Identification of Nepro, a gene required for the maintenance of neocortex neural progenitor cells downstream of Notch

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In the developing neocortex, neural progenitor cells (NPCs) produce projection neurons of the six cortical layers in a temporal order. Over the course of cortical neurogenesis, maintenance of NPCs is essential for the generation of distinct types of neurons at the required time. Notch signaling plays a pivotal role in the maintenance of NPCs by inhibiting neuronal differentiation. Although Hairy and Enhancer-of-split (Hes)-type proteins are central to Notch signaling, it remains unclear whether other essential effectors take part in the pathway. In this study, we identify Nepro, a gene expressed in the developing mouse neocortex at early stages that encodes a 63 kDa protein that has no known structural motif except a nuclear localization signal. Misexpression of Nepro inhibits neuronal differentiation only in the early neocortex. Furthermore, knockdown of Nepro by siRNA causes precocious differentiation of neurons. Expression of Nepro is activated by the constitutively active form of Notch but not by Hes genes. Nepro represses expression of proneural genes without affecting the expression of Hes genes. Finally, we show that the combination of Nepro and Hes maintains NPCs even when Notch signaling is blocked. These results indicate that Nepro is involved in the maintenance of NPCs in the early neocortex downstream of Notch.

KEY WORDS: Neural progenitor cell, Cerebral cortex, Notch, Mouse

INTRODUCTION

Cortical projection neurons are generated from neural progenitor cells (NPCs) residing in the ventricular zone (VZ) of the dorsal telencephalic vesicle (Molyneaux et al., 2007). The first cohort of neurons forms a transient layer called the preplate. Subsequently generated neurons migrate radially and accumulate within the preplate, thereby splitting it into the marginal zone and subplate, and forming the cortical plate (CP). As CP neurons continue to be generated, they migrate past the earlier generated ones and stop beneath the marginal zone. This sequence results in the six layers of the CP having an inside-out pattern of birth dates (Takahashi et al., 1999; Caviness et al., 2008). Cell transplantation studies indicate that NPCs produce distinct types of neurons by changing their differentiation potential (McConnell, 1988; Frantz and McConnell, 1996; Mizutani and Saito, 2005). Thus, molecular mechanisms underlying the maintenance of NPCs are essential for the generation of appropriate numbers of the various types of cortical neurons.

Notch signaling mediates cell-cell interactions during vertebrate and invertebrate development (Louvi and Artavanis-Tsakonas, 2006). Mutations in key components of the Notch signaling pathway have revealed that Notch signaling is required for the maintenance of NPCs (Hitoshi et al., 2002; Yoon and Gaiano, 2005). The intracellular domain of Notch cooperates with the DNA-binding protein Rbpjκ and its co-activator mastermind-like (Maml) to activate transcription of Hes genes, which encode basic helix-loop-helix (bHLH) transcription factors (Kageyama et al., 2007). Hes genes suppress the proneural genes, such as the neurogenins and Mash1 (Ascl1 – Mouse Genome Informatics), which are crucial for neuronal differentiation (Chen et al., 1997; Hatakeyama et al., 2004; Shimojo et al., 2008). It has been shown that Hes1 and Hes5 are required for proper cortical neurogenesis (Ohtsuka et al., 1999; Guillemot, 2007), yet it remains largely unknown whether other types of effectors are required for Notch signaling in the developing neocortex.

To gain insight into the molecular mechanisms underlying the maintenance of cortical NPCs, we identified genes expressed in NPCs at early stages by using digital differential display (DDD) and in situ hybridization. Through functional screening of the genes, we found that one of them, Nepro, exhibited an activity that inhibits neuronal differentiation. To investigate Nepro function in vivo, we analyzed its role in the developing neocortex by gain- and loss-of-function approaches using in vivo electroporation.

MATERIALS AND METHODS

Animals

ICR mice obtained from Clea (Tokyo, Japan) were used for all experiments. The plug date was designated as embryonic day 0.5 (E0.5). All experimental procedures with these mice were conducted in accordance with guidelines established by the Animal Care and Use Committee (Chiba University, Japan).

