A Disintegrin and Metalloprotease 10 in neuronal maturation and gliogenesis during cortex development

Zhixing Ma, Qingyu Li, Zhengyu Zhang, Yufang Zheng

Abstract
The multiple-layer structure of the cerebral cortex is important for its functions. Such a structure is generated based on the proliferation and differentiation of neural stem/progenitor cells. Notch functions as a molecular switch for neural stem/progenitor cell fate during cortex development but the mechanism remains unclear. Biochemical and cellular studies showed that Notch receptor activation induces several proteases to release the Notch intracellular domain (NICD). A Disintegrin and Metalloprotease 10 (ADAM10) might be a physiological rate-limiting S2 enzyme for Notch activation. Nestin-driven conditional ADAM10 knockout in mouse cortex showed that ADAM10 is critical for maintenance of the neural stem cell population during early embryonic cortex development. However, the expression pattern and function of ADAM10 during later cerebral cortex development remains poorly understood. We performed in situ hybridization for ADAM10 mRNA and immunofluorescent analysis to determine the expression of ADAM10 and NICD in mouse cortex from embryonic day 9 (E14.5) to postnatal day 1 (P1). ADAM10 and NICD were highly co-localized in the cortex of E16.5 to P1 mice. Comparisons of expression patterns of ADAM10 with Nestin (neural stem cell marker), Tuj1 (mature neuron marker), and S100β (glia marker) showed that ADAM10 expression highly matched that of S100β and partially matched that of Tuj1 at later embryonic to early postnatal cortex developmental stages. Such expression patterns indicated that ADAM10-Notch signaling might have a critical function in neuronal maturation and gliogenesis during cortex development.

Key Words
neural regeneration; neurogenesis; ADAM10; A Disintegrin and Metalloprotease; Notch; Notch intracellular domain; Tuj1; S100β; Nestin; cerebral cortex; development; neuronal maturation; glial cell; grants-supported paper; photographs-containing paper; neuroregeneration

Research Highlights
(1) A Disintegrin and Metalloprotease 10 (ADAM10) is mainly expressed at the cortical plate and ventricular zone during middle and late embryonic development, and was highly co-localized with Notch intracellular domain.
(2) ADAM10 expression was co-localized to S100β-labeled regions and partially overlapped with Tuj1.
(3) ADAM10-Notch signaling may have a critical function in neuronal maturation and gliogenesis during cortex development.

Abbreviations
VZ, ventricular zone; SVZ, subventricular zone; CP, cortical plate; NICD, Notch intracellular domain; ADAM, A Disintegrin and Metalloprotease; IZ, intermediate zone.
INTRODUCTION

The cerebral cortex has a multiple-layer structure that is important for mediating its functions. Such a multiple-layer structure is tightly controlled during the development process both temporally and spatially. During development, neural stem cells in the ventricular zone (VZ) to subventricular zone (SVZ) differentiate into neuronal intermediate progenitor cells, which further differentiate into mature neurons that migrate along the radial glia fibers to the outer layer of the cortex[1-3]. The late differentiated cells migrate a longer distance to form the upper cortical plate (CP) layer, and this process has been termed an “inside out” development process[1-2]. At the end of the neurogenesis period, neural stem cells undergo gliogenesis to generate glial cells in the cerebral cortex[3-4].

Previous studies have shown that the Notch signaling pathway is a critical molecular switch to determine the fate of neural stem cells during cortex development[5-6]. Notch is a critical molecule in maintaining neural stem cell multipotency by inhibiting differentiation to neurons during early cerebral development[4,7]. However, the Notch signaling pathway can also promote neural stem cell differentiation to astrocytes during late cerebral development stages[8-9], as well as inhibit neural stem cell differentiation to oligodendrocytes[10-12]. In the peripheral nervous system, Notch also controls the differentiation of Schwann cells[13]. It remains unclear how Notch is precisely regulated during these different processes.

Biochemical and cellular studies demonstrated that Notch receptor activation induces several proteases to release Notch intracellular domain (NICD). Of these proteases, A Disintegrin and Metalloprotease (ADAM) 10 and ADAM17 are the critical rate-limiting S2 enzymes during the Notch activation process[14-17]. ADAM10 and ADAM17 have critical functions as secretases for amyloid precursor protein and are involved in the Notch signaling pathway. *In vitro* cell experiments first identified ADAM17 as the S2 enzyme for Notch[18-19]. However, ADAM17 deficient mice have a different phenotype compared with Notch knockout mice[20]. In contrast, ADAM10 knockout mice have a very similar phenotype to Notch knockout mice[21]. Therefore, ADAM10 is considered the physiological enzyme for Notch.

