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DISTRIBUTION OF PROTOCADHERIN 9 PROTEIN IN THE DEVELOPING MOUSE NERVOUS SYSTEM

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Abbreviations: I-X, First to 10th cerebellar lobes; 3N, oculomotor nucleus; 4N, trochlear nucleus; 5n, trigeminal nerve; 5N, motor trigeminal nucleus; 6n, root of abducens nerve; 6N, abducens nucleus; 7n, facial nerve; 7N, facial nucleus; 8n, vestibulocochlear nerve; 8cn, cochlear root of 8th nerve; 8vn, vestibular root of 8th nerve; ac, anterior commissure; Amy, amygdala; AOB, accessory olfactory bulb; APT, anterior pretectal nucleus; AV, anteroventral thalamic nucleus; bic, brachium of the inferior colliculus; BL, basolateral amygdaloid nuclei; CAs, CA regions of Ammon's horn; CAT, nucleus of central acoustic tract; cc, corpus callosum; CD, cochlear duct; Cer, cerebellum; Cg, cingulate cortex; CIC, central nucleus; cp, cerebral peduncle; CPu, caudate putamen; csc, commissure of superior colliculus; Ctx, cortex; DC, dorsal cochlear nucleus; dcw, deep cerebral white matter; DG, dentate gyrus; dhc, dorsal hippocampal commissure; Dk, Darkschewitsch nucleus; DLG, dorsal lateral geniculate nucleus; DLL, dorsal nucleus of the lateral lemniscus; DRG, dorsal root ganglion; DT, dorsal thalamus; DTg, dorsal tegmental nucleus; Epi, epithalamus; Epl, external plexiform layer of olfactory bulb; f, fornix; fi, fimbria; Fl, flocculonodular lobe; fr, fasciculus retroflexus; GCL, ganglion cell layer; Gi, gigantocellular reticular nucleus; Gl, glomerular layer of main olfactory bulb; gl, olfactory glomeruli; GL, granule cell layer of cerebellum; Hb, habenular nuclei; hbc, habenular commissure; IC, inferior colliculus; ic, internal capsule; icp, inferior cerebellar peduncle; InC, interstitial nucleus of Cajal; INL, inner nuclear layer; Int, interposed cerebellar nucleus; IOD, dorsal accessory nucleus of inferior olive; IOM, medial nucleus of inferior olive; IOPr, principal nucleus of inferior olive; IP, interpeduncular nucleus; IPL, inner plexiform layer; Lat, lateral cerebellar nucleus; ll, lateral lemniscus; lot, lateral olfactory tract; LSO, lateral superior olive; ME, mandibular epithelium; Med, medial (fastigial) cerebellar nuclei; MHb, medial habenular nucleus; Ml, mitral cell layer of olfactory bulb; ML, molecular layer of cerebellum; ml, medial lemniscus; mlf, medial longitudinal fasciculus; NE, neuroepithelium; OE, olfactory epithelium; OB, olfactory bulb; OLL, outer limiting layer; on, olfactory nerve; ONL, outer granular layer; OPL, outer plexiform layer; opt, optic tract; OV, olfactory ventricle; pc, posterior commissure; PE, pigment epithelium; PF, parafascicular thalamic nucleus; Pit, pineal gland; Pir, piriform cortex; Pk, Purkinje cell; p1Rt, p1 reticular formation; PMCo, posteromedial cortical amygdaloid nucleus; Pn, pons; Pr, prepositus nucleus; PR, prerubral field; PRL, photoreceptor layer; Pr5, principal sensory trigeminal nucleus; PTg, pedunculopontine tegmental nucleus; R, red nucleus; SC, superior colliculus; SCC, semicircular canal; scp, superior cerebellar peduncle; sm, stria medullaris, thalamus; SNC, compact part of substantia nigra; SNR, reticular part of substantia nigra; SP5, spinal trigeminal tract; SPO, superior paraolivary nucleus; st, stria terminalis; str, striatal axons; Str, striatum; TC, thalamocortical axons; Te, temporal cortex; Tu, olfactory tubercle; tz, trapezoid body; Tz, nucleus of trapezoid body; VC, ventral cochlear nucleus; Ve, vestibular nuclei; VeGn, vestibular nerve ganglion; VG, ventral geniculate nucleus; VL, ventrolateral thalamic nucleus; VLL, ventral nucleus of the lateral lemniscus; VM, ventromedial thalamic nuclei; VMH, ventromedial hypothalamic nucleus; VP, ventral posterior thalamic nuclei; VPL, ventral posterolateral thalamic nucleus; VPM, ventral posteromedial thalamic nucleus; xspc, decussation of the superior cerebellar peduncle; ZI, zona incerta.