Plasmids

For construction of ΔNepro, the BglII-Ncol fragment of mouse Nepro cDNA was deleted to remove the C-terminal half of the Nepro protein. Nepro-HA was constructed by inserting oligonucleotides encoding the hemagglutinin (HA) tag immediately upstream of the translation termination codon of Nepro. For misexpression of genes (see Table S1 in the supplementary material), their entire coding regions were inserted downstream of the second CAG promoter of pcAG-EYFP-CAG (Saito and Nakatsuji, 2001). pcAG-EYFP (enhanced yellow fluorescent protein) was used as a negative control in all studies (Saito and Nakatsuji, 2001).

In vivo electroporation

In vivo electroporation was performed as described (Saito and Nakatsuji, 2001; Saito, 2006). Chemically modified Stealth siRNAs (150 μM in PBS; Invitrogen, Carlsbad, CA, USA) were used. Among three Nepro-specific siRNAs, siRNA 5′-CAUCCCAUGCCUUACUCAAAGAUU-3′, which
corresponds to nucleotides 699-724 of Nepro (GenBank accession NM_145972), successfully repressed Nepro expression (see Fig. 3Z). As a control, Stealth RNA containing a scrambled sequence of the same GC content was used: 5'-CAUACGUCCUCAUCCAAAGCUCU-3'. Rhpkl-specific siRNA (MSS208565) and the corresponding control siRNAs were purchased from Invitrogen. pCAG-EYFP was cotransfected with the siRNAs to visualize transfected cells. L-685,458 (Bachem, King of Prussia, PA, USA) was used at 50 μM. Each electroporation result was reproduced in multiple brains derived from at least three litters.

Immunohistochemistry (IHC) and in situ hybridization (ISH)

IHC and ISH were performed on coronal sections (12-14 μm) that were obtained with a cryostat as previously described (Kawauchi and Saito, 2008), with minor modifications. The following antibodies were used: rabbit anti-class III β-tubulin (RDI, Flanders, NJ, USA), mouse anti-nestin (Pharningen, San Diego, CA, USA), rabbit anti-Tbr1 (Chemicon, Temecula, CA, USA), mouse anti-Ki67 (Pharningen), rabbit anti-GFP (Invitrogen), rat anti-GFP (MBL, Nagoya, Japan), rat anti-HA (Roche Diagnostics, Mannheim, Germany) and Alexa Fluor-conjugated secondary antibodies (Invitrogen). For IHC with anti-HA, anti-Ki67 and anti-Tbr1 antibodies, sections were boiled for 5 minutes in 5 mM Tris-HCl and 1 mM EDTA (pH 8.0) and stained together with anti-GFP antibodies, which also recognize the EYFP protein. cRNA probes were prepared from plasmids listed in Table S1 in the supplementary material.

