense in the somata of neurons in the Purkinje cell layer. During subsequent development (to P80), there was a gradual decrease in the labeling intensity of these neurons, which were readily identifiable as Purkinje cells at these stages. In contrast, the immunoreactivity in the molecular layer increased during the postnatal period. At P5–10, fine granular immunoproducts were present in the inner half of this layer (Figs. 2B, D, 3C). Although the dendritic trunks of the Purkinje cells were not stained, the distribution of PAFAH-45K immunoreactivity appeared to be associated with their arborization (Figs. 2D, E). At P40–80, intense labeling was noted in the entire molecular layer (Figs. 2F, 3D). Immunoreactivity was weak in the external and internal granular cells, which were recognizable at E17–P10 and P5–P80, respectively. The staining of blood vessels was weak at P5 and minimal at P10–P80. The white matter was unlabeled at all stages.

In negative control experiments involving preimmune or preabsorbed antisera, no positive staining was observed.

Immunoreactivity for calbindin D-28k was confined to the Purkinje cells. Their cell bodies were positively stained at P5–80, and their dendrites at P10–80 (Figs. 2C, E, G). At P5, the distribution of cell bodies stained positively for PAFAH-45K was similar to that for calbindin D-28k (Figs. 2B, C), which confirmed the identification of PAFAH-45K-immunoreactive cells as Purkinje cells.

