Necl-4/SynCAM-4 Is Expressed in Myelinating Oligodendrocytes but Not Required for Axonal Myelination

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Abstract

The timing and progression of axonal myelination are precisely controlled by intercellular interactions between neurons and glia in development. Previous in vitro studies demonstrated that Nectin like 4 (Necl-4, also known as cell adhesion molecule Cadm-4 or SynCAM-4) plays an essential role in axonal myelination by Schwann cells in the peripheral nervous system (PNS). However, the role of Necl-4 protein in axonal myelination in the developing central nervous system (CNS) has remained unknown. In this study, we discovered upregulation of Necl-4 expression in mature oligodendrocytes at perinatal stages when axons undergo active myelination. We generated Necl4 gene knockout mice, but found that disruption of Necl-4 gene did not affect oligodendrocyte differentiation and myelin formation in the CNS. Surprisingly, disruption of Necl-4 had no significant effect on axonal myelination in the PNS either. Therefore, our results demonstrated that Necl-4 is dispensable for axonal myelination in the developing nervous system.

Introduction

In vertebrate nervous system, internodal axons are wrapped by compact myelin sheaths, the specialized cellular membranes elaborated by myelinating glial cells. As myelin sheaths provide insulation for axons, action potentials propagate from node (of Ranvier) to node, and this saltatory conduction mechanism dramatically increases the transmission velocity of electrical impulses.

In the central nervous system (CNS), myelin sheaths are formed by oligodendrocytes. During development, oligodendrocytes originate from the neuroepithelium of the ventricular zone and then migrate to the surrounding white matter regions [1–3], where they contact target axons and subsequently differentiate into mature myelinating oligodendrocytes. The progression of axonal myelination involves multiple steps, including adhesion of oligodendrocytes to axons, spiraling of oligodendrocyte process around axons and the formation of compact myelin sheath [4]. Each of these steps is precisely regulated by the reciprocal communication between glial cells and neurons [4,5].

The molecular mechanisms that mediate the axonal-glial interaction and myelin formation in the CNS remain elusive. Recently, it was reported that cell adhesion molecules of the nectin-like (Necl) family are likely to be involved in axonal myelination process [6,7]. The NECL proteins belong to the immunoglobulin/Ig-like CAM superfAMILY and contain three extracellular domains, a single transmembrane domain and a cytoplasmic domain with characteristic FERM- and class II PDZ-binding motifs [8–11]. Through their homophilic or heterophilic interactions, NECL proteins regulate a wide spectrum of biological processes including cell adhesion, cell proliferation, synaptic assembly, and myelin formation [12,13]. In the PNS, neurons express Necl-1, Necl-2, Necl-4 and a low level of Necl-3, whereas Schwann cells only express Necl-2 and Necl-4. Notably, Necl-1 and Necl-4 are located on the apposing sides of axonal-glial contact interface along the internodal region, with Necl-1 on the axonal membrane and Necl-4 on the glial membrane [6,7]. There is a strong heterophilic interaction between Necl-1 and Necl-4 [12]. Disruption of Necl-4 expression or its interaction with Necl-1 abolished axonal myelination of dorsal root ganglion (DRG) neurons by Schwann cells in culture [6,7], suggesting the critical role of Necl-4 in mediating axonal-glial interaction and PNS myelination.

However, it remains unknown whether Necl-4 has a similar role in axonal myelination in the developing CNS, and whether it is required for PNS myelination in vivo. In this study, we showed that Necl-4 is expressed in both CNS neurons and myelinating oligodendrocytes at postnatal stages when axons undergo active...
myelination. However, disruption of Necl-4 alone had little effects on myelin formation in either the CNS or the PNS.

Materials and Methods

In Situ RNA Hybridization and Double Labeling Experiments

Mouse spinal cord and brain tissues from postnatal stages were perfused and fixed in 4% paraformaldehyde in PBS at 4°C overnight. Following fixation, tissues were transferred to 20% sucrose in PBS overnight, embedded in OCT media, and then sectioned on a cryostat. For double labeling experiments, tissues were first subjected to RNA in situ hybridization (ISH) with Necl4 (GenBank accession no. NM_001047107) riboprobe, followed by anti-Olig2, anti-APC or anti-NeuN immunohistochemical staining with ABC kit, respectively. Rabbit anti-Olig2 (a gift from Dr. Charles Stiles) was used at 1:2,000; mouse anti-APC (Ab-7, Oncogene Inc, Cat# ab167994) at 1:3,000; and mouse anti-NeuN (Chemicon Inc, Cat# MAB377) at 1:4,000.

