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Expression of CCDC85C, a causative protein for hydrocephalus, and intermediate filament proteins during lateral ventricle development in rats

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Abstract: Coiled-coil domain containing 85c (*Ccdc85c*) is a causative gene for genetic hydrocephalus and subcortical heterotopia with frequent brain hemorrhage. In the present study, we examined the expression pattern of CCDC85C protein and intermediate filament proteins, such as nestin, vimentin, GFAP, and cytokeratin AE1/AE3, during lateral ventricle development in rats. CCDC85C was expressed in the neuroepithelial cells of the dorsal lateral ventricle wall, diminishing with development and almost disappearing at postnatal day 20. By immunoelectron microscopy, CCDC85C was localized in the cell-cell junction and apical membrane. The expression of nestin and vimentin was decreased in the wall of the lateral ventricle in manner similar to CCDC85C, but GFAP expression started immediately after birth and became stronger with age. Moreover, cytokeratin expression was found at postnatal day 13 and increased at postnatal day 20 in conjunction with the disappearance of CCDC85C expression. Taken together, CCDC85C is expressed in the cell-cell junctions lining the wall of the lateral ventricle and plays a role in neural development with other intermediate filaments in the embryonic and postnatal periods. Our chronological study will help to relate CCDC85C protein with intermediate filaments to elucidate the detailed role of CCDC85C protein during neurogenesis.

Key words: *Ccdc85c*, intermediate filaments, rat, ventricle

Introduction

The lateral ventricles are lined by the ependymal epithelium, which derives from the neuroepithelium [1, 2]. During the development of the central nervous system (CNS), the neuroepithelium undergoes many mitotic divisions before maturing into bipotent progenitor cells, which give rise to either neuronal or glial progenitor cells. This developmental specification is accompanied by a significant change in the gene expression of intermediate filament (IF) proteins, including nestin, vimentin and glial fibrillary acidic protein (GFAP) [3].

IF proteins are a mostly static set of proteins playing a crucial role during neurogenesis through self-renewal/proliferation, differentiation, migration, and structural and functional maintenance of neural cells [4, 5]. Nestin is a type VI IF protein expressed by stem/progenitor cells in the ventricular/sub-ventricular zone (V/SVZ) of the wall of the lateral ventricle and helps in the proliferation, differentiation, and migration of neural stem cells in the V/SVZ during the development of the neocortex [4]. Vimentin and GFAP are type III IF proteins expressed by radial glia and astrocytes during brain development. Vimentin is mainly expressed by radial glia and immature astrocytes at the early stage of development. With the advancement of gliogenesis, GFAP becomes the main IF protein expressed by astrocytes [6]. Cytokeratins are type I and type II IF proteins that are abundant in epithelial cells [7]. During neuronal development, ependymal cells lining the lateral, third, and fourth ventricle

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express keratin in rats, mice [8], and humans [9].

Coiled-coil domain containing 85C (CCDC85C) is a protein that belongs to the delta-interacting protein A (DIPA) family along with CCDC85A and CCDC85B [10]. Our previous study revealed that *Ccdc85c* is a causative gene for genetic hydrocephalus and subcortical heterotopia with frequent brain hemorrhage in hemorrhagic hydrocephalus (*hhy*) mice and *Ccdc85c* knockout rats [11–13], and meshwork-like CCDC85C protein expression was detected predominantly at the apical junctions of the radial glia in the wall of the lateral ventricle of the developing mouse brain [12]. CCDC85C is expressed in various simple epithelia but not in stratified epithelia, and its expression is localized at the cell-cell junction [14]. Thus, it is important to assess the detailed role of CCDC85C protein during brain development. Multiple studies have elucidated, in part, the role of CCDC85C during lateral ventricle development, but the detailed functions remain to be clarified, such as a complete understanding of the relations of CCDC85C protein expression with IF proteins during lateral ventricle development. To definitively determine the function of *Ccdc85c*, studies in different species of animals would be useful. For this reason, we established a *Ccd85c* knockout rat with an F344 genetic background [13]. We are conducting the studies to clarify the relationships between CCDC85C protein and IFs during the development of the ventricles. The *Ccdc85c* knockout rats lack the expression of CCDC85C, so we analyzed CCDC85C expression using wild-type F344 rats to obtain basic data.

