

The expression pattern of Adam10 in the central nervous system of adult mice: Detection by *in situ* hybridization combined with immunohistochemistry staining

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Abstract. ADAM10 (a disintegrin and metalloprotease 10) is a member of the ADAMs family, which is key in the development of the nervous system, by regulating proliferation, migration, differentiation and survival of various cells, including axonal growth and myelination. Previous studies have investigated the embryonic or postnatal expression of *ADAM10*, however, detailed information regarding its cellular distribution in the adult stage, to the best of our knowledge, is not available. The present study investigated the expression pattern of the *ADAM10* gene in the adult mouse central nervous system (CNS) using an ADAM10 complementary RNA probe for *in situ* hybridization (ISH). Immunohistochemical staining was used to identify the type of the ISH staining-positive cells with neuron- or astrocyte-specific antibodies. The results of the current study demonstrated that the *ADAM10* gene was predominantly expressed in the neurons of the cerebral cortex, hippocampus, thalamus and cerebellar granular cells in adult mouse CNS.

Introduction

ADAM10 (a disintegrin and metalloprotease 10) is a member of the ADAMs family, which contain two important

structures, a matrix metalloprotease (MMP) domain and disintegrin domain (DI). The MMP domain is important for cleavage-dependent activation of proteins, including various cell signaling molecules, such as Notch receptors and ligands (1), cadherins (epithelial (E)-cadherin and neural (N)-cadherin) (2,3), epidermal growth factor (EGF) (4) and the adhesion molecule L1 (5). ADAM10 may also act as an α -secretase to cleave amyloid precursor protein and decrease amyloid β protein production (6). ADAM10 may also be important for the development of the nervous system, where it regulates proliferation, migration, differentiation and survival of various cells, including axonal growth and myelination (7). It is clear that ADAM10 is important for neural development, however, it may also be important in certain nervous system diseases, including Alzheimer's disease and inflammatory responses (7,8), the spatial and temporal expression patterns of ADAM10 during vertebrate brain development remain to be fully elucidated. Previous studies have determined that the expression of ADAM10 mRNA was restricted to specific brain regions, including developing blood vessels, neuroepithelial regions and differentiating gray matter (9,10); however, the exact cellular localization of ADAM10 in the adult brains remains unclear.

The present study constructed an ADAM10 complementary RNA (cRNA) probe to investigate the expression pattern of the *Adam10* gene in the central nervous system (CNS) of adult mice by *in situ* hybridization (ISH). Immunohistochemical staining was used to identify the types of ISH staining-positive cells with neuron or astrocyte-specific antibodies. The results demonstrated that the expression of the ADAM10 gene was predominantly in the neurons of the cerebral cortex, hippocampus, thalamus and cerebellar granular cells in the CNS of adult mice.

Materials and methods

Animals. A total of 10 healthy C57/BL6 mice (age, 10 weeks; 5 female, 5 male; weight, 250-300 g), were provided by the Animal Experiment Center of Zhejiang University (Hangzhou, China). They were randomly divided into a control group (a

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Abbreviations: Par, parietal cortex; Pir, piriform cortex; Hip, hippocampus; Tha, thalamus; DG, dentate gyrus; IGL, granule cell layer; ML, molecular layer; PC, purkinje cell layer; GFAP, glial fibrillary acidic protein