Generation of Necl-4 mutant mice

The BAC clone containing the genomic DNA of Necl-4 was purchased from Invitrogen. The gene target vector was constructed by replacing the first exon with inducible Cre recombinase gene (Cre-ERT2) and the neomycin resistance gene. Linearized targeting vector was electroporated into mouse ES cells. Following selections, the genomic DNA of ES clones was digested with SpeI and subjected to Southern hybridization using 3’ flanking probe. The wild type allele yields a band of 8.9 kb and the mutant allele a band of 7.3 kb. 198 independent ES clones were screened by Southern blot genotyping with the 3’ flanking probe. Five clones with homologous recombination were identified and two were injected into blastocysts to produce chimera mice for germline transmission to produce the F1 heterozygous mice. The homozygous mutant animals derived from two independent ES clones exhibited the same phenotype. Germline transmission was confirmed by both Southern hybridization and PCR. The primers N4 neo-UP (5’ CGTTGGCTACCCGTGATATTGCTGAGG-3’) and N4 DP (5’ GGGACAAAGGCGGCGTGGAGAAACG-3’) were to detect the mutant allele (1150 bp); PCR conditions were 95°C for 5 min; 35 cycles of 95°C for 30 s, 60°C for 45 s, 72°C for 1 min 20 sec, followed by incubation at 72°C for 10 min. The primers N4 WT-UP (5’ GCGGAGCA-GAGGGGGAGACTGGACT-3’) and N4 DP (5’ GGGA-CAAAGGCGGCGTTGAAGAG-3’) were used to detect the wild type allele (725 bp); PCR conditions were 95°C for 5 min; 35 cycles of 94°C for 30 s, 63°C for 30 sec, 72°C for 45 sec, followed by incubation at 72°C for 10 min.

Genotyping of Nkx2.2 and Olig1 mutant mice

All of the mice used in this study were handled according to the protocols approved by Institutional Animal Care and Use Committee (IACUC), University of Louisville (IACUC: 12034). The homozygous pups were obtained by interbreeding heterozygous animals. Genomic DNA extracted from tails was used for genotyping by Southern analysis or by PCR. Genotyping methods of Olig1 and Nkx2.2 loci were described earlier [14–16].

Figure 1. Necl-4 expression in embryonic and postnatal spinal cords. A–H: Spinal cord sections from E16.5, E18.5, P0, P4, P7, P15, P30 and P64 were subjected to ISH with Necl-4 riboprobe. Necl4 expression was detected in spinal cord white matter after E18.5, and persisted till adulthood. Scale bar, 100 μm. doi:10.1371/journal.pone.0064264.g001

Figure 2. Co-expression of Necl-4 with neuronal and oligodendroglial markers. Spinal cord sections from P15 were subjected to Necl-4 ISH (in blue), followed by immunohistochemical staining for NeuN and APC, respectively. The arrows and arrowheads indicate representative double stained neurons and mature oligodendrocyte in the ventral white matter, respectively. Scale bar, 50 μm. doi:10.1371/journal.pone.0064264.g002
RT-PCR

Total RNA was prepared from the brain of wild type and mutant mice at P7 with the RNA easy kit (Roche) and reverse-transcribed to cDNA with the first strand synthesis kit (Sigma). Primers Necl4Exon1UP 5'-GGG AGG TGC AGG TGC CGG G-3' and Necl4Exon2DP 5'- GTG CCA TTG AAA AAG AGG GT -3', which were respectively located in the first exon and the second exon, were designed to detect the 5'-end cDNA; Necl4Exon1UP 5'-GGG AGG TGC AGG TGC CGG G-3' and Necl4 3-UTR DP 5'- CCA GGC ATC CAA CAC CC -3', which were respectively located in the first exon and the 3' end untranslated sequence, were used to detect the full length of cDNA. The PCR conditions were 95°C for 5 min; 30 cycles of 94°C for 30 s, 55°C for 30 sec, 72°C for 1 min, followed by incubation at 72°C for 10 min. GAPDH was the control.

Western blotting

Brain tissues were lysed in tissue lysis buffer (Sigma) with protease inhibitor cocktail (Sigma). 30 mg protein from control and mutant tissues was loaded for SDS-PAGE electrophoresis and subsequently detected with anti-Necl-1 (developed in Peking Union Medical University), anti-Necl-2 (Proteintech, Cat# 14335-1-AP), anti-Necl-3 (Abcam Inc, Cat# ab133393) and anti-Necl-4 (UC Davis/NIH NeuroMab Facility, Cat#73-247), and mouse anti-β-actin (Sigma, Cat# A5316) antibodies according to the standard protocol. The integrated density of blots on films was assessed with the analysis tool in Adobe Photoshop CS5 software and the relative densitometric values were used for statistical analyses on the expression level of target proteins.