In this study, we first demonstrated the expression pattern of CCDC85C protein with various IF proteins during lateral ventricle development in the rat in the embryonic and early postnatal stages. We showed the localization of CCDC85C using immunoelectron microscopy. Interestingly, we also showed for the first time that the regression of CCDC85C protein is correlated with cytokeratin; when CCDC85C started to regress from lining the dorsal lateral ventricle wall at postnatal day 13 (P13), cytokeratin expression began in the dorsal lateral ventricle wall and became stronger when CCDC85C expression had almost disappeared at P20. This indicated that there are close relationships between CCDC85C and IF proteins, including cytokeratin, during lateral ventricle development.

Materials and Methods

Animals

F344/DuCrlCrlj rats from Charles River Laboratories Japan (Shiga, Japan) were maintained in a conventional area with a controlled temperature and 12:12-h light-dark cycle at the Animal Facility of Osaka Prefecture University with access to food and water *ad libitum*. All animal care was in accordance with the Guidelines for Animal Experimentation of Osaka Prefecture University. Rats were euthanized with isoflurane on embryonic days 13 (E13), E15, E17, and E19 and on postnatal days 0 (P0), P2, P6, P13, and P20, and brains were collected on each the respective days. Fresh frozen samples were collected using PrestoCHILL (Milestone Medical, Sorisole, Italy) and embedded in TISSU MOUNT® (Chiba Medical, Saitama, Japan) for immunofluorescence. Brain samples were collected, fixed with modified Zamboni (0.1% glutaraldehyde in Zamboni solution) at P2 for immunoelectron microscopy, and frozen at −80°C. We also prepared SUPER FIX (KURABO, Osaka, Japan) fixation samples for immunohistochemistry.

Immunofluorescence

Fresh-frozen brain samples from E13 to P20 were used. Using a cryostat, brain sections were coronally sliced at 10 μm and postfixed with Zamboni's fixative (0.21% picric acid and 2% paraformaldehyde) for 15 min at room temperature (RT). Subsequently, sections were washed with washing buffer (0.3% Triton X-100 in PBS) for 15 min and blocked with 10% normal goat serum (Thermo Fisher Scientific, Waltham, MA, USA) at RT for 30 min. Brain sections were then incubated with a rabbit polyclonal CCDC85C antibody, the immunogen for which was produced by expressing 174 amino acids from the C-terminus of rat CCDC85C (as GST-fusion protein in *Escherichia coli* DH5α) [14], at 1:50,000 dilution at 4°C overnight. After washing with PBS, the sections were incubated with an Alexa 488-labeled secondary antibody against rabbit IgG (1:500, Thermo Fisher Scientific) at RT for 45 min. Sections were coverslipped with Fluoro-KEEPER Antifade mounting medium with DAPI (Nacalai Tesque, Kyoto, Japan). Images were taken using an Olympus FV 3000 confocal microscope (Olympus, Tokyo, Japan).

Immunoelectron microscopy

Frozen samples were cut into 10 μm sections using a cryostat. After drying, the sections were rinsed in PBS containing 0.3% Triton X-100 for 15 min and treated with 10% normal goat serum (Thermo Fisher Scientific) in PBS for 30 min. They were then incubated with a rabbit polyclonal CCDC85C antibody (1:50,000) [14] at 4°C overnight and incubated in 3% hydrogen peroxide (H_2O_2) for 30 min to quench endogenous peroxidase activity. After washing in PBS, the sections were treated with a peroxidase-conjugated secondary antibody (Histofine Simple Stain MAX-PO®, Nichirei Biosciences,

Tokyo, Japan) at RT for 1 h. Then, they were treated with 1% glutaraldehyde for 10 min at RT followed by washing in PBS. After preincubation with 3,3-diaminobenzidine (DAB) without H_2O_2 for 20 min, signals were visualized with a DAB substrate kit (Nichirei Biosciences). Thereafter, sections were postfixed with 1% osmium tetraoxide for 90 min at RT, dehydrated, and embedded in epoxy resin. Ultrathin sections were cut and examined with an H-7500 electron microscope (Hitachi, Tokyo, Japan).

Immunohistochemistry for IFs

Immunohistochemistry was performed on coronally sliced brain sections from E13 to P20. Brains were cut and fixed using SUPER FIX for 24 h and embedded in paraffin. Paraffin-embedded tissues were cut into 4 μm sections and deparaffinized in serial xylene-ethanol washes. Following antigen retrieval by autoclaving in 0.01 M citrate buffer (pH 6.0) for 10 min at 121°C, tissue sections were processed in a Histostainer™ (Nichirei Biosciences). Briefly, sections were treated with 5% skimmed milk in PBS for 15 min and incubated with an anti-nestin (1:200, EMD Millipore, Temecula, CA, USA), anti-vimentin (1:5, Dako, Glostrup, Denmark), anti-GFAP (1:1,000, Cell Signaling Technology, Danvers, MA, USA), and anti-cytokeratin AE1/AE3 (1:5, Dako) for 1 h. After incubation in 3% H_2O_2 for 15 min, a horseradish peroxidase-conjugated secondary antibody (Histofine Simple Stain MAX PO®, Nichirei Biosciences) was applied for 1 h. Positive reactions were visualized with DAB. Sections were counterstained with hematoxylin. Images were observed using an Olympus VS 120 virtual slide system (Olympus).

