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Neural cell adhesion molecule (NCAM) and polysialic acid–NCAM expression in developing ICR mice

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Abstract

Background: Coexpression of polysialic acid (PSA)–neuronal cell adhesion molecule (NCAM) with immature neuronal markers is used to indicate the developmental state of neurons generated in the subgranular zone (SGZ) of adult hippocampus. PSA–NCAM is highly expressed throughout the embryonic and juvenile mammalian brain, but heavily downregulated in adult brain.

Objective: To visualize the expression profiles of NCAM/PSA–NCAM in the dentate SGZ of the hippocampus in developing ICR mice.

Methods: Cellular distribution, expression, and developmental changes of NCAM/PSA–NCAM were studied in ICR mice at embryonic age 17 days (E17); and similarly at postnatal ages P3, P5, and P7. The SGZ was studied using NCAM and PSA–NCAM immunoreactive staining with or without hematoxylin counterstaining. Western blotting was used to confirm protein expression levels.

Results: NCAM expression was localized to the surface of neurons and glia and was higher in postnatal mice than it was in embryonic mice. PSA–NCAM was found in cytoplasm and membrane of neural cells, more densely staining in the dentate SGZ at P7, but no staining found at E17. Western blotting of brain tissues also showed expression of both PSA–NCAM and NCAM increased significantly at P5 and P7 compared with expression at P3.

Conclusions: Progressive increase in NCAM expression occurs in the SGZ during embryogenic and postnatal development. PSA–NCAM was not expressed in embryonic ICR mice, but was increased after birth and highly localized in the SGZ at P7. This NCAM expression pattern in the developing brain indicating structural plasticity and neurogenesis may be useful for study of brain repair.

Keywords: hippocampus; NCAM; postnatal developing mice; PSA–NCAM

Cell adhesion molecules (CAMs) play crucial roles in the architecture of the developing central nervous system (CNS) to modulate cellular interactions and stabilize neural circuitry [1]. Neural CAM (NCAM) is the most widely present CAM in the CNS [2, 3] and comprises a family of cell surface glycoproteins that are closely related structurally and belong to the

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immunoglobulin superfamily of adhesion molecules encoded by a 26-exon-containing gene found at single locus in the genome [4].

The alternative splicing and posttranslational modifications can generate at least 20–30 distinct forms of NCAM from the single-copy gene, including the transmembrane form (NCAM 180), cytoplasmic form (NCAM 140), and cell membrane domain form (NCAM 120) attached to the cell surface via a glycosylphosphatidylinositol anchor [5]. In vertebrate animals, polysialic acid (PSA) is found as a major biomolecule in the brain [6]. In mammals, PSA is a linear homopolymer of α2-8-linked *N*-acetylneuraminic acid. As distinct from other cell-surface carbohydrates, specific polysialylation of NCAM occurs and leads to a helical conformation due to the presence of the polyanion in an aqueous environment [7], thus polysialylation can account for up to 30% molecular mass because of hydration. In NCAM-deficient mice, almost complete absence of PSA–NCAM exists because of a lack of sialylation [8]. Two tissue- or cell-specific enzymes, ST8SiaIV (PST) and ST8SiaII (STX), functioning as polysialyltransferases are responsible for the sialylation of NCAM in the Golgi of eukaryotes [9, 10]. In rodents, these enzymes are highly expressed from an embryonic age of 8 to 9 days, but the expression is then repressed during adulthood [11].

During development, cell–cell interactions mediated by CAMs play key roles in the control of cell aggregation and migration, and also influence cell proliferation and differentiation. Due to the function of NCAM in neuronal differentiation and plasticity of neuronal synapse during neuronal development and regeneration, formation, and retention of memory, it is conceivable that the modulation of NCAM functions might be beneficial for clinical treatments [12]. Experiments in vitro and in vivo have shown that interference with NCAM binding or expression causes marked changes in brain morphogenesis [13], but little is known about its expression pattern in mammalian embryos. Therefore, the aim of the present study was to visualize the expression profiles of NCAM/PSA–NCAM in the subgranular dentate (SGZ) of the hippocampus of developing brains in mice. We hypothesized that PSA–NCAM is increased after birth at the periods of increased migration and the proliferation for structural plasticity in brain development.

