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Role of Neph2 in pontine nuclei formation in the developing hindbrain

Kazuhiko Nishida *, Kazuhide Nakayama, Saori Yoshimura, Fujio Murakami

Graduate School of Frontier Biosciences, Osaka University, Yamadaoka 1-3, Suita, Osaka 560-8531, Japan

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ABSTRACT

Nuclei are anatomical units of the central nervous system (CNS). Their formation sets the structural basis for the functional organization of the brain, a process known as nucleogenesis. In the present study, we investigated the role of the transmembrane immunoglobulin superfamily molecule Neph2 in the nucleogenesis of the pontine nucleus (PN). Neph2 expression is turned on in migrating PN neurons only after they enter the presumptive nuclear region. Neph2 knockdown disrupted the nuclear organization of PN presumably by changing the migratory behavior of PN neurons inside the nuclear region. Moreover, overexpression of the cytoplasmic region of Neph2, which can sequester intracellular signaling of endogenous Neph2, resulted in similar phenotypes. Overall, these results suggest Neph2 is involved in the nucleogenesis of the PN through the control of neuronal migration inside the nucleus.

Introduction

Neurons sharing similar physiological features or function tend to form anatomical units such as layers and nuclei, which in turn serve as functional units for brain wiring. In contrast to layers, nuclei are three-dimensional structures with distinctive shapes that are present widely throughout the CNS. Nucleogenesis describes the formation of such nuclei. This process involves the dynamic movement of neurons within the presumptive nuclear region in order to form structures of defined morphology. To date, still little is known about the cellular and molecular mechanisms that drive nucleogenesis.

The pontine nucleus (PN) in the hindbrain represents a good model system for studying nucleogenesis because of its well described ontogeny as well as its feasibility for in vivo gene introduction experiments (Altman and Bayer, 1987; Kawauchi et al., 2006; Rodriguez and Dymecki, 2000; Taniguchi et al., 2006; Wang et al., 2005). PN is comprised of the pontine gray (PGN) and reticulotegmental (RTN) nucleus, both of which project mossy fibers toward the cerebellum. During development, nucleogenesis of the PN is preceded by the migration of PN neurons from their birth place to the presumptive nuclear region (Altman and Bayer, 1987; Kawauchi et al., 2006). After PN neurons reach the presumptive nuclear region, however, they initiate a different migratory mode inside the nucleus. In order to distinguish these migratory processes, we refer to the former and latter migrations as the "inter-regional" and "intra-nuclear" migrations, respectively (Fig. 1). The intranuclear migration entails several different migratory behaviors, one of which is exemplified by some PN neurons migrating radially along Nestin-positive radial fibers within the nuclear region (Kawauchi et al., 2006; Watanabe and Murakami, 2009). Previous studies using several knockout mice demonstrated the importance of interregional migration for the development of the PN (Marillat et al., 2004; Yee et al., 1999; Zhu et al., 2009). However, the underlying molecular mechanisms and the significance of intranuclear migration for PN nucleogenesis are two key questions that remain unanswered. To address these questions, it is necessary to identify molecules that specifically function during nucleogenesis.

In an attempt to identify molecules involved in the development of the hindbrain, we have screened for genes that express interesting spatial patterns in this region. The transmembrane protein Neph2 is one such molecule we identified (data not shown). Neph2 belongs to the Neph subfamily of the immunoglobulin superfamily which comprises of Neph1, 2, and 3 (also called as Kirrel, Kirrel3/mKirre, and Kirrel2/Filtrin, respectively), all of which are structurally related to Nephrin (Sellin et al., 2003). Neph family members interact homophilically through their extracellular region, with some of these interactions seemingly inducing cell adhesion (Gerke et al., 2005; Minaki et al., 2005; Nishida et al., 2010; Serizawa et al., 2006). The biological importance of this family of molecules has been best illustrated in the formation of the slit diaphragm of the kidney (Patrakka and Tryggvason, 2007). Recently, Neph expression in the developing nervous system has been reported in mammals (Gerke et al., 2006; Komori et al., 2008; Minaki et al., 2005; Mizuhara et al., 2010; Morikawa et al., 2007; Nishida et al., 2010; Tamura et al., 2005).