Results

Expression of CCDC85C during lateral ventricle development

We performed immunofluorescence for CCDC85C chronologically in the embryonic and early postnatal rat brain to evaluate its expression pattern. Meshwork-like expression of CCDC85C was observed lining the wall of the lateral ventricles (Fig. 1). This meshwork-like immunoexpression was similar to that previously reported [12] in mice in the walls of the lateral and third ventricles. As can be seen in Fig. 1, CCDC85C expression became stronger during embryonic development up to E19, gradually decreased from P0, and then almost disappeared at P20. The expression pattern was identical to that in an earlier report in the rat brain ventricle wall, which showed CCDC85C expression immediately before birth and during the postnatal development period [14]. Additionally, Fig. 1 also shows the embryonic expression pattern from a very early stage, starting at E13. These data showed that CCDC85C is expressed diffusely in the embryonic stage, is downregulated after birth, and almost disappears at P20.

Immunoelectron microscopy for CCDC85C

Immunoelectron microscopy was performed to exam-

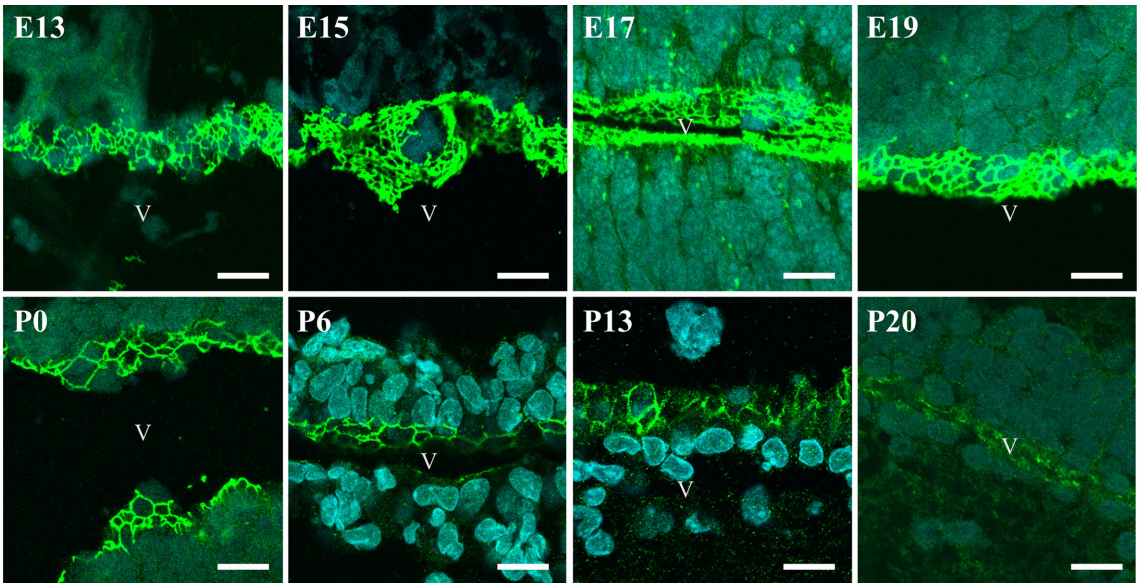

Fig. 1. CCDC85C immunoreactivity in the dorsal lateral ventricle of the rat. The images are from embryonic day 13 (E13), E15, E17 and E19 and postnatal day 0 (P0), P6, P13, and P20. Meshwork-like expression of CCDC85C in lining cells in the wall of the lateral ventricle. Expression increases during embryonic development but decreases during postnatal development, and it almost disappears at P20. V: Ventricular lumen. Bars: 15 μm.

ine the ultrastructural localization of CCDC85C. The results revealed the expression of CCDC85C signals in the cell-cell junctions of the lateral ventricle wall of the rat brain at P2, confirming the localization of this protein (Fig. 2). Dense immunolabelling was found at the apical membrane compared with the basolateral membrane. As reported previously using immunofluorescence, CCDC85C was expressed throughout the cell surface, and there was strong expression in the tight junctions belonging to the apical membrane [14]. In the present study, we confirmed this ambiguously using immunoelectron microscopy.