Materials and methods

Animals

Pregnant 10-week-old mice, Japanese Society for Laboratory Animal Resources: Institute of Cancer Research (USA) JcL:ICR (ICR) mice, were obtained in the laboratory at Faculty of Veterinary Science, Mahidol University (from the National Laboratory Animal Center of Mahidol University, Nakhon Pathom, Thailand). Fetal and postnatal mice were included from 2 or 3 litters at the embryonic age of 17 days (indicated as E17) and 3, 5, and 7 days after birth (postnatal day, indicated as P3, P5, and P7). The mice were housed in a controlled environment, 25°C, 50%–60% humidity, and allowed for food and tap water ad libitum. Room lights were on between 7:00 and 19:00 h. All experiments were performed in compliance with local and international ethical standards and had been approved by the Ethical Committee for Animals Care and Use of the Faculty of Veterinary Science, Mahidol University (Institutional Animal Care and Use Committee No. MUVS-2016-09-35) in compliance with The Animals for Scientific Purposes Act, BE 2558 (AD 2015) (Thai Government Gazette, Vol. 132, Part 18 a, 13th March 2015) and the revised Institute of Laboratory Animal Resources, Commission on Life Sciences, National Research Council "Guide for the Care and Use of Laboratory Animals" Washington, D.C.: National Academy Press; 1996, and were conducted under licences to use animals for scientific purposes from the Institute of Animals for Scientific Purpose Development (IAD) National Research Council of Thailand.

Tissue collection

The pregnant mice were anesthetized intramuscularly with a combination of ketamine (35 mg/kg) and xylazine (2.4 mg/kg) at the embryonic age of 17 days. Mice were perfused with a normal saline flush, then followed with 4% paraformaldehyde (PFA) in 0.1 M phosphate-buffered saline (PBS), pH 7.4. Fetuses (at E17) were removed after perfusion and immersed in the same fixative at 4°C for 1 h, then processed and embedded in paraffin.

The P3, P5, and P7 pups were perfused transcardially. The perfusion is not easy to perform without any practice because these animals are so small. Dry ice (CO_2) was used for anesthesia, then a sharp 28-G needle was inserted into the apex of the heart (left ventricle), the right atrium was nicked. A 10 mL syringe full of 4°C cold PBS was used to perfuse the pup followed by 10 mL of cold PBS with or without 4% PFA. After perfusion, brains of postnatal mice were removed and put into cold PBS with or without 4% PFA. All samples that were put into 4% PFA for postfixation overnight at 4°C were washed in PBS and stored in 70% ethyl alcohol at 4°C for processing to paraffin sections and immunoperoxidase staining. After perfusion without 4% PFA for postfixation, brains of postnatal mice were stored at –80°C for western blotting.

Brain weight, body length, and body weight were recorded to indicate the normal growth and development of experimental mice.

Immunohistochemistry

For immunoperoxidase staining, 3-µm-thick brain sections mounted on slide were placed in 60°C oven overnight with molten paraffin, then deparaffinized with xylene, rehydrated with alcohol, and incubated with 10 mM sodium citrate buffer (pH 6.0) in a 95°C water bath for 40 min to retrieve antigens. Following 3 washes with PBS, sections were incubated with 3% hydrogen peroxide to block endogenous peroxidase and 2% bovine serum albumin (BSA) to block nonspecific binding. The sections were then immunostained at 4°C overnight with primary antibodies: rabbit anti-NCAM antibody (1:100; AB5032; EMD Millipore) and mouse anti-PSA–NCAM antibody (1:200; MAB5324; EMD Millipore). After primary antibody incubation, sections were incubated with goat IgG secondary antibody and visualized by peroxidase activity in Envision kit (K8002-40; Dako), then counterstained with Harris hematoxylin at room temperature, 30 s to stain the nuclei. Stained sections were examined under a light microscope (Olympus). Negative controls were prepared by omitting the primary antibody and showed no notable staining.

Image analysis

Using image analysis software (ImageJ, version 1.46 r; National Institutes of Health, Bethesda, MD, USA), in the coronal sections at the level of dorsal hippocampus, the cortical thickness of all groups was measured from the surface of cerebral cortex to superior border of corpus callosum as shown in **Figure 1**.

The NCAM and PSA–NCAM immunoreactivity in SGZ of mice brains was also measured in a blinded manner using the image analysis software. The area of interest is the SGZ; therefore, cropped images sampling the SGZ and the polymorphic (po) layer of dentate in hippocampus are shown in **Figure 2**. The software was initiated with settings for measuring area (µm2), integrated intensity, and area fraction. The global scale for the image analysis was set as 1,600 pixels, 1 mm, in a pixel ratio of 1. We used 3 images from the representative areas. Area fraction (%area) in thresholded images is the percentage of pixels or staining in the images that were highlighted in red after setting as an 8 bit image [14].