Abbreviations: CNS, central nervous system; PN, pontine nucleus; AEMS, anterior extramural migratory stream; HA, hemagglutinin; shRNA, short hairpin RNA.

* Corresponding author. Present address: Department of Medical Chemistry, Kansai Medical University, Fumizono 10-15, Moriguchi, Osaka 570-8506, Japan. Fax: +81 6 6992 1781.
E-mail address: nishidka@takii.kmu.ac.jp (K. Nishida).

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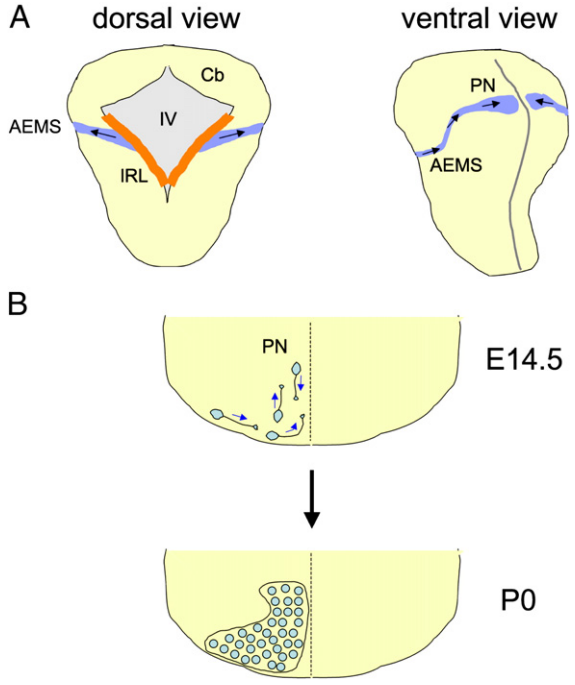

Fig. 1. Migration of PN neurons (A) Interregional migration of the pontine nucleus (PN) neurons. Dorsal and ventral views of the migratory route are shown in blue. PN neurons are born in the neuroepithelium of the lower rhombic lip between E11.5 and E16.5 in mice (shown in orange line). These neurons first migrate tangentially along the pial surface to reach the presumptive nuclear region in the ventral brainstem. (B) Intranuclear migration of PN neurons. At E14.5, PN neurons migrate not only tangentially but also radially in the presumptive nuclear region. Around P0, the nucleogenesis is almost completed (Kawauchi et al., 2006). PN neurons in the left side were shown.

Moreover, Neph homologs in *Drosophila* and *C. elegans* play indispensable roles in neural development (Ramos et al., 1993; Shen and Bargmann, 2003; Wolff and Ready, 1991), raising the possibility of a similar role in mammals.

In the present study, we examined the expression and function of Neph2 in developing PN neurons. *Neph2* began to be expressed in PN neurons after these neurons reached the presumptive nuclear region. *Neph2* knockdown disrupted the nuclear organization of PN, a possible consequence of disrupted intranuclear migration. In general, we found that Neph2 is involved in the nucleogenesis of the PN.

Results

Neph2 is expressed in migrating PN neurons

To analyze the role of Neph2 in the development of the PN, we first examined its spatio-temporal expression in developing PN neurons by in situ hybridization. No *Neph2* signal was found in the anterior extramural migratory stream (AEMS) at E13.5, at which stage PN neurons initiate interregional tangential migration underneath the pial surface (Fig. 2A, arrowheads). However, *Neph2* expression was observed in PN neurons entering the presumptive nuclear region (Fig. 2C). *Neph2* expression in the PN declined at P0, which is when PN nucleogenesis is almost completed (Fig. 2E; Kawauchi et al., 2006). Neph2 expression in the PN was also detected by immunostaining with an antibody specific for Neph2 (Fig. 2H, J–L). These results suggest that *Neph2* is involved in certain developmental processes of PN neurons migrating inside the PN. In the hindbrain, *Neph2* was expressed in the external cuneate nucleus, cerebellar Purkinje cells, the trapezoid body, and the trigeminal spinal nucleus, whereas faint expression was found in the lateral reticular nucleus and the inferior olivary nucleus from E12 to E16 (data not shown). Other Neph family members and Nephrin were not expressed in developing PN neurons (Fig. S1).