Both ADAM10 and Notch knockout mice die at a very early developmental stage because of heart failure[21], and thus it is too early to study cerebral cortex development as it is only a thin epithelial layer at this stage. In 2010, Jorissen *et al*[22] generated conditional ADAM10 knockout mice using a Nestin-Cre promoter. The conditional ADAM10 knockout mice have a disrupted neocortex that contains more neurons and less astrocytes than normal mice. This phenotype was caused by increased neural stem cell differentiation to neurons. Although this study explored the role of ADAM10 in brain cortex development, it mainly showed the effect of ADAM10 during early embryonic cortex development because of the Nestin promoter used. Nestin-driven Cre protein expression starts at embryonic day 9 (E9) in the mouse brain and E10 for the rest of the central nerve system[22-23]. Therefore, Nestin-Cre conditional knockout mice lose the effect of ADAM10 at the stage of neural epithelial layer development and the phenotypes observed in later cortex development are likely the consequences of the earlier defects. The true function of ADAM10 during late cortex development is still not clear. There are few reports on ADAM10 expression at late embryonic cortex and thus its function during this period is elusive.

In the present study, we focused on the perinatal cortex during the late embryonic stage and used immunohistochemistry and *in situ* hybridization methods to study the expression pattern of ADAM10 in mice from E14 to postnatal day 1 (P1). Our results revealed that ADAM10 was co-localized with NICD during this period in the cerebral cortex at both the VZ and CP layers and marked co-localization with S100β and partially colocalized with Tuj1 in the deep CP layer from the late embryonic to early postnatal stages. This expression pattern of ADAM10 indicated that not only might it function as a critical regulator for neural stem cell fate, but it may also play an important role during neuronal maturation and gliogenesis.

RESULTS

**ADAM10 protein and mRNA were specifically expressed in VZ and CP during E16.5 to P1 cerebral cortex in mice**

To investigate the expression pattern of ADAM10 at late embryonic cortex development stages, we performed *in situ* hybridization and immunofluorescent analysis on mouse cerebral cortex coronal sections from E14.5 to P1. *In situ* hybridization results showed that ADAM10 mRNA was expressed at both the VZ layer and CP layer from E17.5 to P1 with a higher expression at the CP layer (Figure 1).
This expression pattern was confirmed by immunofluorescent analysis for the expression of ADAM10 protein in developing cerebral cortex from E14.5 to E18.5 mouse cortex (Figure 2). At E14.5, ADAM10 was mainly expressed at the margin zone and increased at the CP layer at E16.5 and E18.5 cortex with the highest expression near the subplate region (Figures 2, 3A, B). Interestingly, ADAM10 expression became more diffuse within the gray matter at P1 (Figure 2).

**ADAM10 protein expression correlated with NICD expression**

The expression pattern of ADAM10 and NICD in mouse cerebral cortex coronal sections was determined. Mouse brains at E16.5, E18.5, and P1 were processed for immunofluorescent analysis. As shown in Figure 3, ADAM10 and NICD were both expressed in the VZ and CP layer from E16.5 to P1 but there was no signal at the intermediate zone (IZ) layer. Interestingly, similar to ADAM10 expression, NICD expression also became more diffuse in the gray matter at P1 (Figure 2).

**ADAM10 protein expression partially correlated with Tuj1 and S100β expression during late embryonic cerebral cortex development stages (E16.5 to P1)**

The co-expression of ADAM10 with several other markers, including S100β for glial cells, Tuj1 (beta III-tubulin) for mature neurons and Nestin for neural stem cells, in mouse cortex sections was also examined.

**DISCUSSION**

We examined the expression pattern of ADAM10 and NICD in the late embryonic stage in the perinatal mice cortex from E14 to P1. To identify the expression location of ADAM10 and NICD, double immunohistochemistry staining on the same sections should be performed. However, because of the lack of suitable antibodies, we had to stain adjacent serial brain sections with either ADAM10 antibody or NICD antibody.
Figure 3  ADAM10 expression was co-localized with NICD on E16.5, E18.5, and P1 serial mouse cerebral cortex coronal sections (the number of embryos used for each time point = 4).