Western blotting

Brain samples stored at –80°C were thawed and homogenized in cold radioimmunoprecipitation assay (RIPA) buffer using an RNA isolation kit (Stratec Molecular) and centrifuged at 100,000 ×*g*, and the supernatant was mixed with an equal volume of sodium dodecyl sulfate (SDS) buffer (0.125 M Tris–HCl (pH 6.8), 2% SDS, and 5% (v/v) 2-mercaptoethanol) and placed in a boiling waterbath for 5 min. Total protein concentrations were quantified with a modified Bradford protein

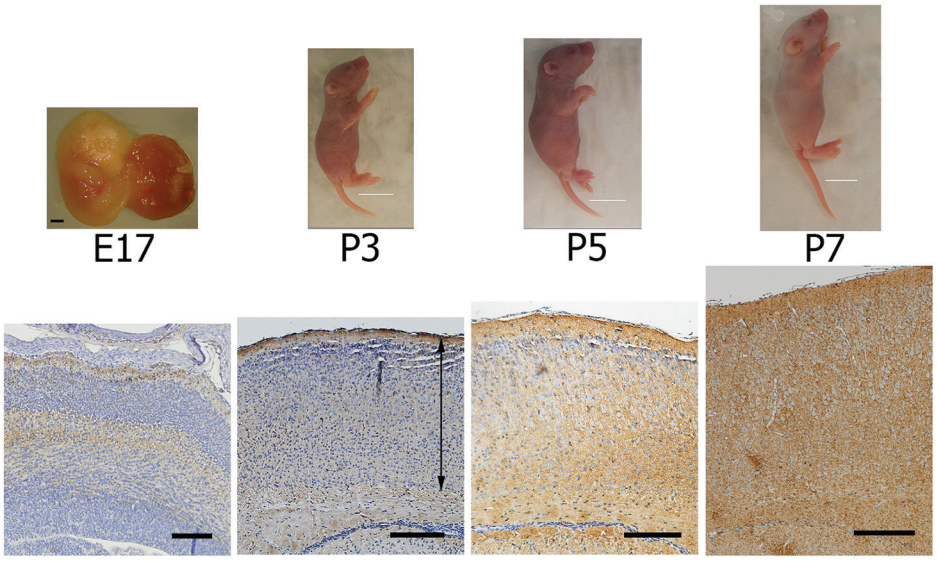

Figure 1. E17 fetal and P3, P5, and P7 postnatal mice with normal appearance. The black scale bar for E17 represents 1 mm. Scale bars in white for the pups represent 1 cm. Neural cell adhesion molecule (NCAM) immunoreactivity (brown) and hematoxylin (purple)-stained coronal brain sections were also compared. Progression of neocortex shows 6 cortical cell layers starting from E17 to P7. At E17, cortical neurons continue to aggregate and separate into layers, but postnatal brains P3, P5, and P7 show the migration of cortical neurons and their location in overlapped layers. NCAM immunoreactivity was increased from E17 to P7. The double-ended arrow indicates the measurement of the cortical thickness. Scale bars on the coronal sections represent 200 µm
E17, fetal mouse at embryonic age of 17 days; P3, P5, and P7, postnatal mice 3, 5, and 7 days after birth respectively

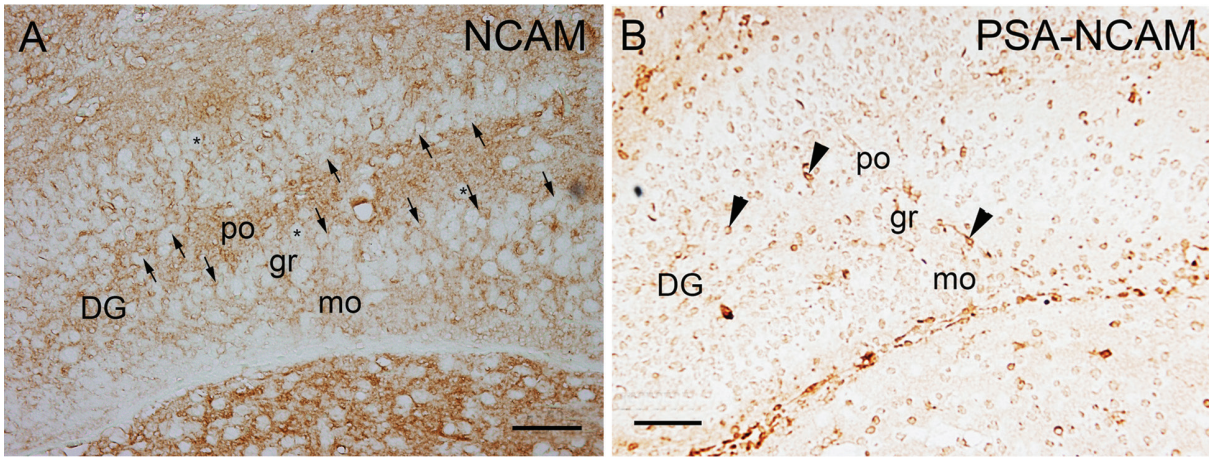

Figure 2. Coronal section through the hippocampus of P3 postnatal mice indicating layers in the DG: po layer, strata gr, and mo. Immunoperoxidase staining with NCAM (**A**) was localized to the extracellular matrix around cell surface of neurons. *Nonstaining neuronal cells and SGZ indicated by arrows for neuronal lining at the base of gr layer. PSA–NCAM (**B**) was localized in the cytoplasm and processes of neural cells (indicated by arrow heads). Scale bar represents 50 µm