Ultrastructural Analyses of Myelin Structures

Wild type and Necl-4 mutant littermates were perfused with 3% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2, and small pieces of tissues from optic nerves, spinal cord (at T6 level), and sciatic nerves were removed and postfixed for three additional hours. Tissues were then washed several times with cacodylate buffer, postfixed in 1% osmium tetroxide for 1 h, washed again with the buffer before dehydration through a series of graded alcohol. Fixed tissues were subsequently embedded in epon plastic and sectioned at 800–1000 Å on a diamond knife and mounted on 200 mesh copper grids. Ultra-thin sections were stained with uranium acetate and lead citrate, and examined under a Philips CM12 EM operating at 80 kV. For statistical analyses of axonal myelination, three or four animals per each genotype were used, and the number or the perimeter of axons was counted with Adobe Photoshop CS5 software.

Statistical analysis

Statistical analyses were performed with two-tailed homoscedastic (unpaired) Student’s t-test. Error bars represented the standard deviations.

Results

Expression of Necl-4 in neurons and oligodendrocytes in the CNS

Recent studies demonstrated that Necl-4 is expressed in myelinating Schwann cells and is required for PNS myelination in cell co-culture [6,7]. However, its expression and function in CNS development has remained unknown. To address this question, we first examined Necl-4 expression in the developing mouse spinal cord tissue by RNA in situ hybridization (ISH). Prior
to the onset of axonal myelination, Necl-4 expression was restricted to the gray matter of spinal cord (Figure 1A). Starting from E18.5, expression of Necl-4 was also detected in the white matter (Figure 1B), and the number of Necl-4+ cells gradually increased with time and reached the maximum between P7 and P15 (Figure 1E–F), indicating the lack of Necl4 transcription in the null mutants (Figure 5D), indicating the lack of Necl4 transcription in the null mutants. Western immunoblotting with anti-Necl-4 antibody revealed that the expression of Necl-4 protein was reduced in Necl4 heterozygous tissues and completely absent in the homozygous mutants (Figure 3, p<0.01).

Expression of Necl-4 in the brain

To investigate whether there is a regional difference in Necl-4 expression along the rostrocaudal axis, we carried out double-staining (ISH for Necl4 and immunohistochemical staining for Olig2) in early postnatal brain tissues. Similar to our observations in the spinal cord, Necl-4 expression in the forebrain was initially detected in neurons and later in oligodendrocytes. Necl-4 expression in cerebral oligodendrocytes started to be detectable in corpus callosum at P7 (Figure 4A), but became more obvious at P15 (Figure 4B, C). In the cerebellum, Necl-4 was strongly expressed in the gray matter at P7, especially the Purkinje cell layer, and in the white matter oligodendrocytes as well (Figure 4D). Later, strong expression of Necl-4 was maintained in neurons, whereas its expression in the white matter glia was gradually down-regulated (data not shown). These results suggested that Necl-4 is sequentially expressed in neurons and white matter oligodendrocytes in the rostral regions of the CNS as well, and its expression is also temporally coincident with the myelination process in the brain.

Generation of Necl-4 knockout mice

To examine the in vivo role of Necl-4 in axonal myelination, we constructed a gene-targeting vector to replace the first exon of Necl-4 gene with the Neo cassette in embryonic stem (ES) cells by homologous recombination (Figure 5A). The first exon contains the only in-frame starting code (ATG) of the entire Necl-4 coding sequence. Following electroporation and neomycin selection, two independent ES clones with homologous recombination were injected into blastocysts to produce chimera mice. Germ line transmission of the mutant allele in the offspring was confirmed by Southern blotting and PCR (Figure 3B and data not shown).

Homozygous mice were viable after birth and morphologically indistinguishable from their littermates. Disruption of Necl-4 expression was confirmed by several molecular and biochemical approaches. RT-PCR and in situ hybridization were performed to detect Necl-4 transcription. Two pairs of primers were designed to detect the 5’- and the full open reading frame (ORF), respectively. The results indicated that Necl-4 transcription is disrupted in the null mutants (Figure 5C). Consistently, in situ hybridization with Necl-4 probe in spinal cord sections could not detect the mRNA transcription of Necl-4 gene in the N4–P– mice (Figure 5D), indicating the lack of Necl4 transcription in the null mutants. Western immunoblotting with anti-Necl-4 antibody revealed that the expression of Necl-4 protein was reduced in Necl-4 heterozygous tissues and completely absent in the homozygous mutants (Figure 5E). These results suggested that the expression of Necl4 was successfully deleted in Necl4 homozygous mutants.

