The protocadherin alpha cluster is required for axon extension and myelination in the developing central nervous system

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Graphical Abstract

The protocadherin alpha cluster functions in axon growth and functional repair after adult mammalian spinal cord injury

Abstract

In adult mammals, axon regeneration after central nervous system injury is very poor, resulting in persistent functional loss. Enhancing the ability of axonal outgrowth may be a potential treatment strategy because mature neurons of the adult central nervous system may retain the intrinsic ability to regrow axons after injury. The protocadherin (Pcdh) clusters are thought to function in neuronal morphogenesis and in the assembly of neural circuitry in the brain. We cultured primary hippocampal neurons from E17.5 Pcdhα deletion (del-α) mouse embryos. After culture for 1 day, axon length was obviously shorter in del-α neurons compared with wild-type neurons. RNA sequencing of hippocampal E17.5 RNA showed that expression levels of BDNF, Fmod, Nrp2, OGN, and Sema3d, which are associated with axon extension, were significantly down-regulated in the absence of the Pcdhα gene cluster. Using transmission electron microscopy, the ratio of myelinated nerve fibers in the axons of del-α hippocampal neurons was significantly decreased; myelin sheaths of P21 Pcdhα-del mice showed lamellar disorder, discrete appearance, and vacuoles. These results indicate that the Pcdhα cluster can promote the growth and myelination of axons in the neurodevelopmental stage.

Key Words: nerve regeneration; spinal cord injury; axons; protocadherin α cluster; hippocampal neurons; RNA sequencing; real-time quantitative polymerase chain reaction; transmission electron microscopy; neural regeneration

Introduction

During neuronal wiring, axons establish a framework that is dependent on a series of guidance events during neural development. Axon outgrowth is crucial for the assembly of neuronal circuitry to ensure proper synaptic connectivity.
et al., 1996). Axon regeneration after injury requires axonal outgrowth from the soma, similar to that during normal development. Injured axons in the adult mammalian spinal cord ordinarily fail to spontaneously regenerate and do not recover functionality. Axon demyelination further increases the difficulty of regeneration after injury (Xu et al., 2014; Kim et al., 2017). A number of factors are thought to be responsible for this phenomenon, including extracellular matrix inhibitors, myelin inhibition, cell death, insufficient growth factor support, and the lack of the intrinsic growth capacity of adult central nervous system neurons (Beattie et al., 2000; Neumann et al., 2002; Filbin, 2003; Fawcett, 2006; Liu et al., 2011; McKerracher and Rosen, 2015).

Many researchers have tried to characterize the environmental inhibitory molecules in the adult central nervous system (Hu and Selzer, 2017; Nathan and Li, 2017). However, removing the inhibitory molecules genetically or pharmacologically only results in limited sprouting and is insufficient for long-distance axon regeneration (Yiu and He, 2006; Filbin, 2008; Fitch and Silver, 2008; Yang et al., 2010). Many studies have attempted to explore the intrinsic regenerative ability of mature central nervous system neurons to promote axon regeneration (Kadoya et al., 2009; Sun and He, 2010; Yang and Yang, 2012). Intrinsic growth activity is gradually repressed in the transition process from embryonic to adult neurons (Abe and Cavalli, 2008; Lu et al., 2014). Thus, an important step in elucidating the mechanisms mediating this activity is to identify the critical genes that promote neurite outgrowth.