DG, dentate gyrus; gr, stratum granulosum; mo, stratum moleculare; po, polymorphic; SGZ, subgranular zone

assay (Bio-Rad). The proteins were separated by polyacrylamide gel electrophoresis (PAGE) using a discontinuous gel and buffer system as described elsewhere [15] (SDS-PAGE), using 7% (w/v) polyacrylamide in the separating gel. After electrophoresis, proteins were transferred to a nitrocellulose membrane using a semidry electroblotting apparatus. Equal loading was confirmed by Ponceau S staining. Nonspecific binding sites on the blots were blocked with 5% fat-free milk powder in PBS with 0.05% Tween 20 for 1 h and the blots probed with primary antibodies. The nitrocellulose membrane was incubated overnight with rabbit anti-NCAM polyclonal antibody (AB5032; EMD Millipore) and mouse anti-PSA–NCAM monoclonal antibody (MAB5324; EMD Millipore) at 1:1,000 dilution and then incubated for 1 h with donkey anti-rabbit IgG affinity purified, peroxidase-conjugated secondary antibody (AP182P; EMD Millipore), and rabbit anti-mouse IgG secondary antibody, horseradish peroxidase (HRP) conjugate (AP160P; EMD Millipore) at 1:1,000 dilution.

The glyceraldehyde 3-phosphate dehydrogenase (GAPDH) primary antibody (ab9485, Abcam; diluted 1:1500) was also probed as a loading control (30 µg protein) to normalize the values for NCAM and PSA–NCAM.

After 5–15 min of color development, the nitrocellulose membranes were washed and recorded photographically. The intensity of the bands was quantified with image analysis software.

Statistical analyses

Statistical analysis was performed using Statistical Package for the Social Sciences (SPSS) version 18.0. The data set from each region of interest was first tested for normal distribution. A one-way ANOVA followed by a post hoc Tukey test was then performed to assess differences. Results are presented as mean ± standard error of the mean (SEM) and differences with $P < 0.05$ were considered significant in tests of statistical inference.

Results

In the present study, fetal (E17) and postnatal mice (P3, P5, and P7) showed a normal external appearance (**Figure 1**) with normal sensory and motor function. Normal growth development is indicated by measurements as shown and compared in **Table 1**. Cortical thickness, brain weight, body weight, and body length were compared between P3, P5, and P7 and increased significantly at $P < 0.05$ when compared with P3. In addition, as shown in **Figure 1**, normal brain development was indicated by the 6 cortical cell layers in all groups of mice. Progression of the neocortex shown by NCAM immunoperoxidase staining with hematoxylin-counterstained coronal brain sections demonstrated 6 cortical cell layers starting from E17 to P7. In E17, cortical neurons still aggregate and separate into layers, but in postnatal brains P3, P5, and P7 the cortical neurons migrate to specific targets and then they show overlapped layers. NCAM immunoreactivity is also shown in **Figure 1**; the intensity increased gradually from E17 to P7 and entirely stained over all layers in the cortex of P7 mice.

The localization of NCAM and PSA–NCAM immunoreactivity in coronal sections through the SGZ of hippocampus is shown and indicated the subfields of the dentate gyrus (DG): po layer, stratum granulosum (gr), and stratum moleculare (mo).

Table 1. Cortical thickness, brain weight, and body weight, and NCAM expression in the cerebral cortex of E17 fetal, and P3, P5, and P7 postnatal mice

Variable (mean ± SD)	E17	P3	P5	P7
Cortical thickness (μm)		541 ± 4.35	574.14 ± 6.4*	780.96 ± 7.05*
Brain weight (g)	–	0.21 ± 0.01	0.24 ± 0.02*	0.27 ± 0.08*
Body weight (g)		2.77 ± 0.14	3.28 ± 0.21*	4.71 ± 0.41*
Body length (cm)		5.58 ± 0.04	5.64 ± 0.07*	7.39 ± 0.03*
NCAM expression in the cerebral cortex (integrated density)	25.87 ± 2.46	234.75 ± 13.61	259.13 ± 11.71	387.34 ± 5.87

*Significant difference at $P < 0.05$ when compared with P3 (n = 6 each group)
E17, mouse at embryonic age of 17 days; NCAM, neural cell adhesion molecule; P3, P5, and P7, postnatal mice 3, 5, and 7 days after birth respectively