Key words: ADAM10, CNS, neuron, astrocyte, ISH

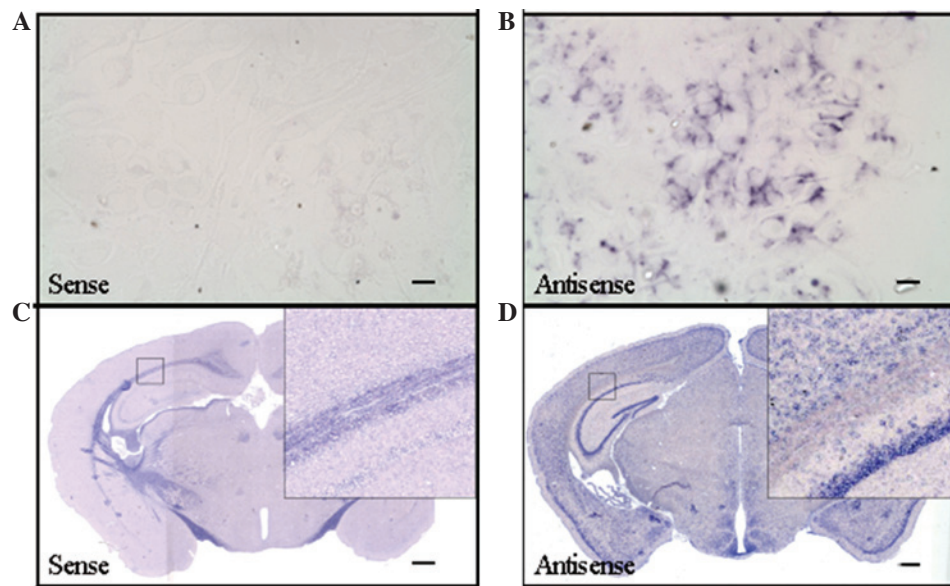


Figure 1. Effectiveness detection of Adam10 cRNA probe. (A and B) Mixed cultures of neurons and glial cells (scale bar, 10 μ m); (C and D) C57BL6 mouse brain slices (scale bar, 500 μ m) with higher magnification of C and D shown in the boxed areas. (A and C) *In situ* hybridization results using sense Adam10 cRNA probes. (B and D) *In situ* hybridization results using antisense Adam10 cRNA probe. This indicates that the hybridization signal was detected only in antisense probe slices. Adam10, a disintegrin and metallopeptidase domain 10; cRNA, complementary RNA.

total of six mice, three mice for mixed cultures of neurons and glial cells, three for tissue sections) and experimental group (four mice, two mice were used for mixed culturing of neuron and glial cells, and two for tissue sections). The mice were housed at an ambient temperature of 37°C with 50% humidity under a 12-h light/dark cycle. All mice had access to food and water *ad libitum*. After 2 weeks, all the mice were sacrificed via cervical dislocation, and the tissues were immediately used for the experiments or stored at -80°C. The current study was approved by the Animal Advisory Committee at Zhejiang University. All the animals were treated in accordance with national and institutional guidelines on the care of animals in research.

In situ hybridization. To investigate the expression pattern of the Adam10 gene in the CNS of adult mice, an ADAM10 complementary RNA probe was constructed. Cell type-specific antibody immunohistochemical staining was applied to identify the types of the ISH staining-positive cells.

Generation of Adam10 cRNA probes.

The method for generation of the ADAM10 cRNA probe was described previously with a few modifications (9,10). More detailed protocols are described below.

RNA extraction and cDNA cloning. Total RNA from adult C57/BL6 mice brains was prepared using the TRIzol reagent, according to the manufacturer's protocol (cat. no. 15596-026, Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and a glass homogenizer, this was incubated subsequently with DNase I for 1 h at 37°C to remove any contaminating DNA. RNA was reverse transcribed to cDNA using a M-MLV RTase cDNA Synthesis kit (cat. no. D6130, Takara Bio, Inc., Otsu, Japan) according to the manufacturer's protocol. The polymerase chain reaction (PCR) amplification of ADAM10 cDNA was performed

Table I. Distribution of Adam10 mRNA expression in the adult mouse brain using *in situ* hybridization.