RESULTS AND DISCUSSION

Identification of Nepro, a gene expressed in the early neocortex

To identify genes that are specifically expressed in the mouse brain at early stages, we compared mouse expressed sequence tag (EST) libraries from early head tissues containing many NPCs with those from older head tissues with fewer NPCs, using DDD (see Table S2 in the supplementary material). In the early head libraries, 139 genes were over-represented. Among them, we focused on five genes, the functions of which are unknown and that encode proteins containing a nuclear localization signal (NLS), as many nuclear proteins are involved in cellular activity. Three of the five genes were expressed in the VZ of the early neocortex (see Table S3 in the supplementary material). To explore their function, we transfected the genes into NPCs using in vivo electroporation. Only one gene, which we termed Nepro, exhibited an activity that inhibits neuronal differentiation (Fig. 2B). Nepro encodes a 564 amino acid protein that contains a NLS but no other known structural motif, such as a HLH. Database analysis revealed that each vertebrate species contains three conserved regions: QVEQC, a hydrophobic amino acid-rich region and DDIDDFI (Fig. 1A; see Fig. S1 in the supplementary material). In the early head libraries, 139 genes over-represented. Among them, we focused on five genes, the functions of which are unknown and that encode proteins containing a nuclear localization signal (NLS), as many nuclear proteins are involved in cellular activity. Three of the five genes were expressed in the VZ of the early neocortex (see Table S3 in the supplementary material). To explore their function, we transfected the genes into NPCs using in vivo electroporation. Only one gene, which we termed Nepro, exhibited an activity that inhibits neuronal differentiation (Fig. 2B). Nepro encodes a 564 amino acid protein that contains a NLS but no other known structural motif, such as a HLH. Database analysis revealed that each vertebrate species contains three conserved regions: QVEQC, a hydrophobic amino acid-rich region and DDIDDFI (Fig. 1A; see Fig. S1 in the supplementary material). In the early head libraries, 139 genes over-represented. Among them, we focused on five genes, the functions of which are unknown and that encode proteins containing a nuclear localization signal (NLS), as many nuclear proteins are involved in cellular activity. Three of the five genes were expressed in the VZ of the early neocortex (see Table S3 in the supplementary material). To explore their function, we transfected the genes into NPCs using in vivo electroporation. Only one gene, which we termed Nepro, exhibited an activity that inhibits neuronal differentiation (Fig. 2B). Nepro encodes a 564 amino acid protein that contains a NLS but no other known structural motif, such as a HLH. Database analysis revealed that each vertebrate species contains three conserved regions: QVEQC, a hydrophobic amino acid-rich region and DDIDDFI (Fig. 1A; see Fig. S1 in the supplementary material).

Nepro inhibits neuronal differentiation in the early neocortex

To examine Nepro function, we transfected Nepro into neocortex NPCs at E13.5, by which stage endogenous Nepro expression has declined. To visualize transfected NPCs and their daughter cells, Nepro was co-expressed with the Eyfp gene using a double promoter vector that carries both Nepro and Eyfp. As a control, NPCs transfected with Eyfp alone gave rise to neurons, and EYFP-positive neurons migrated out of the VZ into the CP (Fig. 2A). By contrast, transfection of Nepro greatly reduced the number of EYFP-positive neurons in the CP, and the majority of EYFP-positive cells remained in the VZ (Fig. 2B). This phenotype resembled that obtained by transfection of Hes genes (Fig. 2C). Indeed, the vast majority of Nepro-misexpressing cells continued to proliferate and were maintained as NPCs, as shown by the expression of the proliferation marker Ki67 and the nuclear localization signal (NLS) (see Fig. 2G,H). None of the Nepro-misexpressing cells in the VZ was positive for the neuronal marker βIII-tubulin (Fig. 2S2 in the supplementary material).

To examine whether Nepro inhibits neuronal differentiation at a later stage, we transfected Nepro into NPCs at E15.5, at which stage Nepro expression was almost absent. In contrast to the transfection at E13.5, cells transfected with Nepro migrated out of the VZ into the CP, similar to when cells were transfected with Eyfp alone as a control (Fig. 2D,E). Conversely, the majority of cells that were transfected with Hes genes at E15.5 were maintained as NPCs in the VZ (Fig. 2F). These results indicate that Nepro exhibits an activity that inhibits neuronal differentiation only at early stages.

Nepro is required for proper differentiation of cortical NPCs

To determine whether Nepro is necessary for the maintenance of NPCs, we knocked down Nepro expression by transfecting Nepro-specific siRNA with Eyfp into neocortex NPCs at E11.5, at which stage Nepro is strongly expressed. Nepro mRNA levels were greatly reduced by Nepro siRNA, in contrast to control siRNA (Fig. 3Y,Z). When Nepro was knocked down, an increased fraction of EYFP-positive neurons was found in the CP, as shown by expression of...
βIII-tubulin and the CP neuronal marker Tbr1 (Hevner et al., 2001), compared with transfection of Eyfp with or without control siRNA (Fig. 2J,M; data not shown). The number of EYFP-positive NPCs in the VZ was greatly reduced by Nepro siRNA, and none of the EYFP-positive cells in the CP was immunolabeled for nestin (see Fig. S3 in the supplementary material). These findings suggest that NPCs are not properly maintained in the absence of Nepro. We next examined whether the C-terminal part of Nepro is important for its activity by transfecting a deletion mutant (ΔNepro; Fig. 1A) into NPCs. In contrast to full-length Nepro, the mutant did not inhibit neuronal differentiation (Fig. 2K,N), suggesting that the C-terminal part is required for Nepro function. Furthermore, misexpression of ΔNepro mimicked the effect of Nepro siRNA (Fig. 2J,M), suggesting that ΔNepro can act as a dominant-negative form of Nepro.