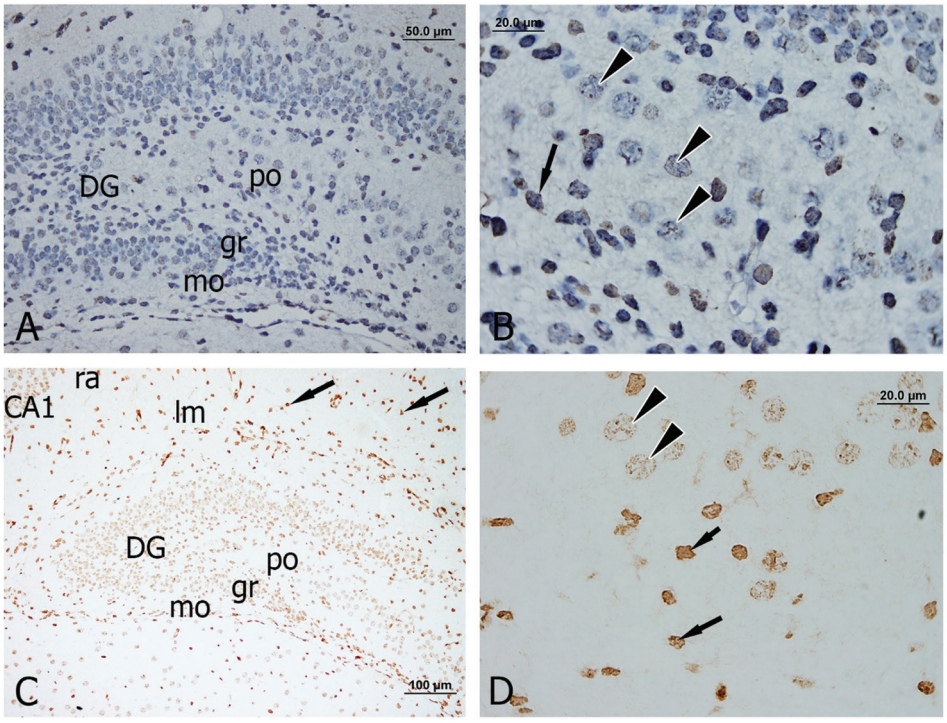

Figure 3. Coronal sections through DG of mice at postnatal day 5 (P5) (**A** and **C**) indicating layers in the DG: po layer, strata gr, and mo. PSA–NCAM immunoperoxidase staining with (**A** and **B**) or without (**C** and **D**) hematoxylin was localized in the cytoplasm and nucleus of neurons in gr (arrow heads) and intense staining in the small nucleus (arrows) is found in lm and ra layers of CA1. Scale bars are 100 μm (**C**), 50 μm (**A**), and 20 μm (**B, D**)
CA1, cornu ammonis 1 area of the hippocampus; DG, dentate gyrus; gr, stratum granulosum; lm, stratum lacunosum moleculare; mo, stratum moleculare; NCAM, neural cell adhesion molecule; po, polymorphic layer; PSA, polysialic acid; ra, stratum radiatum

In **Figure 2A**, NCAM immunoreactivity is localized to the surface of neurons and glia and is more intense in postnatal mice than it is in embryonic mice. In **Figure 2B**, PSA–NCAM is localized in cytoplasm and membrane of neural cells. In **Figure 3**, PSA–NCAM immunoperoxidase staining with or without hematoxylin counterstaining is demonstrated. PSA–NCAM immunoreactive neural cells are localized in the 3 layers of dentate and in the lacunosum moleculare (lm) and radiatum (ra) layers of the cornu ammonis 1 area (CA1) of the hippocampus, and the higher magnification in the po layer of the dentate showed PSA–NCAM immunoreactivity in the cytoplasm and nucleus of neural cells. In **Figure 4**, the localization of NCAM/PSA–NCAM immunoreactivity in SGZ was compared between groups, intense PSA–NCAM staining is seen in the SGZ of dentate in P7, but no or less staining found in E17. Results from image analysis seen in **Figure 5** indicated a gradual increase in NCAM and PSA–NCAM immunoreactivity from E17 to P7; the immunoreactivity was significantly higher when compared with that at E17. The western blot analysis showed similar results, and the NCAM