Brain region	Adam10 mRNA abundance
Piriform cortex	+++
Frontoparietal cortex	+++
Septal nucleus	++
Hippocampus	
CA1-CA3	+++
Dentate gyrus	+++
Thalamus	++
Hypothalamus	++
Striatum	+
Cerebellum	
Molecular layer	++
Purkinje cell layer	+++
Internal granular cell layer	+++
White matter	++

Adam10 mRNA abundance for each anatomical region was determined from optical density measurements. Density values for each parameter are presented according to their respective percentile distributions: +++, strongly positive (>50% above background); ++, moderately positive (>25% above background); +, weakly positive (<25% above the background). Only the regions in which signal for Adam10 was detected are shown. Adam10, a disintegrin and metallopeptidase domain 10.

using the KOD-Plus kit (Toyobo Co., Ltd., Osaka, Japan) and the following primers, sense, 5'GGTGAAACGCATAAG AATC-3' and antisense, 5'CACTGAACTGCTTGCTCC-3' under conditions as follows: Denaturation at 94°C for 4 min;

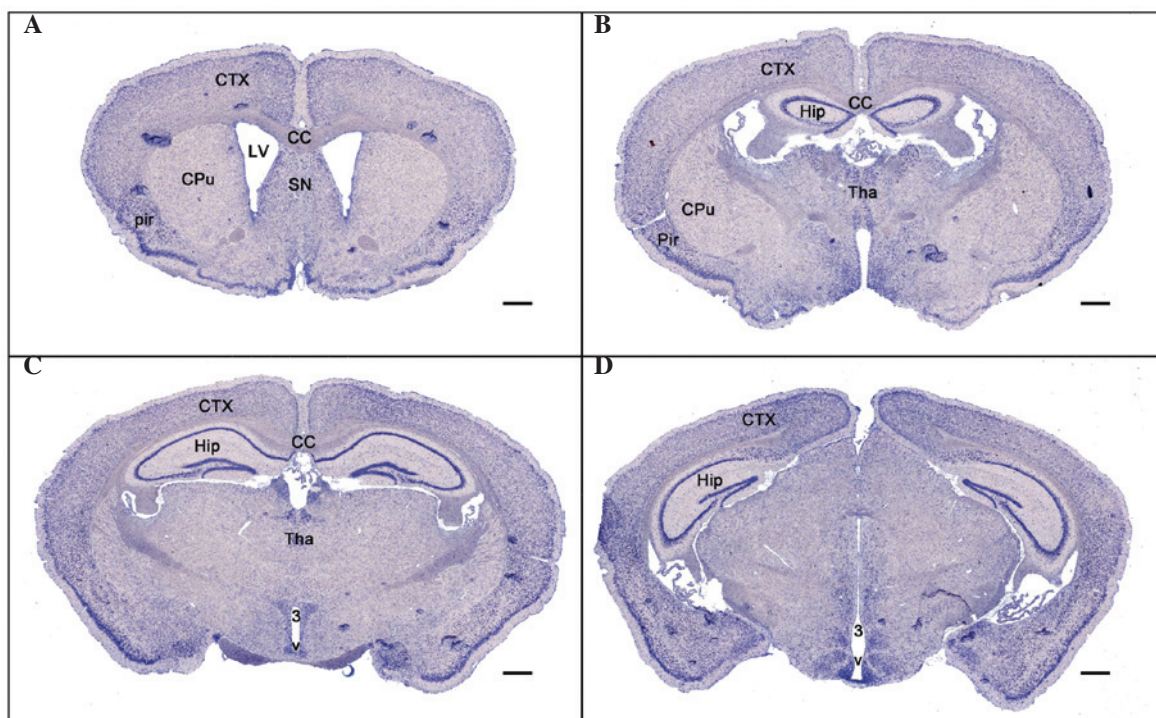


Figure 2. Distribution of Adam10 mRNA expression in the adult mouse brain. (A-D) Adam10 cRNA probe *in situ* hybridization results in cerebral coronal sections from (A) rostral to (D) caudal. Scale bar, 500 μ m. CTX, cortex; Pir, piriform cortex; CPu, caudate putamen; LV, lateral ventricle; SN, septal nucleus; CC, corpus callosum; Hip, hippocampus; Tha, thalamus; 3 V, the third ventricle; Adam10, a disintegrin and metallopeptidase domain 10.

