Tight junction (TJ)–like structures have been reported in Schwann cells, but their molecular composition and physiological function remain elusive. We found that claudin-19, a novel member of the claudin family (TJ adhesion molecules in epithelia), constituted these structures. Claudin-19–deficient mice were generated, and they exhibited behavioral abnormalities that could be attributed to peripheral nervous system deficits. Electrophysiological analyses showed that the claudin-19 deficiency affected the nerve conduction of peripheral myelinated fibers. Interestingly, the overall morphology of Schwann cells lacking claudin-19 expression appeared to be normal not only in the internodal region but also at the node of Ranvier, except that TJs completely disappeared, at least from the outer/inner mesaxons. These findings have indicated that, similar to epithelial cells, Schwann cells also bear claudin-based TJs, and they have also suggested that these TJs are not involved in the polarized morphogenesis but are involved in the electrophysiological “sealing” function of Schwann cells.

Introduction

In vertebrates, a process called saltatory conduction causes action potentials to travel along myelinated axons faster than along nonmyelinated axons. Myelinated axons are tightly enwrapped by the continuous membrane layers of individual glial cells; i.e., Schwann cells in the peripheral nervous system (PNS) or oligodendrocytes in the central nervous system (CNS). Compaction of these layers leads to the formation of the myelin sheath, which electrically insulates axons. Along axons, these sheaths are interrupted at regularly spaced nodes of Ranvier, where axonal membranes can propagate action potentials. Saltatory conduction of action potentials occurs by jumping from node to node over individual myelin sheaths, and, for it to work efficiently, electrical insulation by Schwann cells/oligodendrocytes must be tightly established.

For this insulation, two distinct types of paracellular pathways must be electrically sealed: the axo-glial junctions at the paranode flanking the node of Ranvier and the intermembranous spaces within individual glial cells. Interestingly, the axo-glial paranodal junctions resemble septate junctions that are thought to be responsible for electrical sealing in invertebrates in general (Rosenbluth, 1976; Einheber et al., 1997); thus, this paranodal junction has attracted much interest in the past few years and the molecular architecture of this junction is being rapidly unraveled (Pele and Salzer, 2000; Pedraza et al., 2001; Spiegel and Peles, 2002; Poliak and Peles, 2003). On the other hand, the intermembranous spaces within individual glial cells have been considered to be mostly sealed by compact myelin, but, in addition, old electron microscopic observations indicated the existence of tight junction (TJ)–like structures that might also be involved in the intermembranous sealing of glial cells (Dermietzel, 1974; Mugnaini and Schnapp, 1974; Reale et al., 1975; Schnapp and Mugnaini, 1976; Sandri et al., 1977; Tabira et al., 1978; Dermietzel and Kroczek, 1980; Shinowara et al., 1980; see Fig. 2). However, a knowledge of the molecular components of these TJ-like structures in glial cells has been lacking for some time.

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Abbreviations used in this paper: CAP, compound action potential; Caspr, contactin-associated protein; CMT, Charcot-Marie-Tooth; CNS, central nervous system; ES, embryonic stem; OSP, oligodendrocyte-specific protein; PNS, peripheral nervous system; TJ, tight junction.
TJs have been identified and characterized in detail, mainly by using simple epithelial cells. TJs seal the paracellular routes of epithelial cells to create a primary barrier to the diffusion of solutes across the cellular sheet, and they also function as a boundary between the apical and basolateral membrane domains to produce their polarization (Anderson and Van Itallie, 1995; Balda and Matter, 1998; Tsukita et al., 2001; Anderson et al., 2004; Schneeberger and Lynch, 2004). On ultrathin section electron microscopy, TJs appear as a series of discrete sites of apparent fusion, involving the outer leaflets of the plasma membranes of adjacent cells (Farquhar and Palade, 1963). On freeze-fracture electron microscopy, TJs appear as a set of continuous, anastomosing intramembranous particle strands (TJ strands; Staehelin, 1974). These observations led to our current understanding of the three-dimensional structure of TJs; each TJ strand associates laterally with another TJ strand in apposing membranes of adjacent cells to form “paired” TJ strands where the intercellular space is completely obliterated (Tsukita et al., 2001).

To date, three distinct types of integral membrane proteins have been shown to localize at TJs: occludin (Furuse et al., 1993), junctional adhesion molecules (Martin-Padura et al., 1998), and claudins (Furuse et al., 1998a). Among them, claudin is now believed to be a major constituent of TJ strands (Tsukita and Furuse, 1999; Tsukita et al., 2001; Turksen and Troy, 2004). Claudins with molecular masses of ~23 kDa bear four transmembrane domains and comprise a multigene family consisting of 24 members in mice/humans (Furuse et al., 1998a; Morita et al., 1999a,b,c; Simon et al., 1999; Tsukita and Furuse, 1999; Van Itallie and Anderson, 2004). Interestingly, when each claudin species was overexpressed in mouse L fibroblasts lacking endogenous claudins, exogenously expressed claudin molecules were polymerized within the plasma membrane to reconstitute paired TJ strands in cell–cell contact regions (Furuse et al., 1998b).