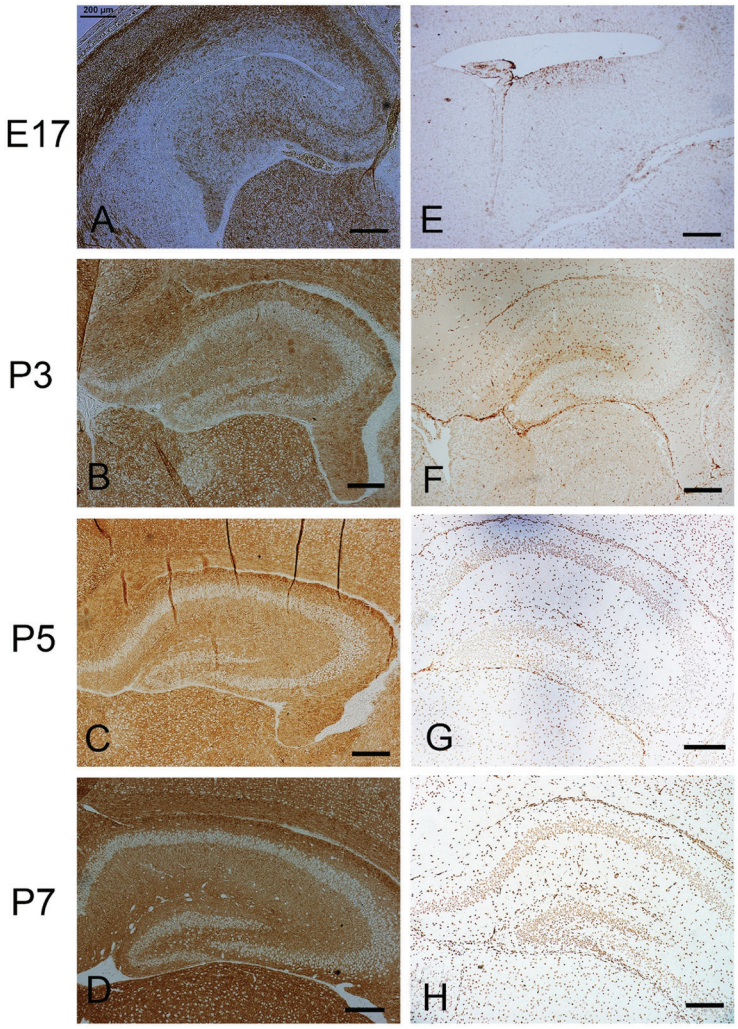

Figure 4. Coronal sections through hippocampus of E17 fetal mice (**A** and **E**), and P3 (**B** and **F**), P5 (**C** and **G**), and P7 (**D** and **H**) postnatal mice after immunoperoxidase staining for NCAM (**A–D**) and PSA–NCAM (**E–H**). Scale bars represent 200 μm
E17, mouse at embryonic age of 17 days; NCAM, neural cell adhesion molecule; P3, P5, and P7, postnatal mice 3, 5, and 7 days after birth respectively; PSA, polysialic acid

and PSA–NCAM immunoreactivity normalized to that of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was significantly greater in P5 and P7 when compared with P3 as shown in **Figure 6**. The result of E17 was not included in Western blot analysis, because whole body of E17 was collected.

Discussion

The present study found all parameters measured indicated normal ICR mice brain development from embryonic stage E17 to postnatal stages P3, P5, and P7 including the increase in cortical thickness related to the increase in brain and body weights. In addition, the 6 cortical cell layers have been shown in all groups of mice with some differences, dense aggregation of neurons in layers found in the embryonic brain at E17, but more homogeneity of cortex was found in postnatal brains at P3, P5, and P7. In agreement with findings reported previously [16], the cytoarchitecture was established during embryonic development through the coordinated processes of neurogenesis and cell migration. During mammalian embryonic development, newly generated neurons leave the proliferative areas to reach their place of final maturation in precise locations within the cortex. The present study established that most of the migration occurred after birth; in postnatal mice, neurons underwent disaggregation and migrated to specific targets or layers, where they would form contacts or synapses.

Neuronal migration is a complex process involving intrinsic and extrinsic factors including transcription factors, adhesion molecules, secreted cues, and extracellular matrix

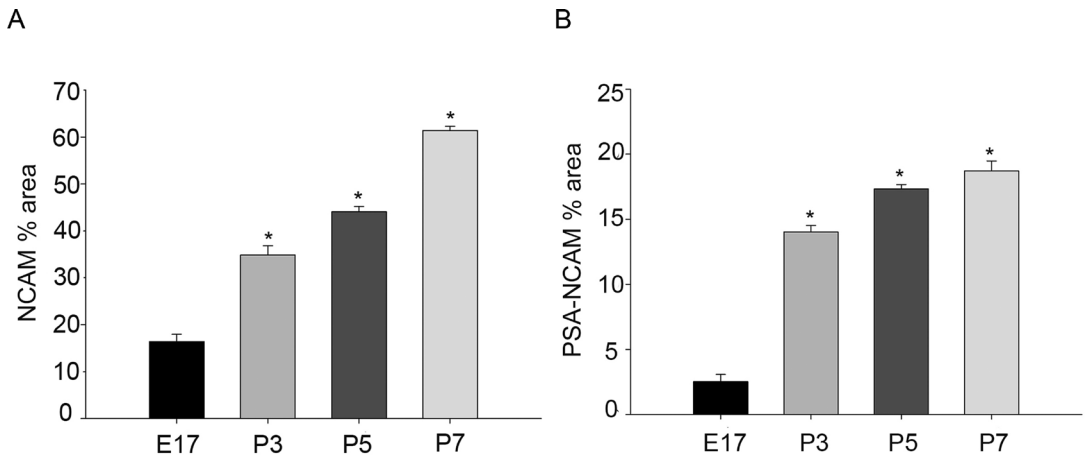

Figure 5. Image analysis of NCAM (**A**) and PSA–NCAM (**B**) immunoreactivity in the SGZ of dentate expressed as % area. The immunoreactivity in the brains of fetal E17 and postnatal P3, P5, and P7 mice was compared. Significant differences (*$P < 0.05$) were found at P3, P5, and P7 when compared with E17. Bars indicate mean % area and error bars indicate SEM