35 cycles of denaturation at 94°C for 30 sec, annealing at 56°C for 40 sec, and extension at 68°C for 90 sec; and extension at 68°C for 5 min. The amplified PCR fragments were analyzed on agarose gels. The identity of the PCR products was confirmed with restriction analysis. For restriction reactions, the electrophoresed PCR products were purified from agarose gels using QIAquick Gel Extraction kit (Qiagen GmbH, Hilden, Germany) and cloned into pGEM-T Easy plasmid (Promega Corporation, Madison, WI, USA) following the manufacturer's protocol.

Probe synthesis. For probe synthesis, digoxigenin-labeled antisense and sense cRNA probes were transcribed *in vitro* from the purified pGEM-T Easy plasmids according to the manufacturer's protocol (Promega Corporation). Sense cRNA probes served as negative controls for *in situ* hybridization. Probes were purified with a mini Quick Spin RNA Column (cat. no. 11814427001, Roche Diagnostics GmbH) and were then precipitated by sodium acetate. Incorporation of label and correct probe size was identified by RNA formaldehyde denaturing gel electrophoresis and blotting.

Combined *in situ* hybridization and immunohistochemistry on cryosections. *In situ* hybridization was performed as described previously with minor modifications (11,12). Briefly, cryostat sections (thickness, 15 μ m) were fixed with 4% formaldehyde in phosphate-buffered saline (PBS), pretreated with proteinase K and acetic anhydride and hybridized overnight at 65°C with cRNA probes at ~1-2 μ g/ml in hybridization solution (50% formamide, 5X saline sodium citrate, 1X Denhardt's solution, 100 μ g/ml herring sperm DNA (KPL, Inc., Gaithersburg, MD, USA), 300 μ g/ml yeast tRNA and 5 mM EDTA). Subsequently, the sections were washed

and the uncombined cRNA was removed by RNase. The sections were incubated with alkaline phosphatase-coupled sheep anti-digoxigenin antibody (1:5,000; Roche Diagnostics GmbH; cat. no. 11093274910) for 1 h at room temperature. To visualize the labeled mRNA, a solution of 4-nitroblue tetrazolium chloride (NBT; Boehringer Ingelheim, Ltd., Ingelheim, Germany) and 5-bromo-4-chloro-3-indoyl-phosphate (BCIP; Boehringer Ingelheim, Ltd.) was added.

Immunohistochemistry. Following *in situ* hybridization, the sections were processed for immunohistochemistry following the immunostaining protocol described by Tiveron *et al* (12), with a few modifications. Briefly, following the blocking step with 10% bovine serum albumin (Beyotime Institute of Biotechnology, Haimen, China) for 1 h at room temperature, post-hybridized slides were incubated with anti-RNA binding protein, fox-1 homolog 3 (NeuN) antibody (diluted 1:100 in 0.1 M PBS; EMD Millipore, Billerica, MA, USA; cat. no. MAB377) and anti-glial fibrillary acidic protein (GFAP) antibody (diluted 1:200 in 0.1 M PBS; EMD Millipore; cat. no. AB5804) overnight at 4°C. Subsequent to three washes for 10 min with 0.01 M PBS, the slides were incubated with horseradish peroxidase (HRP)-conjugated rabbit anti-mouse (1:1,000; Gene Tech, Shanghai, China; cat. no. GP016129) and pig anti-rabbit (1:1,000; Gene Tech, Shanghai, China; cat. no. GP021729) secondary antibodies for 1 h at room temperature and then washed twice for 10 min in 0.01 M PBS. Color development was performed by using the DAB kit (OriGene Technologies, Inc., Beijing, China) according to the manufacturer's protocol. ADAM10 mRNA abundance in each anatomical region was determined from optical density measurements. The measurement was performed by use of the Image-Pro Plus 6.0 (Media Cybernetics, Inc., Rockville, MD,