Nepro represses the expression of proneural genes

We next examined whether Nepro is able to repress proneural genes. Misexpression of Nepro decreased mRNA levels of Ngn2 and Mash1, as did Hes and the constitutively active form of Notch (caNotch), whereas no change in these mRNA levels was seen with transfection of Eyfp alone (Fig. 3E-H; see Fig. S4 in the supplementary material). These results suggest that Nepro inhibits neuronal differentiation, presumably by repressing proneural genes.

Nepro expression is regulated by a Notch receptor

We next examined whether Notch signaling involves Nepro expression. Misexpression of caNotch induced expression of Nepro as well as of Hes5, whereas misexpression of Eyfp alone did not

Fig. 2. Nepro is involved in the maintenance of NPCs. (A-F) The neocortex 3 days after transfection of Eyfp alone as a control (A,D) and Eyfp with either Nepro (B,E) or Hes1 (C,F) at E13.5 (A-C) and E15.5 (D-F). (G,H) Immunostaining with anti-Ki67 (G) and anti-nestin (H) antibodies in the VZ of the Nepro-transfected brain. (I-N) The neocortex 2 days after transfection of Eyfp alone as a control (I,L) and Eyfp with either Nepro-specific siRNA (J,M) or ΔNepro (K,N) at E11.5. Sections were immunostained with an anti-Tbr1 antibody. Apoptosis was not significantly increased by transfection of Nepro, Nepro siRNA or ΔNepro (data not shown). Scale bars: 100 μm in F,N; 25 μm in H.

Fig. 3. Genetic interaction between Nepro and Notch. (A-P) The neocortex 12 hours after transfection of Eyfp (A,E,I,M) as a control and Eyfp with Nepro (B,F,J,N), Hes5 (C,G,K,O) or caNotch (D,H,L,P) at E13.0. The expression of Ngn2, Nepro and Hes5 in the neocortex was analyzed by ISH. Hes1 also repressed the expression of Ngn2 and Mash1, as previously described (data not shown) (Shimojo et al., 2007). (Q-V) The neocortex 5 hours after injection of DMSO (Q,S,U) as a control and L-685,458 (R,T,V) at E11.5. In contrast to Nepro and Hes5, Ngn2 mRNA levels were not decreased (U,V). (W-Z,a,b) The neocortex 16 hours after transfection of Eyfp together with control or Nepro siRNA at E11.5. Nepro mRNA expression was clearly reduced by Nepro siRNA, whereas expression of Hes5 was unchanged. Scale bars: 200 μm.
Influence its expression (Fig. 3I-P). Conversely, a γ-secretase inhibitor, L-685,458, which blocks cleavage and activation of Notch (Martys-Zage et al., 2000), drastically reduced mRNA levels of Nepro and Hes5, compared with the control solvent dimethylsulfoxide (DMSO), which did not affect mRNA levels (Fig. 3Q-V). Furthermore, Nepro expression was decreased by Rbpjk-specific siRNA (Fukushima et al., 2008) and the dominant-negative form of Maml1 (DN-Maml1) (Weng et al., 2003; Maillard et al., 2004) (see Fig. S5 in the supplementary material). These results indicate that Nepro is activated downstream of canonical Notch signaling.