Normal differentiation of oligodendrocytes in Necl-4 mutant spinal cord

Previous studies showed that knockdown of Necl-4 in Schwann cells and DRG co-culture resulted in loss of myelin gene expression and diminished expression of two transcription factors, Oct-6 and Krox-20, which are required for Schwann cell differentiation [6,7]. As Necl-4 started to be expressed in differentiated oligodendrocytes at early perinatal stages, it may be required for oligodendrocyte differentiation. Immunostaining with antibodies against mature oligodendrocyte markers APC and...
MBP was performed to examine differentiation in oligodendrocytes. The results revealed a similar expression pattern in the wild-type and the Necl4 null mutant spinal cord tissues at P7 and P15 (Figure 6A–E and data not shown). The number of APC+ cells in the ventral white matter was counted, and there was no significant difference between the controls (518±70) and the mutants (444±35) (Figure 6E, p=0.11). These results suggested that oligodendrocytes differentiate normally in the Necl4 null mutants.

Normal axonal myelination in Necl-4 knockout

We next investigated whether Necl-4 is required in the CNS myelination by examining myelin structures in the spinal cord at various postnatal developmental stages. At P7, a large number of axons were myelinated in the ventral white matter (at the position of corticospinal tract at T6) (Figure 6F–G). The density of axons in the mutants (304,157±25,470/mm²) was comparable with that in the controls (309,145±22,474/mm²) (Figure S1A, n=3, p=0.61). The myelin structures were found normal in Necl4 null mutants, and the percentage of myelinated axons in the mutant mice (43.74±3.04%) was slightly lower, but not significantly different from that in the wild-type littersmates (46.25±2.53%) (Figure 6H, p=0.34). Therefore, CNS myelination was not significantly affected by the Necl-4 mutation.

Recent studies suggested that reciprocal interaction between NECL-4 and NECL-1 was essential for the ensheathment and myelin wrapping of axons by Schwann cells in the DRG-Schwann cell co-culture [6,7]. Therefore, we also examined the PNS myelination in sciatic nerves at P7. The g-ratio (the ratio of axon diameter to the diameter of axon and myelin sheath) was calculated to analyze the thickness of myelin sheaths in sciatic nerves. The densities of myelinated axons were comparable between the controls (57,412±4,992/mm²) and mutants (50,903±14,464/mm²) (Figure S1B). The average thickness of myelin sheath of Necl-4−/− (g-ratio = 0.70±0.03) was slightly, but not significantly thinner than that of the controls (g-ratio = 0.68±0.04) (Figure 6I–K, p = 0.39). These results indicated that myelination proceeded normally in the Necl4−/− sciatic nerves. Therefore, Necl-4 appears to be dispensable for the PNS myelination by Schwann cells during in vivo development.
Lack of functional compensation between Necl4 and other Necl molecules

The lack of apparent phenotype in Necl-4 mutant animals raised the possibility of potential functional redundancy between Necl-4 and other Necl proteins. Therefore, we examined the expression of Necl1-3 with ISH and western blot (Figure S2, Figure S3 and Figure 7). We had described earlier that Necl1 is only expressed by neurons in the CNS [16]. Our ISH results suggested that Necl2 was also robustly expressed in the gray matter of the spinal cord from E16.5 to P0. However, its expression was down-regulated soon after birth. At P30, little Necl2 staining was detected in the gray matter (Figure S2). Unlike other Necl genes, Necl-3 only had weak expression in the gray matter of the spinal cord at embryonic stages, and no expression was detectable by ISH after birth (Figure S3). Thus, both Necl-2 and Necl-3 are not significantly expressed by oligodendrocytes at all stages. Western blotting results confirmed their relative expression levels in the CNS, and revealed that the expression of Necl-1, Necl-2 and Necl-3 was not significantly altered in the Necl-4 mutants (Figure 7. n = 3. Necl1, p = 0.70. Necl2, p = 0.72. Necl3, p = 0.69). These results suggested