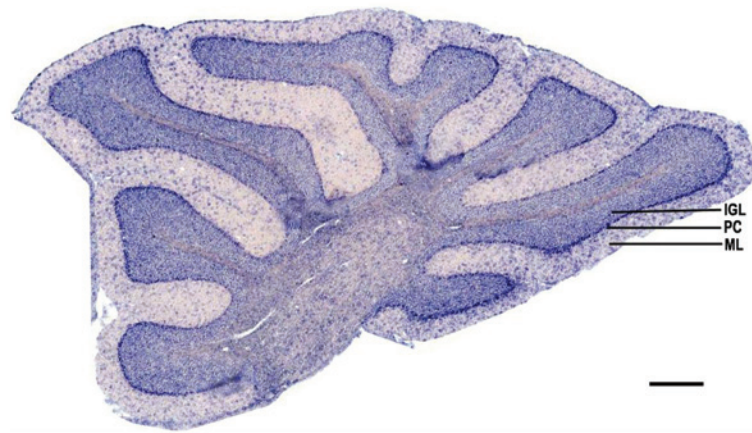


Figure 3. *In situ* hybridization results of Adam10 cRNA probe in adult mouse cerebellum. *In situ* hybridization results indicated that highest expression levels of Adam10 mRNA are in the cerebellum. Scale bar, 300 μ m. ML, molecular layer; IGL, internal granular cell layer; PC, purkinje cell layer; Adam10, a disintegrin and metalloproteinase domain 10.

