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CEREBELLAR CORTICAL-LAYER-SPECIFIC CONTROL OF NEURONAL MIGRATION BY PITUITARY ADENYLATE CYCLASE-ACTIVATING POLYPEPTIDE

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Abstract—Migration of immature neurons is essential for forming the cortical layers and nuclei. Impairment of migration results in aberrant neuronal cytoarchitecture, which leads to various neurological disorders. Neurons alter the mode, tempo and rate of migration when they translocate through different cortical layers, but little is known about the mechanisms underlying this process. Here we show that endogenous pituitary adenylate cyclase-activating polypeptide (PACAP) has short-term and cortical-layer-specific effects on granule cell migration in the early postnatal mouse cerebellum. Application of exogenous PACAP significantly slowed the migration of isolated granule cells and shortened the leading process in the microexplant cultures of the postnatal day (P)0-3 cerebella. Interestingly, in the cerebellar slices of P10 mice, application of exogenous PACAP significantly inhibited granule cell migration in the external granular layer (EGL) and molecular layer (ML), but failed to alter the movement in the Purkinje cell layer (PCL) and internal granular layer (IGL). In contrast, application of PACAP antagonist accelerated granule cell migration in the PCL, but did not change the movement in the EGL, ML and IGL. Inhibition of the cAMP signaling and the activity of phospholipase C significantly reduced the effects of exogenous PACAP on granule cell migration. The PACAP action on granule cell migration was transient, and lasted for approximately 2 h. The duration of PACAP action on granule cell migration was determined by the desensitization of its receptors and prolonged by inhibiting the protein kinase C. Endogenous PACAP was present sporadically in the bottom of the ML, intensively in the PCL, and throughout the IGL. Collectively, these results indicated that PACAP acts on granule cell migration as "a brake (stop signal) for cell movement." Furthermore, these results suggest that endogenous PACAP slows granule cell migration when the cells enter the PACAP-rich PCL, and 2 h later the desensitization of PACAP receptors allows the cells to accelerate the rate of migration and to actively move within the PACAP-rich IGL. Therefore, endogenous PACAP may provide a cue that regulates granule cell migration in a cerebellar cortical-layer-specific manner.

Key words: cerebellar development, granule cell, neuronal cell migration, cAMP, rate of cell movement, desensitization.

In the developing brain, the majority of neurons migrate from their birthplace to their final destinations (Rakic, 1990; Hatten, 1999). This active movement of immature neurons plays a crucial role in establishing the normal neuronal cytoarchitecture (Rakic, 1990; Komuro and Yacubova, 2003). Impairment of this process results in either cell death or improper positioning of neurons, leading to deficiency in a wide variety of brain functions (Rakic, 1988; Flint and Kriegstein, 1997; Gressens, 2006). Recent advances in the real-time observation of neuronal cell migration in the natural cellular milieu reveal that neurons exhibit different modes and speed of migration when they move through different cortical layers (Rakic et al., 1994; Rakic and Komuro, 1995; Komuro and Rakic, 1998b; Komuro et al., 2001; Nadarajah and Parnavelas, 2002; Marin and Rubenstein, 2003; Noctor et al., 2004). For example, in the developing cerebellum, upon completion of their final mitosis, granule cells migrate tangentially in the external granular layer (EGL), then change the direction, and migrate radially along the process of Bergmann glial cells through the molecular layer (ML) (Komuro et al., 2001). After entering the Purkinje cell layer (PCL), the cells detach from the glial cells and slow down their movement (Komuro and Rakic, 1998a). Two hours later the cells speed up their movement and cross the border between the PCL and the internal granular layer (IGL). In the IGL, the cells migrate radially until attaining their final position (Komuro and Rakic, 1998a). Interestingly, the average speed of granule cell migration in the cortical layers decreases in the following order: EGL>ML>IGL>PCL (Komuro and Rakic, 1995, 1998a; Komuro et al., 2001; Komuro and Yacubova, 2003; Yacubova and Komuro, 2003). To date, several molecules, which affect the direction of migration, have been discovered, but little is known about the mechanisms by which

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Abbreviations: E, embryonic day; EGL, external granular layer; Erk, extracellular signal-regulated; GalR1, galanin receptor 1; GRK, G-protein-coupled receptor kinase; IGL, internal granular layer; MAPK, mitogen-activated protein kinase; ML, molecular layer; NMDA, *N*-methyl-D-aspartate; P, postnatal day; PACAP, pituitary adenylate cyclase-activating polypeptide; PBS, phosphate buffer saline; PCL, Purkinje cell layer; PKA, protein kinase A; PKC, protein kinase C; PKI, protein kinase A inhibitor fragment 14–22 myristoylated trifluoracetate salt; PLC, phospholipase C.

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the speed of migration is controlled in a cortical-layer-specific manner.