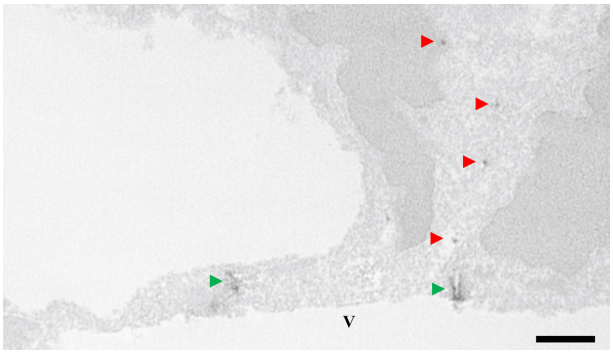

Fig. 2. Immunoelectron microscopy for CCDC85C in the dorsal lateral ventricle of the rat at postnatal day 2 (P2). Expression of CCDC85C (marked by arrowheads) is observed in the cell-cell junctions lining the lateral ventricle, and immunolabelling is stronger in the apical membrane (green arrowheads) compared with the basolateral membrane (red arrowheads). V: Ventricular lumen. Bar: 2 μm.

Expression of cytokeratin AE1/AE3 during lateral ventricle development

The cerebral ventricle is lined by ependymal cells and by type B1 cells with a small apical ending (a subset of V/SVZ astrocytes) that retain key epithelial properties of radial glia [15]. We stained the brain using cytokeratin AE1/AE3, but no immunoreactivity was found in the embryonic and early postnatal lateral ventricle. However, beginning at P13, cytokeratin expression began lining the wall of the lateral ventricle of the rat brain, and it was increased at P20 (Fig. 3).

Nestin expression during lateral ventricle development

Nestin immunostaining was detected in the neural stem/progenitor cells of the V/SVZ in the wall of the lateral ventricle in the embryonic and early postnatal stages (Fig. 4). Nestin expression was found in the processes of radial glia, extending from the ventricular zone towards the cortical zone. Immediately after birth, nestin expression in the radial processes and ventricular lining cells lining the ventricle wall dropped steadily. At P20, no glial expression was seen except in some discrete round cells lining the lateral ventricle (Fig. 4). From this, it was inferred that nestin expression is stronger in the