E17, mouse at embryonic age of 17 days; NCAM, neural cell adhesion molecule; P3, P5, and P7, postnatal mice 3, 5, and 7 days after birth respectively; PSA, polysialic acid; SEM, standard error of the mean; SGZ, subgranular zone

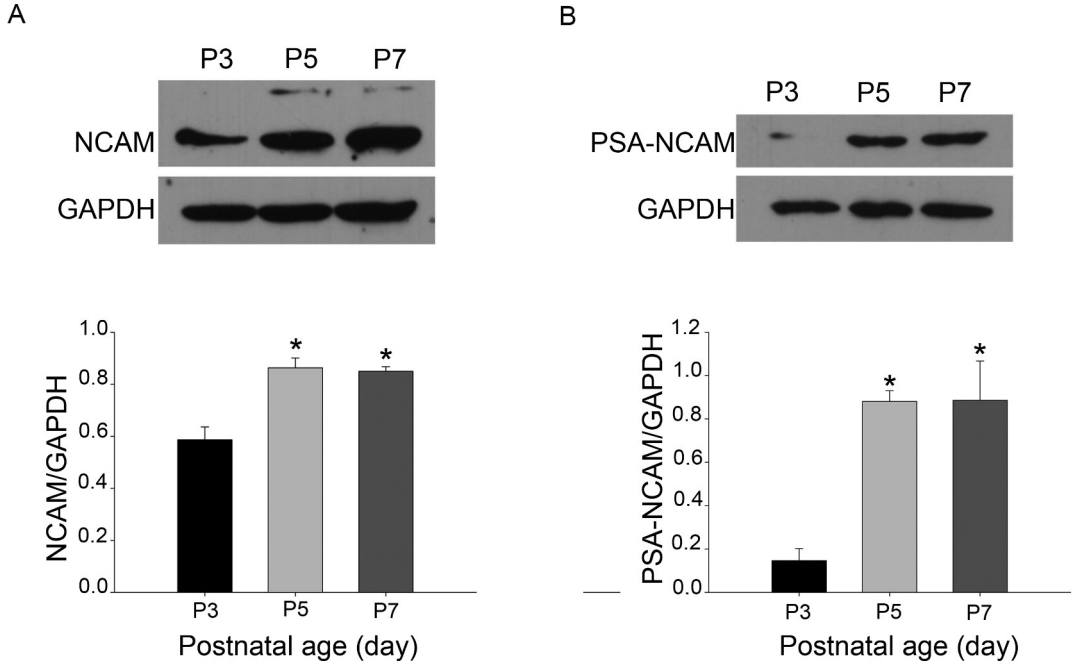

Figure 6. Western blot analysis of NCAM (**A**) and PSA–NCAM (**B**) immunoreactivity in brain tissue (homogenates) from individual P3, P5, and P7 postnatal mice (n = 3). Bars indicate mean values of NCAM and PSA–NCAM immunoreactivity levels normalized to that for GAPDH. Significant differences (*$P < 0.05$) were found for P5 and P7 when compared with P3. Error bars indicate SEM

GAPDH, glyceraldehyde 3-phosphate dehydrogenase; NCAM, neural cell adhesion molecule; P3, P5, and P7, postnatal mice 3, 5, and 7 days after birth respectively; PSA, polysialic acid; SEM, standard error of the mean

components that control the different phases and aspects of cell movement. These factors can regulate the cytoskeleton in the control of cell migration in brain including radial, tangential, and axonophilic migration [17].

Previous studies have found a variety of NCAM expressions in the brain development of different species. NCAM was found to appear early in development in homologs of all 3 germ layers in amphibian and avian embryos [18]. However, at later stages, NCAM is also dramatically upregulated in the neural plate and neural tube forming in response to neural induction. Moreover, NCAM is ubiquitously distributed in the neural tube and developing CNS [19].