Protocadherins (Pcdhs) are a large group of calcium-bind ing transmembrane cell-adhesion and signaling proteins, belong to the cadherin superfamily, and are subgrouped into “clustered” and “non-clustered” Pcdhs based on their respective genomic structures (Yagi and Takeichi, 2000; Morishita and Yagi, 2007; Kim et al., 2011; Hayashi and Takeichi, 2015). In mammals, more than 50 clustered Pcdh genes are organized into three sequentially-linked clusters known as Pcdha, Pcdhp, and Pcdhy (Wu and Maniatis, 1999; Wu et al., 2001). The clustered Pcdh genes are expanded in species with rich behavior repertoires such as zebrafish and octopus but not in Drosophila (Wu et al., 2001; Albertin et al., 2015). Some non-clustered protocadherins, such as Pcdh17, Pcdh18b, and NF-Pcdh, participate in axon extension and arborization (Biswas et al., 2014; Hayashi et al., 2014; Leung et al., 2015). Recent studies have indicated that mice with complete deletion of the Pcdha cluster or its constant region are viable and fertile, although they exhibit abnormal axonal projections from olfactory sensory neurons, defects in dendritic branching, and altered spine morphogenesis and oligodendrocyte development (Hasegawa et al., 2008; Hasegawa et al., 2012; Suo et al., 2012; Yu et al., 2012). Furthermore, Pcdha may function in the establishment and maintenance of appropriate synaptic connections (Zipursky and Sanes, 2010; Chen and Maniatis, 2013). We predicted that the Pcdha cluster may be key in the outgrowth of axons because of these characteristics.

This study investigated the molecular functions of the Pcdha cluster, focusing on its relationship with axon growth and myelination. The aim of this study was to investigate the axon growth defects and myelin sheath deficiency in hippocampal neurons of Pcdha knockout mice.

Materials and Methods

Animals

Pcdha knockout (Pcdha-del) mice were prepared previously (Wu et al., 2007, 2008) and housed in the Experimental Animal Center of Shanghai Jiao Tong University of China. Mice were able to reproduce. Male and female mice aged approximately 3 months were used (Animal use license No. SYXK (Hu) 2013-0052). Animals were maintained at 23.8°C under a 12-hour light dark cycle (lights on from 07:00–19:00). All experiments complied with the guidelines of the Institutional Animal Care and Use Committee of Shanghai Jiao Tong University (approval No. 1602029).

Mice genotyping

DNA extraction from tail biopsies and genotyping was performed according to a previous study (Truett et al., 2000). Primers used were as follows: ConF1: 5’-AGG CTG AAT AAC GTG CAC AGC TAA G-3’; GFPmutF: 5’-CCC CCT GAA CCT GAA ACA AGA TAA G-3’; ConR1: 5’-GCA GAT TGC TTC AAT GGA GTC TTT-3’.

Hippocampal neuron culture

Heterozygous Pcdha-del female mice were crossed with heterozygous Pcdha-del male mice. Embryonic day 17.5 (E17.5) embryos were collected from a pregnant dam and genotyped. Wild-type and Pcdha-del embryos were prepared for neuronal culture. Hippocampi were collected in Hanks’ Balanced Salt Solution containing 10 mM Hepes (Gibco, Grand Island, NY, USA), 0.5% glucose and 100 μg/ml penicillin/streptomycin. Tissues were digested with 0.25% trypsin for 15 minutes at 37°C. After terminating the reaction with trypsin inhibitor (0.5 mg/ml) for 3 minutes at room temperature, tissues were gently triturated in the plating medium, containing minimum essential medium (Gibco, Grand island, NY, USA), 10% fetal bovine serum (Gibco), 1 mM glutamine (Sigma, St. Louis, MO, USA), 10 mM Hepes (Gibco), and 50 μg/ml penicillin/streptomycin (Gibco). The number of viable cells was counted using 0.4% trypan blue in a hemocytometer (QIUJING, Shanghai, China). Cells were plated at a density of 1,000 cells/mm² on poly-L-lysine/Laminin coated coverslips (Becton, Dickinson, and Company BD Corning, Corning, NY, USA) in 24-well culture dishes (Thermo, Waltham, MA, USA). Cells were incubated in an atmosphere of 5% CO₂ at 37°C. After 3–4 hours, the plating medium was replaced with a serum-free culture medium, supplemented with neurobasal medium (Gibco), 2% B27, 0.5 mM glutamine, 50 mg/mL penicillin/streptomycin, and 25 mM glutamate (Sigma).