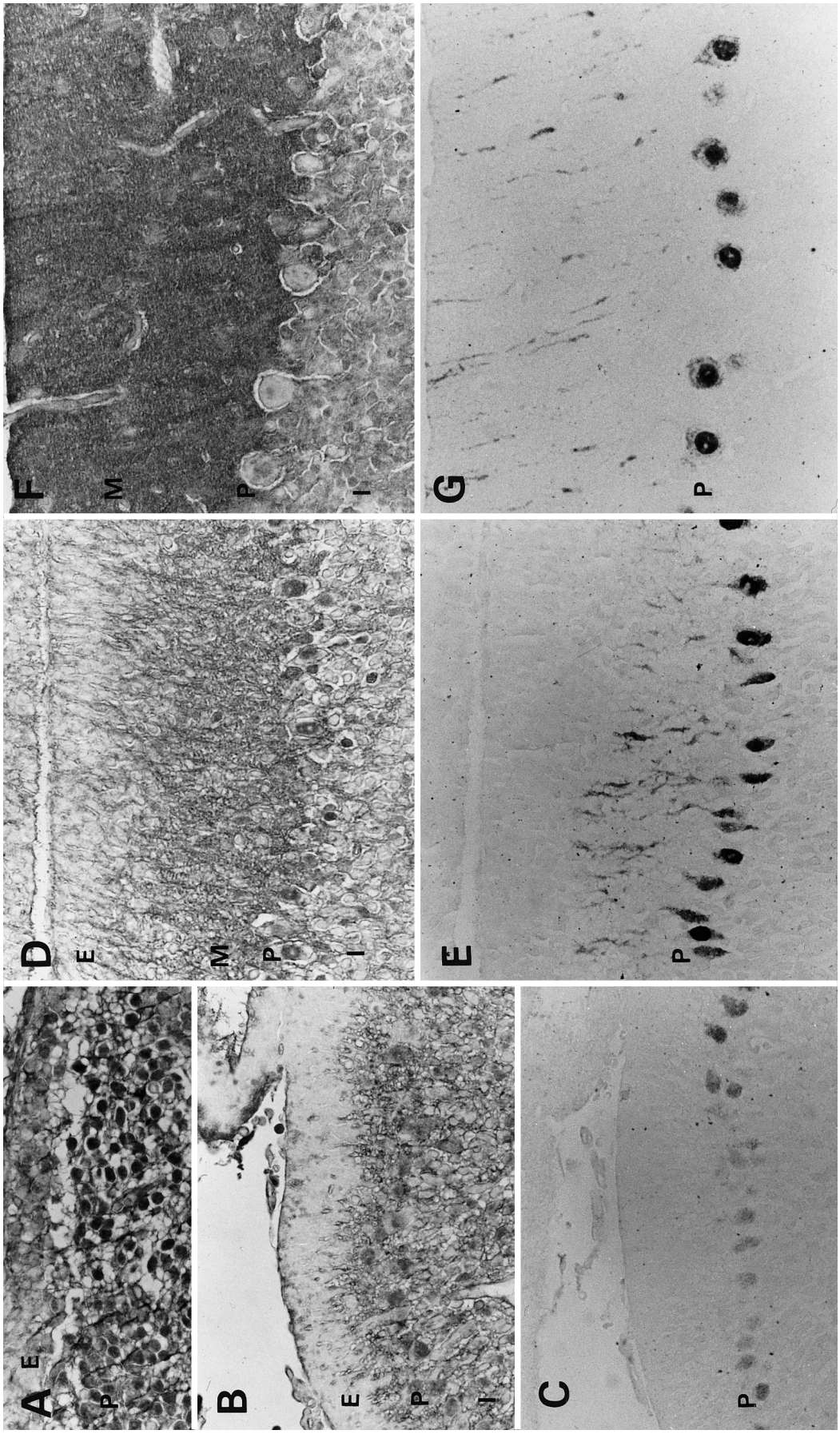
Discussion

Intracellular PAFAH is a member of the calcium-independent phospholipase A_2 family in mammalian tissues (Hattori et al. 1993). The isoform of PAFAH present in bovine brain is a heterotrimer comprising three subunits with molecular masses of 45K, 30K and 29K. The complementary DNA for the 45K subunit exhibits striking identity (99%) with a protein encoded by the putative causative gene (LIS-1) for Miller-Dieker lissencephaly. The amino acid sequence of PAFAH-45 K, or LIS-1, is highly conserved among mammalian species (Péterfy et al. 1994). On the other hand, cerebellar anomalies, such as a reduction in size and defects in convolutional folding, have been described in some lissencephaly patients (Miller 1963; Stewart et al. 1975). These findings suggest the functional importance of brain-type PAFAH, and its possible involvement in the histogenesis of the cerebellum as well as of the cerebrum.

In this study, we observed high expression of PAFAH-45K in the developing cerebellum, which is compatible with the results of a previous in situ hybridization study (Reiner et al. 1995), and implicates this protein in cerebellar histogenesis. Interestingly, our immunohistochemical studies demonstrated significant changes in the localization of brain PAFAH during development. Intense labeling shifted from the Purkinje cells in fetuses to the molecular layer in postnatal pups. On the other hand, immunoreactivity was not localized to some structures related to neuronal migration, such as granular cells and the processes of Bergmann glial cells. Thus, this study provided evidence that the expression of PAFAH-45K is developmentally regulated. Its distribution during the fetal period suggested that PAFAH-45K regulates the migration of Purkinje cells but not that of granule cells. In the postnatal life, this protein is localized to the molecular layer, implying its possible role in events other than neuronal migration.

PAF acetylhydrolase inactivates PAF by removing the acetyl group at its *sn-2* position. PAF modulates the phosphoinositide second messenger system and mobilizes intracellular calcium (Catálan et al. 1992). Treatment of neuroblastoma cells with PAF results in an increased intracellular calcium level (Kornecki et al. 1988; Yue et al. 1993). Whereas this increase may affect the organization of the cytoskeleton, which could alter the migratory behavior of cells (Komuro et al. 1992; Komuro et al. 1993; Rakic et al. 1994), it probably regulates many other events that occur during development of the CNS. This notion is supported