In this study we hypothesized that pituitary adenylate cyclase-activating polypeptide (PACAP), which is a member of the secretin/glucagon/vasoactive intestinal polypeptide family, controls the migration of cerebellar granule cells. PACAP has two bioactive products, PACAP38 and PACAP27 (Miyata et al., 1989, 1990; Vaudry et al., 2000). PACAP27 is the N-terminal 27-amino acid sequence of PACAP38 (Miyata et al., 1989, 1990). PACAP exerts pleiotropic physiological functions on multiple targets via a family of three receptors (PAC1, VPAC1 and VPAC2), which belong to the class B G-protein-coupled receptor superfamily (Ishihara et al., 1992; Pisegna and Wank, 1993). The roles of PACAP in the brain development have been suggested from the clinical studies. For example, gain of function of chromosomal regions including genes in the PACAP signaling pathway leads to developmental disorders of the brain. The *PACAP* gene (*ADCYAP1*) resides at 18p11. Fetuses with trisomy 18 develop microcephaly and spina bifida (Salihu et al., 1997). Patients with tetrasomy 18p suffer from microcephaly, mental retardation, and congenital hydrocephalus (Takeda et al., 1989). The 18p11 region is also associated with an increased susceptibility to schizophrenia (Faraone et al., 2005). The *PAC1* gene (*ADCYAP1R1*) is situated at 7p15. A patient with 7p15 duplication exhibits severe mental deficiency with communicating hydrocephalus (Miller et al., 1979). Although these chromosomal regions encode a large number of genes, these lines of evidence indicate that alterations of the PACAP signaling may contribute to some of these neurologic manifestations. Furthermore, recent studies showed that transgenic mice, which overexpress the human PAC1 receptors, develop hydrocephalus-related phenotypes (Lang et al., 2006). Collectively, these studies suggest that PACAP plays a critical role in the formation and function of the brain.

Interestingly, it has been reported that there are specific patterns of PACAP and its receptor expression throughout the developing brain: PACAP is present intensively in differentiating and postmigratory neurons, while neuronal precursors and premigratory neurons express PACAP receptors (Basille et al., 1993, 1994, 2000, 2006; Nielsen et al., 1998a,b; Skoglosa et al., 1999). In the case of developing rodent and human cerebellum, high levels of PACAP are present in differentiating cells and axonal terminals in restricted cortical layers, and granule cells express PACAP receptors, especially PAC1 receptors, prior to an initiation of their migration (Basille et al., 1993, 2000, 2006; Nielsen et al., 1998a; Falluel-Morel et al., 2005). Furthermore, it has been shown that the activation of PAC1 receptors, which are highly present in the immature neurons, including premigrating granule cells, alters the intracellular cAMP- and Ca^{2+}-signaling pathway (Zerr and Feltz, 1994; Favit et al., 1995; Gonzalez et al., 1997; Villalba et al., 1997; Mei, 1999; Vaudry et al., 2000). This is very intriguing because the speed of granule cell migration is sensitive to the changes in these second messenger signaling pathways (Komuro and Rakic, 1998b; Kumada and Komuro, 2004; Komuro and Kumada, 2005; Kumada et al., 2006, 2007; Jiang et al., in press). These lines of evidence suggest that endogenous PACAP may control granule cell migration at the specific cortical layers by activating the PACAP receptors. To examine this possibility, in this study, we examined whether and how exogenous and endogenous PACAP affects the cortical-layer-specific changes in granule cell migration in the early postnatal mouse cerebellum.

EXPERIMENTAL PROCEDURES

All animal procedures were approved by the Internal Animal Care and Use Committee of the Cleveland Clinic Foundation and University of Rouen and conformed to the U.S. National Institutes of Health guidelines and the European Community Council Directive on the ethical use of animals. All efforts were made to minimize the number of animals used and their suffering.

Migration of isolated granule cells in microexplant culture

Cerebella of postnatal day (P)0–3 mice (CD-1) were placed in ice-chilled Hanks' balanced salt solution, and freed from meninges and choroid plexus (Komuro and Rakic, 1996, 1999; Yacubova and Komuro, 2002a; Kumada and Komuro, 2004; Kumada et al., 2006). Cerebellar slices were then made with a surgical blade, from which white matter and deep cerebellar nuclei were removed. Rectangular pieces (50–100 μm) were dissected out from the remaining tissue, which mainly consisted of cerebellar gray matter, under a dissecting microscope. Small pieces of cerebellum were placed on 35 mm-glass bottom microwell dishes (MatTec Corporation, Ashland, MA, USA) which had been coated with poly-L-lysine (100 μg/ml)/laminin (20 μg/ml), with 50 μl of the culture medium. We used poly-L-lysine and laminin as substrata, since these materials provide scaffold for migrating granule cells and promote their movement (Yacubova and Komuro, 2002a). We were aware that the rate of neuronal cell movement depends on the concentrations of laminin coated on coverslips. Higher (50–100 μg/ml) or lower (1–5 μg/ml) concentrations of laminin significantly reduced the rate of granule cell movement (Yacubova and Komuro, 2002a). Therefore, we used a concentration of 20 μg/ml of laminin that allows granule cells to migrate at the fastest rate. Each dish was put in a CO_2 incubator (37 °C, 95% air, 5% CO_2). One to 2 h after plating, 1 ml of the culture medium was added to each dish. The incubation medium consisted of DMEM/F12 (Invitrogen, Carlsbad, CA, USA) with N2 supplement, 90 U/ml penicillin and 90 μg/ml streptomycin. In these cultures more than 95% of the migrating neurons were granule cells, which were easily distinguished from other neurons by the small size of their cell bodies (Komuro and Rakic, 1996; Yacubova and Komuro 2002a). Although granule cells were prepared from the EGL and the IGL of all lobules of the cerebellum, the vast majority of granule cells were derived from the EGL, since at the age of P0–P3 the IGL contains only very small numbers of postmigratory granule cells (Yacubova and Komuro 2002a). Therefore, the majority of granule cells were at the same developmental stage. One day after plating, dishes were transferred into the chamber of a micro-incubator (Harvard Apparatus, Holliston, MA, USA) attached to the stage of a confocal microscope (TCS SP, Leica, Exton, PA, USA). Chamber temperature was kept at 37 °C, and the cells were provided with a constant gas flow (95% air, 5% CO_2). The transmitted images of migrating granule cells at 488 nm were collected every 60 s for up to 5 h. To examine the effects of PACAP on granule cell migration, PACAP27 or PACAP38 was added to the culture medium.