Immunofluorescent staining

Cultured primary hippocampal neurons were washed once with 1× PBS, and then fixed in 4% paraformaldehyde for 20 minutes at room temperature. Neurons were permeabi-
lized and blocked with 0.3% Triton X-100 and 5% bovine serum albumin for 10 minutes, followed by incubation with mouse anti-Tau-1 primary antibody (monoclonal, 1:5,000; Millipore, Billerica, MA, USA) at 4°C overnight and then with a secondary antibody, donkey anti-mouse IgG (1:5,000; Jackson ImmunoResearch, West Grove, PA, USA). F-actin was stained with rhodamine phalloidin (Thermo) at 4°C overnight. Cell nuclei were visualized with 4',6-diamidino-2-phenylindole (DAPI). Images were collected with a Nikon confocal microscope (Tokyo, Japan) (AISI) under a 20× objective for axon analysis. For each mouse embryo, nine fields of vision were randomly selected and pictures taken. The neuronal culture and immunofluorescent staining were performed at least three times.

RNA sequencing of hippocampal tissues
Hippocampal tissue was collected from E17.5 embryos according to the above-mentioned method. Three wild-type embryos and three Pcdhα-del embryos were prepared for RNA sequencing. Total RNA was prepared from embryonic hippocampal tissue using TRIzol Reagent (Ambion, Austin, TX, USA) according to the manufacturer’s protocol. The quality and yield of the isolated RNA were assessed using a microplate reader and 1% agarose gel electrophoresis. Prior to synthesizing cDNA, 2 μg RNA was purified using oligo (dT) magnetic beads. ProtoScript II reverse transcriptase and random primers (Promega, Madison, WI, USA) were used for reverse transcription, and second-strand cDNA synthesis was then performed. The polymerase chain reaction (PCR) product was purified using AMPure XP beads. Total cDNA was used to prepare the sequencing library according to the method outlined in the NEBNext Ultra RNA Library Prep Kit for Illumina (NEB E7530, Beverly, MA, USA). The cDNA libraries were sequenced on an Illumina instrument with 50-base pair single reads. The read was aligned to the mouse genome (National Center for Biotechnology Information) using TopHat and Bowtie, followed by Cufflinks for assembly of the reads into transcripts. Relative abundance of transcripts was measured by Fragments Per Kilobase of exon per Million mapped fragments. The mapping of sequence data to the genome and transcriptome was visualized in Integrative Genomics Viewer, and genes with a maximum P-value of 0.05 and a minimum fold change of ±2 were selected as differentially expressed genes. GO terms were assigned to genes with significant differences in expression according to Gene Ontology.

Real-time quantitative polymerase chain reaction (RT-qPCR)
Hippocampi from E17.5 embryos were collected according to the above-mentioned method. Tissues were homogenized and lysed in TRIzol reagent according to the manufacturer’s protocol (Life Technology). RNA yields were measured using a NanoDrop 2000 (Thermo, Waltham, MA, USA). A reverse transcription system (Promega) was used to obtain cDNA. Real-time PCR was performed on an Applied Biosystems real-time system according to the detailed instructions provided by FastStart Universal SYBR Green Master (Roche, Basel, Switzerland). For normalization of gene expression, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal standard. Primers used are listed in Table 1.

Transmission electron microscopy
Twenty-one days after birth is the peak time for myelin basic protein expression in the myelin sheath of the central nervous system, i.e., the critical period of myelination. Therefore, myelin was observed at this time using electron microscopy (Sánchez et al., 1996). Three postnatal 21-day male wild-type mice and three postnatal 21-day male Pcdhα-del mice were prepared for transmission electron microscopy. Hippocampi were fixed overnight with 2.5% glutaraldehyde in 0.1 M sodium phosphate buffer, pH 7.4. Tissues were then washed with 0.1 M sodium phosphate buffer and postfixed in 1% osmium tetroxide. Tissues were dehydrated in a graded series of ethanol and then embedded in resin. Several semi-thin sections were cut for anatomical orientation under light microscopy before ultrathin sectioning. The samples were ultrathin sectioned and then examined using a transmission electron microscope (Philips CM-120, Amsterdam, The Netherlands).