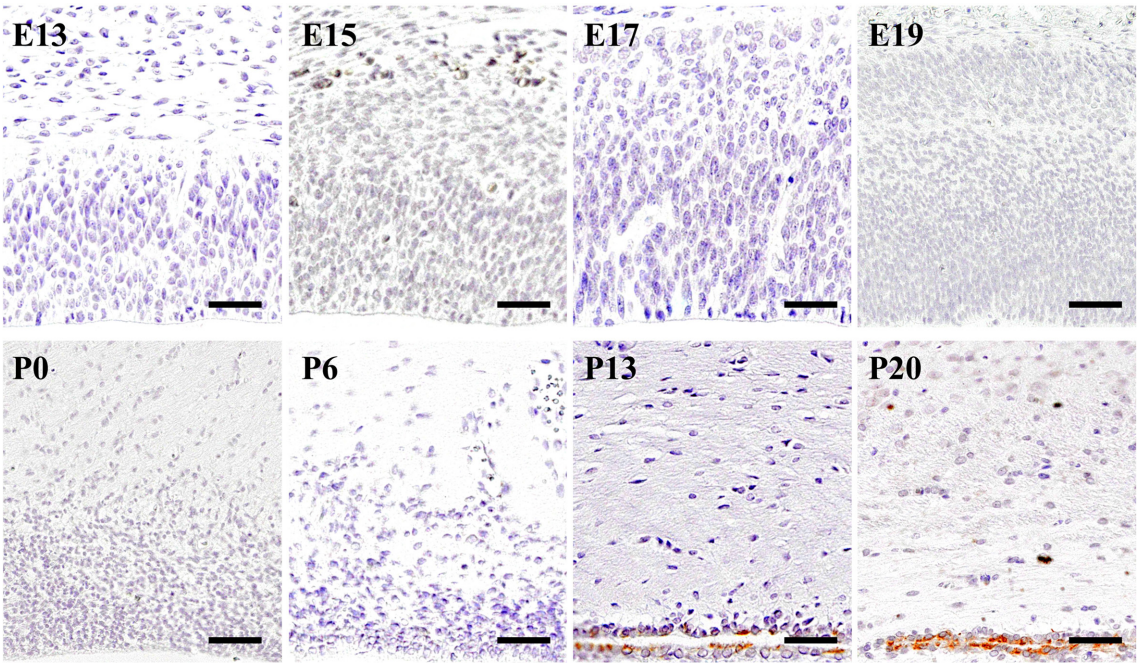

Fig. 3. Cytokeratin AE1/AE3 immunoreactivity in the lateral ventricle of the rat. No immunoreactivity of cytokeratin AE1/AE3 is observed during embryonic and early postnatal development of the lateral ventricle, but expression begins to be observed at P13 lining the ventricle and becomes stronger at P20. In all images, the line at the bottom indicates the ventricular lining. Bars: 50 μm.

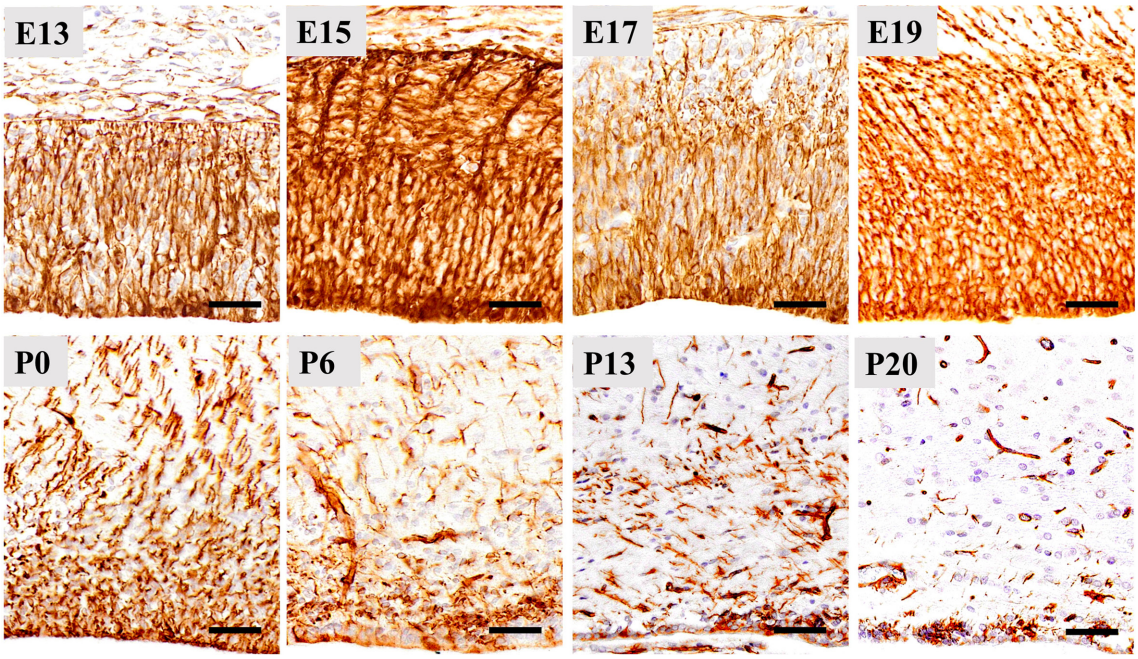

Fig. 4. Nestin immunoreactivity in the lateral ventricle of the rat. Expression of nestin in the neural stem/progenitor cells and processes of the ventricular and subventricular zone in the wall of the lateral ventricle during the embryonic and early postnatal period. In all images, the line at the bottom indicates the ventricular lining. Bars: 50 μm.

embryonic stage, gradually weakens in the postnatal stage, and is restricted to a few ventricular lining cells in the adult stage.

Expression of vimentin during lateral ventricle development

Vimentin immunoreactivity in the wall of the lateral ventricle along the radial process was prominent during perinatal development compared with that in the early embryonic stage and late postnatal stage (Fig. 5). In the early postnatal brain, vimentin was localized in the long fibers of the cerebral cortex. A major portion of the ependymal cells lining the lateral ventricles was also positive for vimentin. At P20, vimentin was localized only in the lining of the lateral ventricle wall (Fig. 5).

Expression of GFAP during lateral ventricle development

No expression of GFAP was found in the dorsal wall of the lateral ventricle before birth (Fig. 6). Immediately after birth, expression was observed in the astrocytes of the V/SVZ. Starting on P6, GFAP expression was upregulated with age throughout the V/SVZ and showed a star-like pattern. Some circular cells lining the ventricle, such as B1 cells (a subset of V/SVZ astrocytes), were also positive for GFAP (Fig. 6, arrowheads).