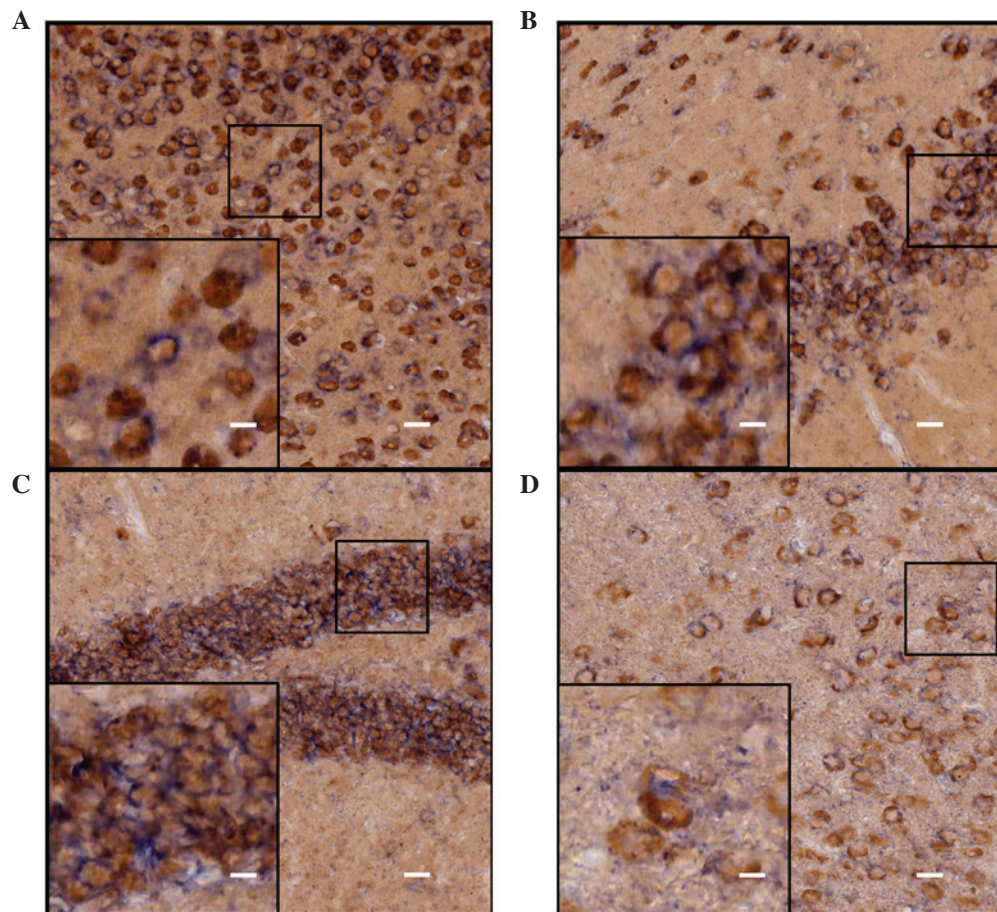


Figure 4. Adam10 *in situ* hybridization-positive cells coexpress NeuN in selected regions of adult mouse cerebrum. (A-D) Double staining results of Adam10 *in situ* hybridization and NeuN immunostaining in (A) the parietal cortex, (B) piriform cortex, (C) hippocampal dentate gyrus and (D) thalamus. Scale bar, 20 μ m. Scale bar in zoom box, 8 μ m. Adam10, a disintegrin and metalloproteinase domain 10; NeuN, RNA binding protein, fox-1 homolog 3.

USA). The mean activity in the tissue fields was calculated. For each field, at least five sections were measured and the mean value was used. Density values for each parameter are presented according to their respective percentile distributions: +++, >50% above background, strong; ++, >25% above background, moderate; +, <25% above the background, low but positive signal.

Image analysis. All sections were viewed and photographed under a light microscope (Olympus BX40; Olympus Corporation, Hamburg, Germany) equipped with a digital camera (Olympus DP70; Olympus Corporation). The measurements were performed using Image-Pro Plus 6.0 software. Photomicrographs were adjusted in contrast and brightness using Adobe Photoshop (Adobe Systems, Inc., San