Figure 6. Oligodendrocyte differentiation and axonal myelination in Necl4 null mutant mice. A-D. Immunofluorescent staining of wildtype and Necl-4 null mutant spinal cord tissues at P15 with mature markers MBP and APC. Scale bar, 100 μm. E. Statistical analysis of the number of APC+ cells at P15 (n = 4). Student’s t-test, p = 0.11. Error bar, standard deviation. F–K. Myelination in spinal cord and sciatic nerves at P7 from the wild type and Necl-4 null mutants was examined under electron microscope. Scale bars, 5 μm. High magnification images of individual axons were shown as the inserts. H. Statistical analysis of the percentage of myelinated axons (n = 3). Student’s t-test, p = 0.34. Error bar, standard deviation. K. Statistical analysis of the G-ratio of sciatic nerves (n = 3). Student’s t-test, p = 0.39. Error bar, standard deviation. SC, spinal cord. SN, sciatic nerve.
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that Necl-4 did not cross regulate the expression of other members of the Necl family, and the loss of Necl-4 in oligodendrocytes was unlikely to be compensated by the up-regulation of Necl-2/3.

Discussion

The reciprocal communication between neurons and glia plays a critical role in the control of myelination process [21]. Previous studies described that Necl-4 is expressed by PNS neurons and Schwann cells, and mediates axonal myelination by heterophilic binding to its axonal partner Necl-1 [6,7]. In this study, we systematically analyzed Necl-4 expression in the developing CNS and found that Necl-4 expression was initially detected in gray matter neurons, but later up-regulated in mature myelinating oligodendrocytes in the white matter (Figure 1–2) at early postnatal stages.

The up-regulation of Necl-4 in oligodendrocytes is spatiotemporally correlated with the myelination events in the spinal cord and the brain. Myelination of axons by oligodendrocytes in the CNS primarily starts at neonatal stages, first in the spinal cord and later in the brain, following a rostral-to-caudal order in the spinal cord or a caudal-to-rostral sequence in the brain [5]. In the spinal cord, Necl-4 started to be transcribed in differentiated oligodendrocytes at perinatal stages when myelination process commences, and reached the maximum at the peak time of myelin formation (from P7 to P15) in the spinal cord [4], followed by gradual down-regulation thereafter (Figure 1). A similar temporal correlation was also noticed between Necl-4 expression and axonal myelination in the cerebellum and the forebrain (Figure 4). Together, these expression studies provided circumferential evidence for its possible involvement in mediating axon-glial interactions and promoting axonal myelination in the CNS.

Surprisingly, our genetic analyses demonstrated that disruption of Necl-4 gene did not cause apparent developmental defects in the CNS. The Necl-4 null mutant mice were viable and fertile, and did not display noticeable motor disorders. The normal expression pattern of mature oligodendrocyte markers in the mutant spinal cords at neonatal stages suggested that Necl-4 is not required for oligodendrocyte differentiation and maturation (Figure 6A–E). The number and percentage of myelinated axons in spinal cord and optic nerve (Figure 6H, Figure S1 and data not shown) were similar in both controls and Necl-4 null mutants. These results suggested that axonal myelination proceeded in a comparable pace in both mutants and the controls.

Contrary to the previous in vitro studies [6,7], Necl-4 homozygous mutants and their wild type littermates displayed similar pattern of myelination and thickness of myelin sheaths in the sciatic nerve (Figure 6I–K, Figure S1B), indicating that Necl-4 does not play an essential role for developmental myelination in the PNS as well. Expression analyses did not support the idea of functional compensation by other Necl proteins, because Necl-4 is the only Necl protein whose expression is upregulated in myelinating oligodendrocytes (Figure 1, Figure S2, S3), and disruption of Necl-4 did not significantly change the expression level of other Necl proteins in the CNS and the PNS (Figure 7 and data not shown). One plausible explanation for the discrepancy between in vitro experiments and in vivo observations is that the initiation of axonal myelination may also involve interactions between other cell adhesion molecules such as laminins and integrins [22–25] and this interaction might be disrupted in dissociate culture and therefore can not compensate for the loss of Necl-1/Necl-4 interaction. Further studies with compound mutants of Necl-4 and other adhesion proteins could delineate the role of various cell adhesion molecules in myelination in the developing nervous system.
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Author Contributions

Conceived and designed the experiments: YZ XP BQ JY MQ. Performed the experiments: YZ HL XZ XH HH. Analyzed the data: YZ XZ. Contributed reagents/materials/analysis tools: KL TA JP YB BQ JY XP. Wrote the paper: YZ XP MQ.

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