In myelinated axons of the CNS, TJ strand–like structures ~10 nm thick were observed between the lamellae of myelin sheaths by freeze-fracture electron microscopy (Dermietzel, 1974; Reale et al., 1975; Schnapp and Mignaini, 1976; Tabira et al., 1978; Dermietzel and Kroczek, 1980). These interlamellar strands run parallel to the axon axis and run radially through the myelin sheath, consisting of the so-called ‘radial component of myelin’ (Peters, 1961, 1964; Dermietzel, 1974), and these radial components were speculated to be directly involved in electrically isolating the extracellular compartment within myelin sheaths (Mignaini and Schnapp, 1974). When we discovered the existence of the claudin gene family, we noticed that claudin-11, which was initially identified as an oligodendrocyte-specific protein (OSP), constitutes these interlamellar strands (Morita et al., 1999b). Further, Gow et al. (1999) reported that mice lacking the expression of claudin-11/OSP lacked interlamellar strands in the myelinated axons of the CNS and exhibited characteristic neurological deficits.

Also in myelinated axons of the PNS, the existence of TJ strand–like structures has been reported in three regions of Schwann cells; strands existed between the apposed membranes of paranodal terminal loops and the Schmidt-Lanterman incisures, as well as those of outer/inner mesaxons (Sandri et al., 1977; Tetzlaff, 1978, 1982; Shinowara et al., 1980; Salzer, 2003; see Fig. 2 A). Interestingly, however, claudin-11 was not expressed in Schwann cells (Morita et al., 1999b), which leads to the intriguing question of whether these strands can also be regarded as TJ strands, and, if so, what species of claudins constitutes them. The identification of such claudin species has been regarded as important for a better understanding of the molecular basis to the physiology of Schwann cells.

In this study, we found that claudin-19 was expressed in large amounts in the PNS, not in the CNS, and constituted the TJ-like structures of Schwann cells that were detectable by electron microscopy. Furthermore, we generated claudin-19–deficient mice and found that they lacked TJs in Schwann cells. Through detailed analyses of these mice, we examined and discussed the functions of claudin-based TJs in Schwann cells.

Results

Claudin-19 as a constituent of TJs in the Schwann cells of peripheral myelinated nerves

Northern blotting revealed that claudin-19 was expressed in large amounts in the PNS. Other tissues, including the brain, did not give strong signals, but the kidney expressed this claudin in fairly large amounts (Fig. 1 A). Similar to other claudin species, claudin-19 showed an ability to polymerize to reconstituted TJ strand–like structures in cell–cell contact regions when overexpressed in mouse L fibroblasts (Fig. 1 B). These findings suggested that claudin-19 constituted TJ strands in some types of cells within peripheral nerves. In peripheral nerves, three types of cells have been reported to bear TJs or TJ-like structures: endothelial cells of blood vessels, mesothelial cells in the perineurium, and Schwann cells of myelinated axons.

To determine which types of cells in peripheral nerves expressed claudin-19, we first attempted to raise a specific pAb in rabbits using keyhole limpet hemocyanin–conjugated peptides that corresponded to the COOH-terminal tail of claudin-19 as an antigen. One of the affinity-purified pAbs appeared to be specific for claudin-19, as examined with Western blotting against GST fusion proteins with the cytoplasmic tails of claudin-1 to -16 (Fig. 1 C). This specificity was later confirmed in the analyses of claudin-19–deficient mice (see Fig. 6).

In contrast to claudin-19, claudin-11 was reported to be expressed in the CNS but not in the PNS (Morita et al., 1999b). We then examined the mutually exclusive expression patterns of claudin-11 and -19 in the nervous system by using immunofluorescence microscopy. Frozen sections of the mouse spinal cord with the ventral root were stained with anti–claudin-11 and anti–claudin-19 pAbs. As shown in Fig. 1 D, the signals for claudin-11 and -19 were completely restricted to the spinal cord (i.e., CNS) and the ventral root (i.e., PNS), respectively. Furthermore, close inspection revealed that claudin-19 was not detected in either the endothelial cells of blood vessels or in the mesothelial cells of the root perineurium, but its distribution appeared to be associated with relatively thick nerve fibers.
We then examined the distribution of claudin-19 in peripheral nerves in more detail. When single fibers teased from sciatic nerves were whole-mount stained with anti–claudin-19 pAb, all of the myelinated axons showed characteristic staining patterns (Fig. 2 B). As schematically drawn in the left panel of Fig. 2 A, TJ strand–like structures have been reported to form a circumferential belt and to occur along incisures on both sides of individual, unrolled flat Schwann cells (Arroyo and Scherer, 2000; Poliak et al., 2002; Spiegel and Peles, 2002; Salzer, 2003). When these cells are rolled around axons, the strand on one side of each Schwann cell has been thought to make a paired strand with that on its other side; i.e., to form TJ-like junctions, the localization of which is very peculiar and three-dimensionally complex in internodal segments (Fig. 2 A, right). Interestingly, the claudin-19 staining pattern of myelinated axons appeared to coincide with this peculiar, complex localization of the TJ-like structures of Schwann cells (Fig. 2 B). In the paranodal region, strong claudin-19 signals were detected, which may correspond to TJ-like structures that were observed between the paranodal terminal loops of Schwann cells. Consistent with this notion, claudin-19 was found to be more outwardly distributed than contactin-associated protein (Caspr)/Paranodin when the paranodal region of single myelinated fibers was double stained for claudin-19 and Caspr/Paranodin (a specific marker for the axo-glial paranodal junctions; Fig. 2 B, bottom). Claudin-19 was also highly concentrated at Schmidt-Lanterman incisures, where TJ-like structures were again shown to occur by electron microscopy. Furthermore, in most of the individual rolled Schwann cells, two continuous claudin-19–positive lines ran parallel to the axon axis between two paranodal regions, sometimes in a spiral manner. These may correspond to TJ-like structures that were observed at the outer and inner mesaxons of Schwann cells. It is safe to say that claudin-19 constitutes the TJ strand–like structures observed in Schwann cells by freeze-fracture replica electron microscopy, and that these structures can be regarded as a variant of the TJ strands found in many other epithelial/endothelial cells.