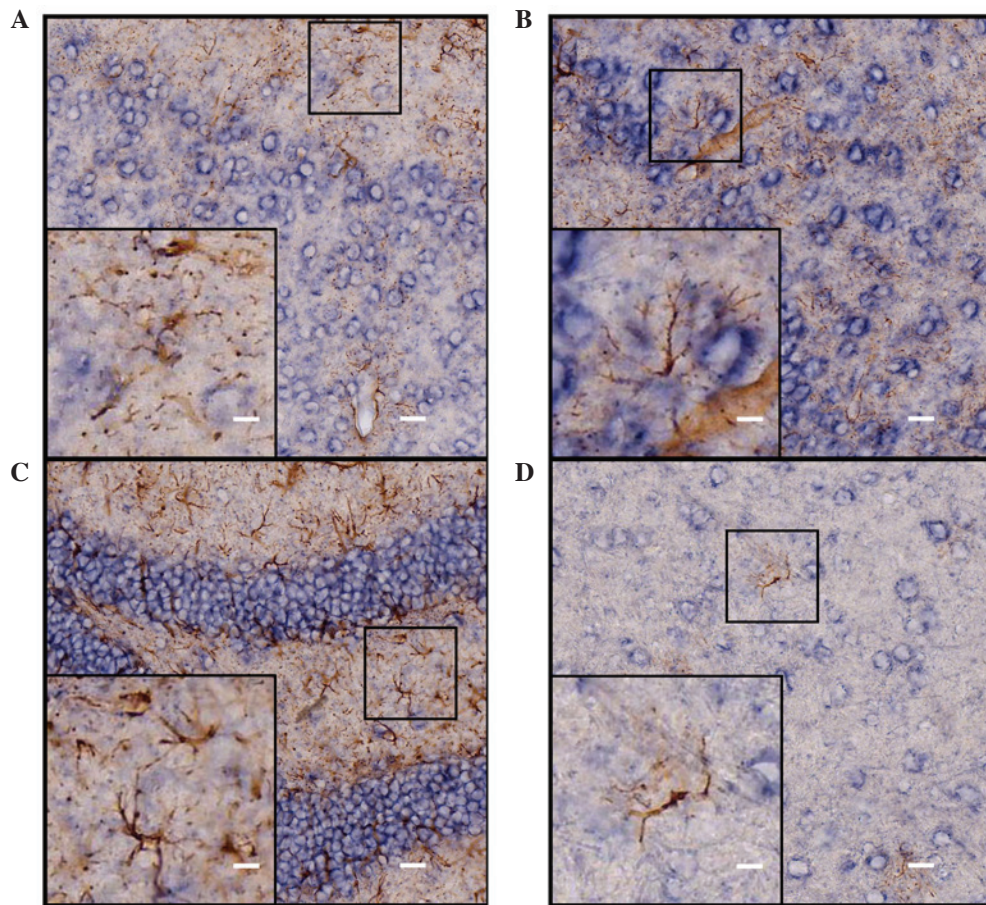


Figure 5. Adam10 *in situ* hybridization-positive cells are GFAP-negative in selected regions of adult mouse cerebrum. (A-D) Double-staining of Adam10 *in situ* hybridization and GFAP immunostaining in (A) the parietal cortex, (B) the piriform cortex, (C) the hippocampal dentate gyrus and (D) the thalamus. Scale bar, 20 μ m. Scale bar in zoom box, 8 μ m. Adam10, a disintegrin and metallopeptidase domain 10; GFAP, glial fibrillary acidic protein.

Jose, CA, USA) for optimal display of the staining patterns in the figures.

Results

The effectiveness of the ADAM10 cRNA probe was indicated by detection of immunoreactivity with the antisense probe. To detect the effectiveness of the ADAM10 cRNA probe for *in situ* hybridization, T7 RNA transcriptase and SP6 RNA transcriptase were used in *in vitro* transcription to obtain the antisense and sense cRNA probes, respectively. Sense cRNA probes served as a negative control for *in situ* hybridization. The *in situ* hybridization results presented in Fig. 1 indicated that hybridization signal may be detected only in the antisense ADAM10 cRNA probe slices using mixed cultures of neurons and glial cells and C57BL6 mouse brain slices (Fig. 1B and D). The immunoreactivity was not detected by use of the sense ADAM10 cRNA probe (Fig. 1A and C).

ADAM10 mRNA expression is distributed in selected regions of the adult mouse brain. *In situ* hybridization determined that ADAM10 mRNA distribution is present in selected regions of the adult mouse brain (Table I). ADAM10 mRNA was markedly expressed throughout the telencephalon, including the parietal and piriform cortex, the hippocampus (CA1-CA3 and the dentate gyrus; Fig. 2). Within the diencephalon, the

hybridization signal was moderate, particularly in the septal nucleus (Fig. 2A), the thalamus and hypothalamus (dorsomedial hypothalamic nucleus; Fig. 2B and C) and surrounding areas of the third ventricle (Fig. 2C and D). In the striatum, a weaker hybridization signal for ADAM10 was also detected (Fig. 2A and B).

ADAM10 mRNA expression is distributed in selected regions of the cerebellum of adult mice. To detect the expression of ADAM10 mRNA in the cerebellum, cerebellar sagittal sections were used for *in situ* hybridization. The hybridization results indicated that the positive signals were predominantly distributed in the internal granular cell layer and purkinje cell layer. There were also scattered positive cells distributed in the molecular layer (Fig. 3).

Identification of the ISH-positive cells in the cerebrum of adult mice. Neurons and astrocytes are the basic components of the CNS, and each performs important functions. In order to further identify cell locations of ADAM10 in neurons or astrocytes, immunohistochemical staining with different cell-specific antibodies (astroglial marker, GFAP and the neuronal marker, NeuN) were used to determine the types of the ISH-positive cells. The hybridization signal was developed by alkaline phosphatase and NBT/BCIP staining, and the positive cells were a blue-purple color following staining.