Data analysis
Statistical analysis was performed using GraphPad Prism5 software (Version X; La Jolla, CA, USA). The axon of each neuron was traced using ImageJ software (NIH, Bethesda, MD, USA). The resulting trace was used to calculate the length. The calculation was carried out in a double-blind fashion with respect to homozygous Pcdhα-del and wild-type littermates. For RT-qPCR, each sample was tested in triplicate and relative gene expression was calculated using the formula 2−ΔΔCt. Percentages of myelinated axons were checked manually. The results were taken in double-blind fashion with respect to homozygous Pcdhα-del and wild-type genotypes. All data were analyzed by two-tailed Student’s t-test to assess the significance of difference from controls.
Results

Axon growth defects in cultured Pcdha-del neurons

To compare axonal growth changes between Pcdha-del and wild-type mouse neurons, we observed cultured hippocampal neurons in a 24-hour period by confocal microscopy through immunofluorescence staining. Mice were genotyped by PCR before hippocampi were collected (Figure 1). Cells were seeded on coverslips coated with poly-L-lysine/laminin and cultured. The morphology of neurons was diverse and neurite outgrowth was remarkable after 24 hours. Cells were stained with DAPI (Figure 2A, E). There was no difference in nuclei morphology. Tau is a microtubule binding protein that stabilizes microtubules in growing axons. An anti-Tau-1 antibody was used to stain the axon, cell bodies, and dendrites of growing hippocampal neurons. The morphology of neurons was identified via Tau-1 staining. A neuron had one axon and multiple dendrites. The axon was always thin and funicular. We found that axon length was significantly decreased in Pcdha mutants compared with controls (Figure 2B, F). This indicates that the Pcdha cluster may function in axon outgrowth in cultured hippocampal neurons. Microfilaments consisting of actin, are widely distributed in neuronal soma and neurites, and can adapt to the physiological activities of neurons with morphological changes. Rhodamine phalloidin was used to stain F-actin, to show the integrity of the neurons (Figure 2C, G). These in vitro data suggest a potential role of the Pcdha cluster in axon development in vivo.

Loss of Pcdha resulted in axon length shortening in cultured hippocampal neurons

To further determine the role of clustered Pcdhs in axonal outgrowth, ImageJ software was used to analyze the length of axons. The axon length of wild-type neurons was 13.75 ± 0.95 μm after in vitro culture for one day. In contrast axon length in Pcdha-del neurons was 10.85 ± 0.50 μm (P < 0.05, vs. control group; Figure 3). These data confirmed that the Pcdha genes play an important role in axonal development.

Pcdha mutants affected transcriptional levels of genes related to multiple axonal activities

To determine changes in transcription of genes related to axon growth and extension in Pcdha-del mice, we compared hippocampal transcriptome profiles between wild-type and Pcdha-del mice using next-generation RNA sequencing. According to a cutoff threshold of > 2 fold change and P value < 0.05, 1,341 RNA transcripts were identified, of which 1,125 were downregulated and 216 upregulated (Figure 4A) in Pcdha-del mice. According to GO analysis, these differentially expressed transcripts are enriched for several cellular components that are crucial in several biological processes, including axon extension, axon guidance and spinal cord development (Figure 4B and Table 2). A group of prom-
Lu WC, Zhou YX, Qiao P, Zheng J, Wu Q, Shen Q (2018) The protocadherin alpha cluster is required for axon extension and myelination in the developing central nervous system. Neural Regen Res 13(3):427-433. doi:10.4103/1673-5374.228724

Eminent neuronal activity-regulated genes, including BDNF, Fmod, Nrp2, OGN, and Sema3d, was down-regulated in the hippocampus in the absence of Pcdha (Figure 4C).

**RT-qPCR verification of the transcriptome sequencing results**

To validate the reliability of the deep sequencing data, we confirmed the alteration of expression using RT-qPCR. Six significantly differentially expressed genes in the GO term axon extension were selected. As shown in Figure 5, the expression pattern of these five genes was in concordance with the deep sequencing results. Among the six genes, the expression of Slit3 was up-regulated, while the other genes, including BDNF, Fmod, Nrp2, OGN, and Sema3d, were down-regulated.
Table 2 Genes in GO terms related to neural development and axonal extension