Fig. 2 Immunoperoxidase staining (low-power views) of mouse cerebella at E17 (**A**), P5 (**B**, **C**), P10 (**D**, **E**) and P40 (**F**, **G**) with the antibodies against PAFAH-45K (I2) (**A**, **B**, **D**, **F**) and anti-calbindin D-28k (**C**, **E**, **G**). In the Purkinje cell layer (*P*), expression of PAFAH-45K was high in the fetal period (**A**), but it decreased thereafter. In the molecular layer (*M*), the labeling increased during postnatal development (**B**, **D**, **F**). At all stages, staining was weak in the external (*E*) and internal (*I*) granular layers. The distribution of calbindin D-28k, an immunohistochemical marker of Purkinje cells, showed a resemblance to that of PAFAH-45K at P5–10 (**B–E**). ×85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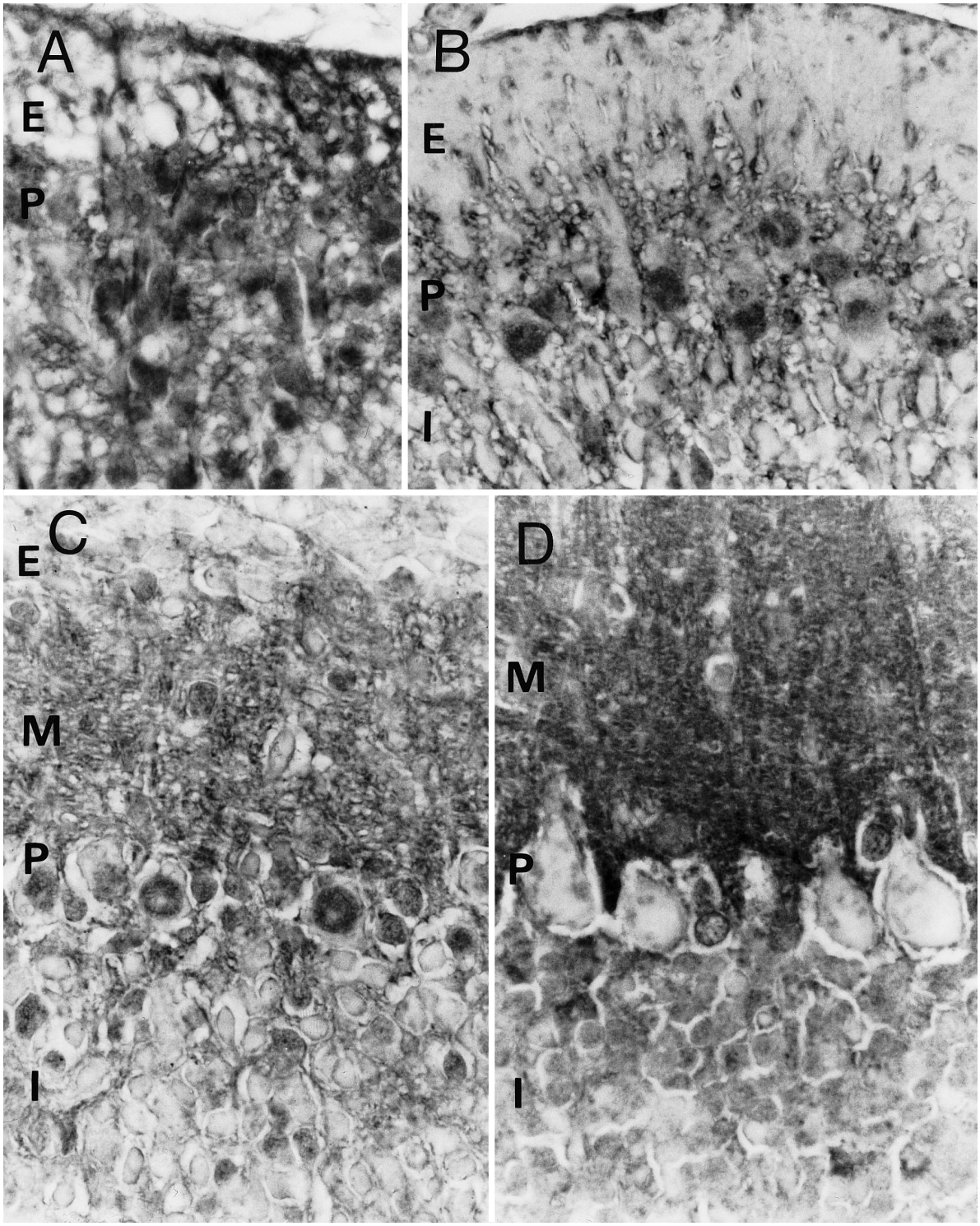

Fig. 3 Immunoperoxidase staining (high-power views) with the anti-PAFAH-45K (I2) antibody of mouse cerebella at E17 (**A**), P5 (**B**), P10 (**C**) and P40 (**D**). The Purkinje cell bodies are stained positively at E17-P10, but not at P40. The density of the fine granular immunoproducts in the molecular layer increased with advancing age. ×250

not only by the localization of PAFAH-45K observed in this study, but also by the presence of this protein in many subcellular fractions of brain tissues (Mizuguchi et al. 1995).