Abstract—Protocadherin 9 (Pcdh9) is a member of the protocadherin family, which includes many members involved in various phenomena, such as cell-cell adhesion, neural projection, and synapse formation. Here, we identified Pcdh9 protein in the mouse brain and examined its distribution during neural development. Pcdh9, with a molecular weight of approximately 180 kDa, was localized at cell-cell contact sites in COS-1 cells transfected with Pcdh9 cDNA. In cultured neurons, it was detected at the growth cone and at adhesion sites along neurites. In the E13.5 brain, prominent Pcdh9 immunoreactivity was detected in the dorsal thalamus along with other regions including the vestibulocochlear nerve. As development proceeded (E15.5-P1), Pcdh9 immunoreactivity became observable in various brain regions but was restricted to certain fiber tracts and brain nuclei. Interestingly, many Pcdh9-positive brain nuclei and fascicles belonged to the vestibular (e.g. vestibulocochlear nerve, vestibular nuclei, and the vestibulocerebellum) and oculomotor systems (medial longitudinal fascicles, oculomotor nucleus, trochlear nucleus, and interstitial nucleus of Cajal). In addition, we examined the distribution of Pcdh9 protein in the olfactory bulb, retina, spinal cord, and dorsal root ganglion. In these regions, Pcdh9 and OL-protocadherin proteins were differentially distributed, with the difference highlighted in the olfactory bulb, where they were enriched in different subsets of glomeruli. In the mature retina, Pcdh9 immunoreactivity was detected in distinct sublaminae of the inner and outer plexiform layers. In the dorsal root ganglion, only certain subsets of neurons showed Pcdh9 immunoreactivity. These results suggest that Pcdh9 might be involved in formation of specific neural circuits during neural development.

Key words: protocadherin, cadherin, cell adhesion molecule, neural circuit formation.

INTRODUCTION

The protocadherin family is a major sub-branch of the cadherin superfamily, whose members have six or seven cadherin motifs in their extracellular domain (Sano et al., 1993; Hulpiau and van Roy, 2009; Hirano and Takeichi, 2012). In mammals, the protocadherin family contains more than 60 members which, according to their genomic organization, are divided into clustered-type protocadherins (α, β, and γ- protocadherins) and non-clustered-type ones (e.g., δ-protocadherins). Delta-protocadherins are further divided into δ1- and δ2-protocadherins based on conserved amino acid sequences in their cytoplasmic domain (Redies et al.,

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2005). Expression of the majority of protocadherins occurs in the nervous system and is developmentally regulated (Sano et al., 1993; Hirano et al., 1999; Redies, 2000; Kim et al., 2007; Hirano and Takeichi, 2012). Their adhesion properties and restricted expression have led to the speculation that they are involved in developmental processes such as brain nucleus formation, axon migration, and synapse formation (Hirano and Takeichi, 2012). In fact, recent studies revealed the involvement of protocadherins in neural development and function. For example, OL-protocadherin (protocadherin 10) is involved in axon growth and neural circuit formation (Uemura et al., 2007). In addition, γ-protocadherins also participate in neural differentiation and synapse formation (Wang et al., 2002; Weiner et al., 2005), whereas α-protocadherins (CNRs) are involved in neural projections (Hasegawa et al., 2008; Katori et al., 2009). Arcadlin (protocadherin 8) functions in long-term potentiation and synaptic transmission (Yamagata et al., 1999; Yasuda et al., 2007).

Protocadherin 9 (Pcdh9) is a δ1-protocadherin that, based on its deduced amino acid sequence, contains 7 cadherin motifs in its extracellular domain. The cytoplasmic region is different from that of other protocadherins except for limited similarities (Hulpiau and van Roy, 2009). The expression pattern of Pcdh9 mRNA was studied by *in situ* hybridization in various species including mouse (Vanhalst et al., 2005; Redies et al., 2005; Hertel et al., 2008; Krishna et al., 2011), rat (Kim et al., 2007, 2010), ferret (Etzrodt et al., 2009), chicken (Lin et al., 2012), and zebrafish (Liu et al., 2009; Blevins et al., 2011). However, the distribution of the protein in the brain and its relation to neural circuits remain unknown. Here, we identified mouse Pcdh9 protein and examined its distribution pattern in the developing mouse nervous system. The protein was localized at the cell-cell contact sites and at the growth cone and neurites in cultured cells. The distribution of Pcdh9 protein was restricted to various brain nuclei and fibers, and many of them belonged to the vestibular and oculomotor systems. Our results suggest that Pcdh9 may be involved in specific neural circuit formation during development.