Adjacent sections were cut and stained with rabbit anti-ADAM10 or rabbit anti-NICD antibodies. Cy3-labeled (red) goat anti-rabbit secondary antibody was used for both stainings. ADAM10 staining was pseudo-colored to green by computer software for easier comparison. Images were captured by Olympus IX71 microscope. At E16.5 (A) and E18.5 (B), ADAM10 was mainly located in the CP layer with the highest expression at the border of CP close to the IZ layer. At P1 (C), ADAM10 was uniformly expressed in the CP layer. The expression pattern of NICD during these stages (D: E16.5, E: E18.5, F: P1) was almost identical to ADAM10. Scale bar: White for A, B, D & E, 100 μm; blue for C & F, 200 μm.

ADAM10: A Disintegrin and Metalloprotease 10; NICD: Notch intracellular domain; VZ: ventricular zone; SVZ: subventricular zone; IZ: intermediate zone; CP: cortical plate; E: embryonic day; P1: postnatal day 1.

Figure 4  ADAM10 expression co-localized with Tuj1 and S100β on E16.5, E18.5, and P1 serial mouse cerebral cortex coronal sections (the number of embryos used for each time point = 4).

Sections were stained with rabbit anti-ADAM10 antibody (A–D) and antibodies for one of the following markers: mouse anti-Nestin antibody for neural stem cells (L–N), mouse anti-Tuj1 antibody for matured neurons (I–K) or rabbit anti-S100β antibody for glia cells (E–H).

Cy3-labeled (red) goat anti-rabbit or anti-mouse secondary antibody was used accordingly. ADAM10 staining was pseudo-colored to green by computer software for easier comparison. Both ADAM10 and S100β were expressed mainly at the CP layer. A higher magnification for panels B & F, which were adjacent sections of E18.5 for ADAM10 and S100β staining, are presented in panels D & H. Images were captured by Olympus IX71 microscope. Scale bar in A–C & E–G & I–N is 200 μm (white); scale bar in D & H is 100 μm (blue).

ADAM10: A Disintegrin and Metalloprotease 10; NICD: Notch intracellular domain; VZ: ventricular zone; SVZ: subventricular zone; IZ: intermediate zone; CP: cortical plate; E: embryonic day; P1: postnatal day 1.
Our results revealed that ADAM10 co-localized with NICD during late embryonic to perinatal stages in the mouse cerebral cortex. Both were highly expressed at the CP layer, expressed at lower levels in the VZ to SVZ layer and were not present in the IZ layer. This expression pattern indicated that ADAM10-Notch signaling is probably a critical regulator for late embryonic cortex development. At the SVZ to VZ layer, ADAM10-Notch signaling is probably required for maintenance of the NSC pool as previously demonstrated[5, 24]. However, the role of Notch signaling in the CP layer is unclear even though this expression pattern was also observed in the human fetal cerebral cortex[25]. One possibility is that ADAM10-Notch signaling in the CP layer is involved in binary fate choice and required for a specific type of neuron maturation. It has been shown that Notch signaling in vertebrates can regulate binary fate choices leading to multiple distinct neuronal cell types[26]. Recently, studies in Drosophila demonstrated that Notch activation can inhibit dopaminergic neurons[27], and RBP-J/Su(H)/Lag1, the main transcriptional mediator for Notch signaling, can promote intermediate neuronal progenitor cell maturation in the cerebellum[28]. Another possibility is that ADAM10-Notch signaling is required for gliogenesis as S100β was also highly expressed in the same region. However, the type of cells and the timing of its developmental processes require further investigation.

Our results indicated that ADAM10-Notch signaling is critical for cerebral cortex development and their expression patterns suggest that it may have different functions in the different layers. How is ADAM10 regulated? It functions as an enzyme, thus its function can also be regulated by its activity. Reversion-inducing cysteine-rich protein with Kazal motifs (RECK) can inhibit ADAM10 activity and modulate Notch signaling during cortical neurogenesis[29] and secreted frizzled-related proteins can also inhibit ADAM10 activity during retinal neurogenesis[30]. However, our results showed that ADAM10 expression is not uniform throughout the VZ to CP layer. Both mRNA and protein expression were down-regulated in the IZ layer during late embryonic stages. Therefore, it is likely that ADAM10 is regulated by a transcriptional mechanism within the developing cerebral cortex. Thus, layer specific upstream factors may regulate transcription of ADAM10. Pax2 can regulate ADAM10 expression in renal carcinoma cells[31-32] and retinoic acid can activate ADAM10 expression in neuroblastoma cells[33]. Whether Pax2 and retinoic acid also play similar roles during cortex development requires further investigation. Besides transcriptional regulation of ADAM10, layer specific miRNA molecules for post-transcription regulation of ADAM10 mRNA such as miRNA may have important roles during cortex development[34]. However, which miRNA molecules and the mechanisms of how ADAM10 is regulated by miRNA remain to be elucidated.