Discussion

In this paper, we report the chronological relationships between the expression of CCDC85C, a causative protein for hydrocephalus, and IF proteins, including nestin, vimentin, GFAP, and cytokeratin AE1/AE3, during lateral ventricle development in the rat brain. We revealed the cell-cell localization of CCDC85C protein in the wall of the lateral ventricle and its strong expression in the apical membrane using immunoelectron microscopy. Our findings clarified that CCDC85C protein expression is directly proportional to nestin and vimentin expression but inversely proportional to GFAP expression in the embryonic and early postnatal development of the brain. Furthermore, our data demonstrated the correlation of CCDC85C and cytokeratin expression during lateral ventricle development.

CCDC85C localization and proliferative activity

Ccdc85c comprises a pair of conserved coiled-coil motifs that are thought to participate in DNA transcriptional regulation [10]. Herein, we showed CCDC85C protein expression lining the dorsal wall of the lateral ventricle from the prenatal to postnatal stages of brain development. Very strong CCDC85C immunoreactivity was detected beginning at E13 in the early embryonic stage. After birth, expression decreased gradually and almost disappeared at P20. Mori *et al.* reported that *Ccdc85c* was disrupted in *hhy* mice showing hydro-

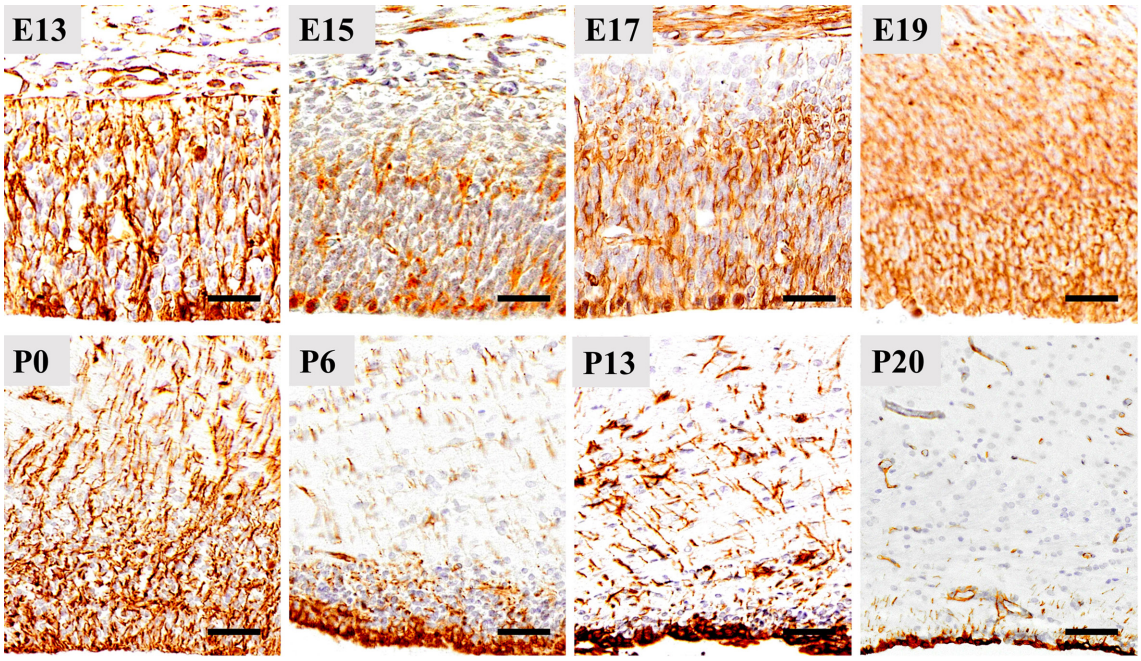

Fig. 5. Vimentin immunoreactivity in the lateral ventricle of the rat. Expression of vimentin by radial glial cells of the ventricular and subventricular zone in the wall of lateral ventricle is observed during embryonic and early postnatal development. Vimentin positivity is also observed in ependymal lining cells during postnatal ventricular development. In all images, the line at the bottom indicates the ventricular lining. Bars: 50 μm.