Knockdown of Neph2 by shRNA

To analyze the function of Neph2 in the development of PN neurons, we performed loss of function analyses using *Neph2*-specific short hairpin RNA (shRNA). To verify its knockdown efficiency, we introduced shRNAs with a Neph2 expression vector into COS7 cells, and analyzed Neph2 expressions by Western blotting. In contrast to control shRNA, Neph2 expression was efficiently knocked down by two shRNAs (Fig. 3A). We then confirmed knockdown efficiency of the shRNA in vivo by introducing it into developing PN neurons. As expected, the Neph2 mRNA level was greatly reduced by Neph2-shRNA, suggesting that this shRNA could efficiently block endogenous Neph2 expression in PN neurons (Fig. 3B). Decreased Neph2 expression in shRNA-introduced PN neurons was also observed by immunostaining using anti-Neph2 antibody (Fig. S2).

Introduction of Neph2-shRNA disturbed localization of PN neurons in the nucleus

To determine whether Neph2 is indispensable for the development of PN neurons, we co-electroporated Neph2-shRNA and EGFP vectors into PN neurons, and analyzed the phenotype of EGFP-positive PN neurons. We first observed the localization of PN neurons at E18.5, at which stage most PN neurons settle in their final positions (Fig. 4). When Neph2 was knocked down by shRNA1, more EGFP-positive neurons were localized near the midline compared to mock- or control shRNA-transfected samples (Fig. 4A–L). A similar phenotype was observed in shRNA2-introduced PN neurons (Fig. S3). Quantitative analyses revealed that more than 20% of Neph2-knocked down PN neurons localized within 100 μm from the midline, whereas less than 5% of mock- or control shRNA-introduced PN neurons localized there (Fig. 5B–D). Apart from the medial PN, neurons in the middle of the PN along the mediolateral axis were reduced when Neph2 was knocked down. This reduction is not attributable to apoptotic cell death, since apoptotic cells were rarely seen in shRNA-introduced PN neurons (Fig. S4). Neph2 knockdown did not cause notable effects on the distribution of PN neurons along the dorsoventral axis in the nucleus (data not shown). Neph2-shRNA-introduced neurons expressed *Barhl1*, a marker for PN neurons, indicating that Neph2 knockdown does not change the cell's overall fate (Fig. 4Q–S). These results suggest that Neph2 plays an important role in the localization of PN neurons within the nucleus.

Introduction of Neph2-shRNA affected intranuclear migration of PN neurons

Spatiotemporal expressions of Neph2 in PN neurons suggest that abnormal localization of PN neurons induced by Neph2 knockdown may be caused by defects in certain developmental processes in PN neurons migrating inside the PN (Figs. 2 and 4). One possibility is that Neph2 knockdown disturbs intranuclear migration, thereby changing the distribution of PN neurons. To address this possibility, we first examined intranuclear migration of wild type PN neurons by analyzing the direction of their leading processes at E15.5 (Fig. 6). Consistent with a previous report focusing on E14.5 mice (Watanabe and Murakami, 2009), we observed PN neurons extending leading processes tangentially forward and radially around the presumptive nuclear region (Figs. 6D, E, and S5). To our surprise, quite a few PN neurons extended leading processes tangentially backward, opposite to the migratory direction of PN neurons entering the nucleus, suggesting that at least some PN neurons move even backward along medio-lateral axis after they enter the nuclear region. We then examined the effect of Neph2