| GO term              | GO category          | Symbols in list          |
|----------------------|----------------------|--------------------------|
| Gap junction          | Cellular component   | Gja1, Calb2, Gjb2, Nov, Gjb6, Gja5 |
| Axon terminus         | Cellular component   | Ntr1, Th, Slc17a8, Ccl2, Casr, Calb2, Cdhd1, Calca, Chrm3, Calb1, Aqp1, Snc |
| Axon extension        | Biological process   | Sema3d, Wnt5a, Epyc, Wnt5a, Bdnf, Ogn, Barhl2, Ntn1, Plxn4, Fmod, Nrp2, Slit3, Nkx6-1 |
| Axon guidance         | Biological process   | Sema3d, Wnt5a, Epyc, Bmp7, Wnt3a, Bdnf, Ogn, Lhx1, Isl1, Ntn1, Atoh1, Otx2, Plxn4, Lhx9, Bmpr1b, Zic2, Lmx1a, Fmod, Robo2, Nrp2, Slit3, Foxd1 |
| Spinal cord development | Biological process     | Wnt1, Wnt3a, Lhx1, Isl1, Lhx5, Olig3, Lbx1, Dmrt3, Nkx6-1, Zic1 |

Pcdha mutants possessed fewer myelinated axons

To assess the central nervous system myelination phenotype in Pcdha knockout mice, hippocampal nerves of P21 Pcdhα-del and wild-type mice were examined using transmission electron microscopy. Strikingly, significantly fewer myelinated axons were observed in Pcdhα-del mice compared with the controls at a low magnification (Figure 6A, B). At high magnification, myelin sheaths around axons displayed alternately dark and bright lamellae, concentric configuration and consistent structural integrity in P21 wild-type mice. By contrast, myelin sheaths showed lamellar disorder, discrete appearance, and vacuoles in P21 Pcdhα-del mice (Figure 6C, D). Quantification revealed a significant decrease in the percentage of axonal myelination in mutants compared with wild-type mice (Figure 6E). These data demonstrated that Pcdha is required for proper myelination at P21.

Discussion

An axon’s main function is to transmit nerve impulses from the soma to other neurons or effector cells. During nervous system development, axons extend to a specific location to establish proper neural wiring with target cells (Ferreira and Paganoni, 2002; Scheiffele, 2003; Gibson and Ma, 2011; Chia et al., 2014). One theory to explain why adult central nervous system axons cannot regenerate after spinal cord injury is because of a nonpermissive environment in the extracellular matrix (Huang and Sheng, 2012; Li et al., 2016). A previous strategy indicated that axonal growth could be promoted by removing this extracellular inhibitory activity; however, recent studies revealed that this is insufficient. Increasing the intrinsic regenerative ability of adult neurons has emerged as a promising strategy after spinal cord injury (Hannila and Filbin, 2008; Smith et al., 2009). However, these findings only provide limited help and do not enable axons to achieve long-distance growth.

This study found that Pcdha proteins may influence axon growth in vivo and that Pcdha-del cultured hippocampal neurons exhibit a significant decrease in axonal length. In addition, Pcdha-del mice exhibited a significant decrease in the degree of axon myelination in vivo. This is consistent with human genetic studies of a 3q21.3 microdeletion syndrome that causes delayed myelination in the human infant central nervous system (Shimojima et al., 2011). Our results show that the Pcdha cluster is important for the growth and maturation of axons. Moreover, axonal expression of Pcdha proteins is gradually repressed in mature mouse neurons (Morishita et al., 2004), indicating that the Pcdha cluster may play a crucial role in axon development. RNA-Seq experiments demonstrated that genes related to axon extension and axon guidance, such as BDNF, Fmod, Nrp2, OGN, and Sema3d are down-regulated in the absence of Pcdha. Many studies have shown that these genes participate in the growth of axons (Liu et al., 2004; Winckler, 2007; McIntyre et al., 2010; Steinhart et al., 2014; Taku et al., 2016; Guo et al., 2017). In summary, the Pcdha cluster functions in axon outgrowth and myelination. Neuron-intrinsic factors regulate axonal development in complex ways. Reactivating the expression of this gene can help repair adult mammalian spinal cord injury. This study focuses on morphological observations and does not clarify the mechanism by which the Pcdha cluster influences axon outgrowth and myelination. Furthermore, the mechanism by which expression of the Pcdha cluster is activated remains unclear, highlighting the need for additional strategies to reactivate intrinsic axonal growth after injury.

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