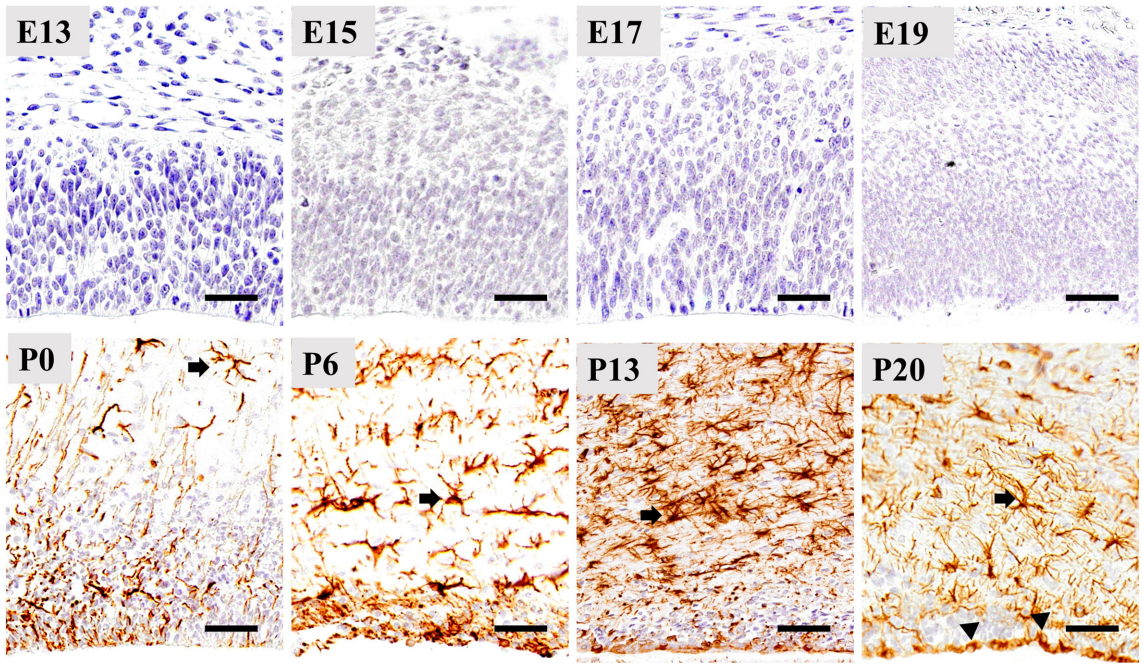

Fig. 6. GFAP immunoreactivity in the lateral ventricle of the rat. Expression of GFAP by astrocytes (arrows) of the ventricular and subventricular zone in the wall of the lateral ventricle. No GFAP expression is found in the dorsal lateral ventricle during embryonic development. Several round cells lining the ventricle similar to B1 cells are also positive for GFAP (arrowheads). In all images, the line at the bottom indicates the ventricular lining. Bars: 50 μm.

cephalus and subcortical heterotopia [12]. In *hhy* mice, the disruption of CCDC85C results in radial glial demise followed by agenesis of the ependymal layer lining the neonatal cortex that disrupts brain development. In the present study, the expression patterns from the embryonic to postnatal stages suggest that there are relationships between CCDC85C protein expression before and after birth and IFs.

Previous research revealed that meshwork-like CCDC85C immunoreactivity is largely overlapped by the immunoreactivity of zonula occludens (ZO)-1, an apical junction marker, on the lateral ventricle wall in mice [12] and the immunoreactivity of cell-cell junctions, especially tight junctions of the simple epithelia in the rat [14]. In this report, we demonstrated the localization of CCDC85C at the cell-cell junctions of the lining cells in the wall of the lateral ventricle using immunoelectron microscopy. Like the earlier reports, the expression of CCDC85C was found more frequently in the apical membrane, than in the basolateral membrane.

In mammals, tight junctions (TJs) are localized to the most apical region of the cell and are composed of transmembrane proteins (claudin and occludin) and adaptor proteins (ZO-1 and ZO-2), which link to the underlying actin cytoskeleton [16]. ZO-1 targets proliferative cell nuclear antigen (PCNA) by binding with the transcription factor ZO-1-associated nucleic acid binding protein (ZONAB) in a cell density-dependent manner and controls cell proliferation [17]. The adherens junction is localized on the lateral membrane and is primarily composed of the transmembrane protein, E-cadherin, and adaptor proteins, beta-catenin, and alpha-catenin, which link to the underlying actin cytoskeleton [16, 18]. The interaction of these transmembrane and adaptor proteins with other signaling molecules is required for the proper localization and organization of apical junctional complexes and to minimize embryonic lethality [19, 20]. As CCDC85C is localized in the cell-cell junction, it may have a crucial role in the proliferation and maintenance of the lining cells in the wall of the lateral ventricle and in the development of the brain.