In a study of early mouse embryo, immunostaining of the cerebral cortex showed NCAM immunoreactivity throughout the width of the telencephalon in E9 [20]. Marín-Padilla [21] described that the prenatal development of mammalian cerebral cortex, including that of humans, is characterized by 2 periods: an early embryonic and a late fetal period [22]. In the present study, NCAM immunoreactivity suggesting expression of the protein in the cerebral cortex and SGZ in the dentate of the hippocampus gradually increases from the late fetal E17 stage and progressively through postnatal stages P3, P5, and P7. However, PSA–NCAM immunoreactivity was coupled with NCAM immunoreactivity after birth in postnatal P3, P5, and P7 suggesting a role in the increase of neuronal migration in both the cerebral cortex and SGZ.

Our findings are consistent with an immunohistological study of motor neuron development [23] that showed the absence of NCAM immunoreactivity in the early development as in E10.5 rat cervical spinal cord and immunoreactivity restricted to motor neurons at E11. Another study of NCAM expression in the early mouse embryo demonstrated positive immunostaining throughout the neuroepithelium in the cross-sections of the E8 and E9 neural tube at the level of the future spinal cord [24]. Our present study confirmed that low levels of both NCAM and PSA–NCAM immunoreactivity in SGZ are detected in the early development as seen here in the embryonic brain at E17 with higher levels gradually increasing in postnatal brains at P3, P5, and P7 as demonstrated by western blotting and histology with immunoperoxidase staining.

The focus on newly generated neurons in the SGZ in hippocampus was studied [25] by demonstrating the coronal section through hippocampus to show subfields in CA1 in the hippocampus, and in the DG. Individual layers in the subfields are indicated as follows: strata oriens (or), pyramidale (py), ra, and lm in CA1; po layer, strata gr, and mo in the DG. As shown in **Figures 2 and 3**, NCAM immunoreactivity was seen on the surface of neuronal cells when cell–cell interactions, aggregation, and migration occur and may influence cell proliferation, differentiation, and the development of synapses. Dityatev et al. [26] reported that PSA–NCAM is highly expressed throughout the embryonic and juvenile mammalian brain, but by contrast we found no or scant immunoreactivity at E17, PSA–NCAM was mostly found after birth in ICR mice. PSA–NCAM is proposed as involved in a range of developmental processes including cell migration, axon/dendrite growth and remodeling, and synaptic reorganization [26]. PSA–NCAM is also considered to mediate neuronal plasticity by facilitating the remodeling of synapses and neuronal structure.

In all vertebrates [27], including mammals [28] and humans [29], the main neurogenic area of the hippocampus is found at the DG, and not at the subventricular zone. The newly produced hippocampal neurons tend to differentiate to granule cells locally, rather than migrate to other areas [30]. Proliferation of the neuronal cells occurs in the SGZ of DG or in the inner area of the granule cell layer. The majority of these newly produced cell bodies translocate to peripheral layers just a few micrometers distant. They then extend their dendrites and axons to the stratum moleculare and the hilar area, respectively [30]. By 3 weeks after neuronal production, their neurites start to proliferate, while the dendritic spines appear from the 3rd week [31]. Substantial PSA–NCAM expression can be demonstrated in these newly proliferated precursors of the granule cells [32]. To investigate the plasticity of the CNS and neuronal production in developing mice, it may be useful to employ NCAM and PSA-NCAM as markers for implicating the pattern of NCAM expression in brain repair.

The present study is limited because E17 fetal brains are too small to be collected. They were collected as whole body for immunoperoxidase staining, but not included in Western blotting of brain tissue. Therefore, PSA–NCAM immunoreactivity at E17 cannot support its low level as shown in staining. Any comparison using immunoperoxidase staining may find it difficult to demonstrate any difference among groups, and the result may be better after immunofluorescent staining. Nevertheless, immunoperoxidase staining can show other nearby cells and related structures.

Conclusions

NCAM is expressed in both embryonic and postnatal mice during neuronal development related to increasing age. Our findings support the hypothesis that PSA–NCAM is not expressed in embryonic mice, but the polysialylated glycoprotein expression increases after birth and is highly localized in the SGZ at P7. The change in NCAM/PSA–NCAM immunoreactivity of SGZ showed similar pattern to western blot analysis of total brain tissue; the pattern of immunoreactivity in the SGZ can be a potential indicator for investigating the alteration in plasticity and neuronal production during CNS development. PSA–NCAM may be useful for implying the pattern of NCAM expression in repairing of brain area that can newly generate like SGZ of hippocampus involving cognitive repair.

Author contributions. CT and SC contributed substantially to the conception and design of this study. CT, WS, PN, GPS, WW, and BT dissected the embryonic and postnatal mice and collected the data. All the authors analyzed and interpreted the data. CT and SC drafted the manuscript, and WS, PN, GPS, WW, BT contributed substantially to its critical revision. All the authors approved the final version submitted for publication and take responsibility for the statements made in the published article.

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Conflict of interest statement. The authors have each completed and submitted an International Committee of Medical Journal Editors Uniform Disclosure Form for Potential Conflicts of Interest. None of the authors disclose any conflict of interest.

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