HRP-labeled chromogenic reagents were used for immunohistochemistry, and the positive cells were stained brown. Results from the double staining indicated that the majority of the ADAM10 ISH-positive cells coexpressed NeuN (Fig. 4), and that a number of cells were not NeuN immunoreactive (Fig. 4D); however, these cells were also negative for GFAP staining (Fig. 5) in various selected regions of adult mouse brain, including the parietal cortex, piriform cortex, hippocampal dentate gyrus and thalamus.

Discussion

In the present study, an ADAM10 cRNA probe for *in situ* hybridization was constructed to investigate the expression pattern of the ADAM10 gene in the CNS of adult mice. Immunohistochemical staining was used to identify the type of cell that was ISH staining-positive. The results demonstrated that expression of the ADAM10 gene was restricted to neurons of the cerebral cortex, hippocampus, thalamus and cerebellar granular cells in the CNS of adult mice.

ADAM10, termed Kuzbanian in *Drosophila* (13) is essential to embryonic development and control of neurogenesis and axon extension in the CNS (14-16). ADAM10 has been confirmed as a candidate α -secretase responsible for cleaving various proteins, including APP, heparin-binding EGF, EGF receptor, E-cadherin, N-cadherin, protocadherin C3 and vascular endothelial-cadherin (2-4,17-20). ADAM10-deficient mice developed only to embryonic day (E)9.5 with multiple defects in the CNS, somites and the cardiovascular system (1). A previous study determined that conditional knock-out of ADAM10 in neural progenitor cells (NPCs), NPC-derived neurons and glial cells in mice, leads to perinatal mortality with a disrupted neocortex and a markedly reduced ganglionic eminence (21). ADAM10 gene knockout results in abnormalities of the cardiovascular system and CNS, suggesting that the ADAM10 gene is important for the development of the CNS. However, its specific roles and precise underlying molecular mechanisms remain to be further elucidated.

Previous studies have demonstrated that the expression of ADAM10 in the brain of adult mice was restricted to specific areas. Within the telencephalon and diencephalon, the expression of ADAM10 mRNA was more widespread. In the mesencephalon, ADAM10 mRNAs was expressed in the inferior colliculus. The highest expression was detected in the cerebral cortex (9). These results are similar to the findings of the present study, that ADAM10 mRNA was distributed in specific regions of adult mouse brain, including notable expression in the parietal and piriform cortex, the hippocampus and the cerebral cortex. Moderate expression was also observed in the septal nucleus, the thalamus, hypothalamus and surrounding areas of the third ventricle. The present study also determined that a weaker hybridization signal for ADAM10 may be detected in the striatum. In a previous study, Lin *et al* (10) indicated that ADAM10 was predominantly expressed by developing blood vessels, restricted neuroepithelial regions and in differentiating gray matter. In addition, ADAM10 was observed to be expressed by oligodendrocytes at later embryonic stages in numerous fiber tracts (10). The present study aimed to detect the ADAM10 expression in neurons and astrocytes in the CNS of adult mice;

thus, it was not determined whether blood vessels expressed ADAM10. However, based on previous studies that identified strong ADAM10 expression in blood vessels persisting at E10 and E12, prior to decreasing at later stages and is no longer detectable in brain at E19 (10), the present study hypothesizes that the mature blood vessels would not express ADAM10 in the CNS of adult mice. In addition, the ADAM10 expression in oligodendrocytes was not investigated in the current study, as there is increasing evidence suggesting that oligodendrocytes express ADAM10 in the developing and adult brain (10,22).

In conclusion, the present study is consistent with previous results by other groups (9,10) that demonstrate ADAM10 is expressed in a number of restricted regions of the brain of adult mice. To the best of our knowledge, this is the first study to demonstrate that the ADAM10 is expressed by neurons in the brain of adult mice. These results may provide the basis for future investigations at the experimental level using Cre/Loxp conditional knockout technology (23).

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