CCDC85C and shifts in intermediate filaments

IF remodeling is important for changes in cellular shape during cell differentiation [21]. In the present study, immunostaining revealed nestin expression in radial glial cells along with their long processes during lateral ventricle development. This is consistent with previous reports indicating that nestin was detected in the projections of radial glial cells extending from the ventricular zone to the pial zone [6, 22, 23]. Between E13 and E16, cortical radial glial cells in the dorsal wall of the lateral ventricles proliferate to produce neurons. Switching from neurogenesis to gliogenesis after E16, radial glial cells in this region produce astrocytes [24].

In the developing CNS, radial glia serve as both structural scaffoldings for migrating neuroblasts and as embryonic neural progenitors [25]. In *hhy* mice, *Ccdc85c* expression is absent, and heterotopic cortical tissue is developed in the dorsal part of the ventricles. The present report reveals the expression pattern of CCDC85C in relation to the nestin-positive radial glia during both prenatal and postnatal development in rats.

In this study, vimentin was found to be expressed strongly in the radial process around perinatal stages and also in the ependymal cells lining the lateral ventricle in postnatal stages. Earlier reports also showed that many ependymal lining cells are vimentin positive in adult rats [8]. Other studies in rodents reported that vimentin is strongly expressed in late gestation, with long, thin fibers in the cerebral cortex [6, 26]. After P6, vimentin expression decreased like nestin; that is, vimentin expression was also directly proportional to CCDC85C expression, like nestin.

GFAP is the key IF III protein responsible for the cytoskeleton structure of glial cells and is uniquely found in astrocytes in the CNS. Earlier research showed that the expression of GFAP in the cerebral cortex of the newborn rats was weak, increased in adult rats and abundant in aged rats [6]. In the present study, no GFAP expression was found in the dorsal wall of the lateral ventricle up to E19; GFAP expression began immediately after birth and gradually increased. Several circular cells lining the ventricle that were similar to B1 cells (a subset of V/SVZ astrocytes) were also positive for GFAP. Earlier research had shown that type B cells in the subventricular zone display ultrastructural characteristics and markers of astroglial cells, including GFAP [27]. Interestingly, we found that the GFAP expression in the lateral ventricle wall was inversely proportional to CCDC85C expression. The dominantly expressed IFs in developing cells change during the embryonic and postnatal periods. These data illustrate that CCDC85C has a direct relationship with the shifts in these three major IFs (nestin, vimentin and GFAP) that play the most critical role in neurogenesis.

CCDC85C and cytokeratin

Cytokeratins are characteristic epithelial intermediate filaments, but little is known about their distribution in neuroectodermal cells. In the current study, we observed cytokeratin expression lining the lateral ventricle at P13, at which point CCDC85C protein expression was diminishing. Expression of cytokeratin increased with age, whereas that of CCDC85C decreased with age. A previous study indicated that the ependymal layers lining the lateral, third, and fourth ventricles in adult rats and late embryonic mice express keratin [8]. In humans, another report showed cytokeratin expression in the spinal leptomeninx and tanycytes of the spinal cord ependyma [28] and in the fetal ependymal cells lining the floor of the fourth ventricle and lateral ventricle [9]. Based on

our data, we speculate that there is a strong relationship between cytokeratin and CCDC85C.

CCDC85C and other brain development pathways

A recent study revealed that human CCDC85C protein interacts with and translocates Yes-associated protein 1 (YAP1), a key downstream regulator for the Hippo pathway from the nucleus to the cytoplasm, and maintains cell proliferation [29]. Interestingly, another recent study, which performed genome-wide single-nucleotide polymorphism (SNP) and gene-based association analyses revealed that CCDC85C is relevant to Alzheimer's disease [30]. Frankly, the number of subjects in the present study was small. Detailed comparative studies are needed in many animals and humans to elucidate the role of CCDC85C.

In conclusion, the present study shows the expression of CCDC85C protein and IF proteins (including nestin, vimentin, GFAP, and cytokeratin AE1/AE3), which have a crucial role in the maintenance of radial glia and astrocytes, during lateral ventricular development in rats. CCDC85C also might have fateful roles in ependymal cells of the lateral ventricles and in the development of the ventricle system in the CNS. Examining the role of *Ccdc85c* in hydrocephalus, especially in the development and maintenance of ependymal cells in the ventricles, may reveal new mechanisms responsible for hydrocephalus. That information would be useful for the prevention and treatment of hydrocephalus. Further studies are needed to clarify the function of the *Ccdc85c* gene in animals and humans.

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