In conclusion, ADAM10-Notch signaling pathway is tightly regulated in different cortical layers and may have an important role during cortex development.

**MATERIALS AND METHODS**

**Design**
A basic study of neural development.

**Time and setting**
The experiments were performed at the School of Life Sciences, Fudan University, Shanghai, China from 2009 to 2012.

**Materials**
Thirty time-pregnant C57BL/6 female mice (mated pregnant mice) and 20 newborn C57BL/6 mice were used. All C57BL/6 mice were purchased from Shanghai SLAC Laboratory Animal Inc, Shanghai, China.

**Methods**

**Constructs for in situ hybridization**

In situ hybridization probe for mouse ADAM10 (NM_007399; bases 208–730 bp, amino acids 68–240) was cloned by PCR and the obtained cDNA fragments were subcloned into pSPT19 vector (Roche Diagnostics (Shanghai) Limited, Shanghai, China) between EcoRI and HindIII sites.

In situ hybridization

Frozen mouse brains were sectioned coronally at 15 μm using a cryostat (Leica CM1900, Solms, Germany). Frozen sections were air dried and fixed in 4% paraformaldehyde/PBS for 10 minutes. The slices were digested with proteinase K. Prehybridization and hybridization were performed with a 5 × SSC–50% formamide solution to avoid evaporation. After incubation, the sections were washed in 2 × SSC (room temperature) for 30 minutes, in 2 × SSC (50°C) for 1 hour, in 0.1 × SSC (50°C) for 1 hour, and equilibrated in Buffer 1 (100 mM Tris-HCl and 150 mM NaCl, pH 7.5) for 5 minutes. The sections were then incubated at room temperature with alkaline phosphatase-coupled anti-digoxigenin antibody (Roche Diagnostics (Shanghai)
Limited, Shanghai, China) diluted 1:5 000 in Buffer 1 containing 0.5% blocking reagent for 2 hours. Excess antibody was removed by two 15-minute washes in Buffer 1, and the sections were equilibrated in Buffer 2 (100 mM Tris-HCl, 100 mM NaCl, and 50 mM MgCl2, pH 9.5) for 5 minutes. Color development was performed at room temperature (30 minutes to 3 days, depending on the amount of transcripts to be detected) in Buffer 2 containing 1.875 mg/mL nitro blue tetrazolium chloride and 0.94 mg/mL 5-bromo-4-chloro-3-indolyl-phosphate. ADAM10 (208–730 bp) was cloned into pSPT19 vector and antisense RNA was transcribed using T7 promoter with Roche DIG RNA labeling Kit. Bright field images were taken by using Axiovert-200 microscope (Carl Zeiss, Oberkochen, Germany).

**Immunofluorescent staining**

Embryonic brains were fixed in 4% paraformaldehyde in PBS overnight and placed in 15% sucrose/PBS for 12 hours and then 30% sucrose/PBS for 24 hours at 4°C. Brains were then embedded in optimal cutting temperature mounting medium and frozen before coronal sectioning at 10 or 20 μm using a cryostat (Leica CM1900, Germany). The following primary antibodies were used: rabbit polyclonal anti-ADAM10 antibody, rabbit polyclonal anti-NICD antibody, rabbit polyclonal anti-S100B antibody, mouse monoclonal anti-Nestin antibody, mouse monoclonal anti-Tuj1 (beta III-tubulin) antibody (all purchased from Abcam, Cambridge, UK) at 1:200 dilution. Sections were then incubated with secondary Cy3-labeled goat antibody (anti-rabbit or anti-mouse, purchased from Beyotime, Suzhou, China) at 1:500 dilution. The negative control was performed using a secondary antibody alone on sections. Fluorescent images were taken using an Olympus IX71 microscope (Olympus, Tokyo, Japan).

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**Author contributions:** Yufang Zheng and Zhixing Ma designed the study. Zhixing Ma, Qingyu Li, and Zhengyu Zhang conducted the experiments. Yufang Zheng wrote the manuscript. All authors approved the final version of paper.

**Conflicts of interest:** None declared.

**Ethical approval:** Animal-related procedures were reviewed and approved by the Animal Care and Use Committee of Fudan University, Shanghai, China.

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