Misexpression of Hes1 and Hes5 did not affect Nepro expression, and misexpression of Nepro did not change Hes1 or Hes5 expression (Fig. 3K,N; see Fig. S6A in the supplementary material). Moreover, Nepro knockdown did not affect the expression of Hes1 or Hes5 (Fig. 3b; see Fig. S6B in the supplementary material). These findings suggest that Nepro is activated in parallel with Hes, but neither downstream nor upstream of Hes.

**Nepro is an essential Notch effector**

To determine whether Nepro is an essential effector for Notch signaling, Nepro siRNA or ΔNepro was transfected together with caNotch. Whereas caNotch maintained the vast majority of cells as NPCs in the VZ, cells transfected with caNotch together with Nepro siRNA or ΔNepro differentiated into CP neurons positive for Tbr1 (Fig. 4A,B; see Fig. S7 in the supplementary material), indicating that Nepro is necessary downstream of Notch for maintenance of NPCs.

We then examined whether misexpression of Nepro and Hes is sufficient to maintain NPCs when Notch activity is blocked (Fig. 4C-H). L-685,458 markedly reduced NPCs in the VZ (Fig. 4D), consistent with the precocious differentiation caused by the blocking of Notch signaling. The number of proliferating NPCs was measured by immunostaining of Ki67 (Fig. 4I; see Fig. S8 in the supplementary material). The reduction of NPCs by L-685,458 was not suppressed by misexpression of Hes1 or Nepro (Fig. 4E,F). Co-transfection of Hes1 and hes-related 1 (Hey1) was also not sufficient to suppress the reduction (Fig. 4G), indicating that the lack of Hes1 and Hey1 heterodimers, which are known to be more stable than homodimers (Iso et al., 2001), is not the reason for the absence of activity. By contrast, misexpression of Hes1 and Hey1 with Nepro suppressed the reduction in NPCs (Fig. 4H). Similarly, whereas overexpression of Hes and Hey genes (Hes1, Hes5, Hey1 and Hey2), which are expressed in the embryonic neocortex, was also not sufficient to suppress the reduction in NPCs, the addition of Nepro was sufficient to suppress the reduction (Fig. 4I; data not shown). Furthermore, the combinatorial effect of Hes1 and Nepro was also observed when Notch signaling was blocked by DN-Maml1 (see Fig. S9 in the supplementary material). These results indicate that when Notch activity is blocked, Nepro and Hes are necessary for the maintenance of NPCs.

In this study, we identified Nepro, which is a novel Notch effector for the maintenance of NPCs in the early stages of neocortex development (Fig. 4J). Nepro is an atypical Notch effector for the following reasons: first, Nepro does not contain an HLH motif, unlike Hes and Hey proteins; and second, although members of the Notch pathway are conserved from invertebrates to vertebrates and unlike Hes and Hey proteins; and second, although members of the Notch pathway are conserved from invertebrates to vertebrates and

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**Fig. 4. Nepro is required to maintain NPCs. (A,B) The neocortex 2 days after transfection of Eyfp and caNotch with (A) or without (B) Nepro siRNA at E11.5. (C-H) The neocortex 2 days after transfection of Eyfp as a control and Eyfp with Hes1, Hey1 and/or Nepro in the presence of L-685,458 at E11.5. The distribution of Eyfp-positive cells was not affected by injection of DMSO as a control (C). (I) Quantification of the percentage of Ki67-positive/EYFP-positive cells in sections transfected with the genes shown along the x-axis. Error bars indicate the s.d., *, P<0.001, **, P<0.005. P-values were calculated by Student’s t-test. (J) The Nepro and Notch pathways in the early neocortex. After cleavage by γ-secretase, activated Notch induces the expression of Nepro and Hes in the early neocortex. Scale bars: 100 μm in B; 50 μm in H.**
indirect interaction may be crucial. $\Delta$NeoPro, which lacks the C-terminal portion, mimicked the NeoPro knockdown phenotype (Fig. 2K), suggesting that NeoPro interacts with key factors. It remains to be determined what proteins interact with NeoPro and how NPCs are maintained by those interactions in the early cortex.

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Supplementary information
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