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References

Baimbridge KG, Miller JJ (1982) Immunohistochemical localization of calcium-binding protein in the cerebellum, hippocampal formation and olfactory bulb of the rat. Brain Res 245: 223–229

Catalán RE, Martínez AM, Aragonés MD, Fernández I, Lombardía M, Miguel BG (1992) PAF-induced activation of polyphosphoinositide-hydrolyzing phospholipase C in cerebral cortex. Biochem Biophys Res Commun 183:300–305

Dobyns WB, Curry CJ, Hoyme HE, Turlington L, Ledbetter DH (1991) Clinical and molecular diagnosis of Miller-Dieker syndrome. Am J Hum Genet 48:584–594

Dobyns WB, Reiner O, Carrozzo R, Ledbetter DH (1993) Lissencephaly: a human brain malformation associated with deletion of the LIS1 gene located at chromosome 17p13. JAMA 270: 2838–2842

Hattori M, Arai H, Inoue K (1993) Purification and characterization of bovine brain platelet-activating factor acetylhydrolase. J Biol Chem 268:18748–18753

Hattori M, Adachi H, Tsujimoto M, Arai H, Inoue K (1994) Miller-Dieker lissencephaly gene encodes a subunit of brain platelet-activating factor acetylhydrolase. Nature 370:216–218

Komuro H, Rakic P (1992) Selective role of N-type calcium channels in neuronal migration. Science 257:806–809

Komuro H, Rakic P (1993) Modulation of neuronal migration by NMDA receptors. Science 260:95–97

Kornecki E, Ehrlich YH (1988) Neuroregulatory and neuropathological actions of the ether-phospholipid platelet-activating factor. Science 240:1792–1794

Ledbetter SA, Kuwano A, Dobyns WB, Ledbetter DH (1992) Microdeletions of chromosome 17p13 as a cause of isolated lissencephaly. Am J Hum Genet 50:182–189

Miller JQ (1963) Lissencephaly in 2 siblings. Neurology 13:841–850

Mizuguchi M, Ikeda K, Asada M, Mizutani S, Kamoshita S (1994) Expression of Bcl-2 protein in murine neural cells in culture. Brain Res 649:197–200

Mizuguchi M, Takashima S, Kakita A, Yamada M, Ikeda K (1995) Lissencephaly gene product: localization in the central nervous system and loss of immunoreactivity in Miller-Dieker syndrome. Am J Pathol 147:1142–1151

Péterfy M, Gyuris T, Basu R, Takács L (1994) Lissencephaly-1 is one of the most conserved proteins between mouse and human: a single amino-acid difference in 410 residues. Gene 150:415–416

Rakic P, Cameron RS, Komuro H (1994) Recognition, adhesion, transmembrane signaling and cell motility in guided neuronal migration. Curr Opin Neurobiol 4:63–69

Reiner O, Carrozzo R, Shen Y, Wehnert M, Faustinella F, Dobyns WB, Caskey CT, Ledbetter DH (1993) Isolation of a Miller-Dieker lissencephaly gene containing G protein β-subunit-like repeats. Nature 364:717–721

Reiner O, Albrecht U, Gordon M, Chianese KA, Wong C, Gal-Gerber O, Sapir T, Siracusa LD, Buchberg AM, Caskey CT, Eichele G (1995) Lissencephaly gene expression in the CNS suggests a role in neuronal migration. J Neurosci 15:3730–3738

Stewart RM, Richman DP, Caviness VS (1975) Lissencephaly and pachygyria. Acta Neuropathol 31:1–12

Yue TL, Lysko PG, Fridman E, Feuerstein G (1993) Platelet activating factor receptor-mediated signal transduction mechanism in neurohybrid cells. In: Shukla SD (ed) Platelet activating factor receptor. CRC Press, Boca Raton, Fl, pp 93–100