EXPERIMENTAL PROCEDURES

Animals

ICR and C57BL/6N SLC mice used for immunostaining were obtained from the same commercial source (Japan SLC, Shizuoka). Because staining patterns were essentially the same in both strains, we primarily used ICR mice. Donryu rats (Japan SLC) were used to produce antibodies as described below. Mice and rats were handled according to the guidelines of the Kochi University. Mice and rats were anesthetized with diethyl ether.

Generation of monoclonal antibodies

Pcdh9 fused to maltose-binding protein (MBP) was used as an immunogen. A cDNA encoding the C terminal region of mouse Pcdh9 (amino acids 958–1237; MGC189939) was subcloned into the pMAL-c4X vector (Biolabs NEB, Massachusetts), and the fusion protein was produced according to the manufacturer's protocol. Seven-week-old Donryu rats were immunized 4 times, at 2-week intervals, with a subcutaneous injection of protein (0.5–0.7 mg) in Freund's adjuvant (DIFCO, BD, New Jersey). Splenocytes from the immunized rats were fused with P3X63Ag8.653 myeloma cells, and culture supernatants were collected and screened for their ability to immunostain Pcdh9-transfected COS-1 cells. Monoclonal antibodies (mAbs) 2D3 and 1E3 specifically stained COS-1 cells transfected with Pcdh9 cDNA. These mAbs were used for immunohistochemistry and Western blot analysis. Because immunohistochemical staining patterns with mAbs 2D3 and 1E3 were essentially the same, but the signal of mAb 2D3 was stronger than that of mAb1E3 (data not shown), we used mAb 2D3 for all the experiments described in this study unless otherwise specified.

Pcdh9 expression in cells cultured *in vitro*

A cDNA fragment that included the full coding region of mouse Pcdh9 was subcloned into the pDEST47 expression vector (Invitrogen, Life Technologies, California) with or without a stop codon before the coding region of GFP. DNA constructs were introduced into COS-1 cells by electroporation using the Neon system (Invitrogen). We detected Pcdh9-expressing cells in transfected COS-1 cell cultures by using mAb 2D3 and anti-GFP antibodies (MBL, #598, Japan), and approximately 10–20% of the cells were positive in transient culture. However, the number of Pcdh9-expressing cells declined over time; and we were unable to isolate stably transformed cell lines (data not shown). Pcdh11x protein was expressed in COS-1 cells by introducing Pcdh11x cDNA (MGC141524) in the pDEST47 expression vector having a GFP tag. Expression of GFP-tagged Pcdh11x protein was confirmed by Western blot analysis with anti-GFP antibody (MBL, #598; Fig. 1B).

Western blot analysis

Standard procedures were used for Western blot analysis. Briefly, Pcdh9-transfected COS-1 cells and dorsal thalami at E15.5 were isolated and dissolved in SDS–PAGE sample buffer containing 5% 2-mercaptoethanol. After electrophoresis, the proteins were blotted onto Immobilon-P membranes (Millipore, Massachusetts). After having been blocked with 5% skim milk (DIFCO, BD, New Jersey) in Tris-buffered salt solution containing calcium (TBS-Ca; 50 mM Tris, 150 mM NaCl, and 1 mM $CaCl_2$ at pH 7.6), the membrane was incubated with mAb 2D3, specific for Pcdh9, or with mAb2G8, specific for OL-protocadherin (Hirano et al., 1999), and anti-GFP antibody (MBL #598) or without antibodies as a negative control, and subsequently with alkaline phosphatase-conjugated anti-rat Ig or anti-rabbit Ig (Promega, Wisconsin). Signals were detected with Western Blue solution (Promega, Wisconsin).

Immunohistochemistry

MAbs 2D3 and 1E3 were used for immunostaining of Pcdh9; whereas we used irrelevant rat mAb 2B4 (anti-denatured OL-protocadherin), which is not reactive with OL-protocadherin in immunohistochemistry as described before (Hirano et al., 1999) or deleted the first antibodies as negative controls. MAb 2H3 was used to detect neurofilaments (Dodd et al., 1988). OL-protocadherin in tissue sections was detected with mAb 5G10