**Generation of claudin-19-deficient mice**

To explore the function of claudin-19 in vivo, we produced mice unable to express it. Nucleotide sequencing, as well as restriction mapping, identified four exons that cover the whole ORF of claudin-19 (Fig. 3 A). We constructed a targeting vector, which was designed to disrupt the claudin-19 gene by replacing all of these exons (exons 1–4) with the neomycin resistance gene. Two distinct lines of mice were generated from distinct embryonic stem (ES) cell clones in which the claudin-19 gene was disrupted by homologous recombination. Southern blotting confirmed the disruption of the claudin-19 gene in heterozygous as well as in homozygous mutant mice (Fig. 3 B), and RT-PCR detected no claudin-19 mRNA from the kidney of homozygous mutant mice (Fig. 3 C). Because both lines of mice showed the same phenotypes, we will mainly present data obtained from one line.

No obvious phenotype was apparent in heterozygous mutant mice, and when these mice were interbred, wild-type (Cld19+/+), heterozygous (Cld19+/−), and homozygous (Cld19−/−) mutant mice were produced in the expected Mendelian ratios. Cld19−/− mice were developed, grew normally in the laboratory environment, and showed no differences in weight, size, or reproductive ability from Cld19+/+ mice up to 2 yr of age.

**Functional analyses of claudin-19-deficient mice**

Cld19−/− mice appeared to walk awkwardly on a smooth surface, especially on a smooth rod. Therefore, we subjected these mice (10–13-wk-old male Cld19+/+ and Cld19−/− mice) to several established behavioral tests (Fig. 4). First, a "beam test" was performed, in which we counted how many times a hindlimb slips while a mouse is walking a given distance on a thin or thick bar. Interestingly, Cld19−/− mice exhibited...
significantly more slips on these bars than Cld19+/+ mice. Second, these mice were subjected to a “rotarod test.” In this test, mice were put on a rotating rod, and how long they remained on the rod was measured. Cld19−/− mice fell from the rod more quickly than Cld19+/+ mice. Importantly, in both tests, but especially in the rotarod test, Cld19−/− mice performed better as the trials were repeated (similar to Cld19+/+ mice), which was consistent with the notion that the neuronal
deficits of Cld19−/− mice observed in these tests were attributable to defects in the PNS. Furthermore, to evaluate CNS functions in Cld19−/− mice, we performed two more behavior tests, the “open field test” and the “prepulse inhibition test” (see Materials and methods). In these tests, no behavioral abnormalities were detected in Cld19−/− mice. Thus, taking the PNS-specific expression of claudin-19 in the nervous system into consideration, we concluded that Cld19−/− mice suffered from a kind of peripheral neuropathy.

To directly evaluate the electrophysiological properties of the peripheral nerves in Cld19−/− mice, we made field potential recordings of isolated sciatic nerves to measure the compound action potentials (CAPs), though this recording was not technically easy mainly because of the short length of isolated mouse sciatic nerves. When nerves were stimulated with sufficiently large currents to elicit the maximal CAP amplitude, CAPs obtained from Cld19+/+ mice demonstrated a single-peak, smooth waveform. In 7 out of 10 Cld19−/− mice, CAP waveforms or population conduction velocities did not appear to be significantly different from those in Cld19+/+ mice, but in three Cld19−/− mice we noticed a significant difference in CAP waveforms. These CAPs showed a characteristic double-peak waveform: a peak around a normal conduction velocity and an additional delayed peak (Fig. 5). Interestingly, under the stimulus intensity to elicit half-maximal CAP amplitude, this double-peak CAP waveform was reproducibly detected in Cld19−/− mice (n = 10) but never in Cld19+/+ mice (n = 10; unpublished data). Although the mechanism responsible for this difference remains unclear, it is safe to say that the claudin-19 deficiency changed the conduction parameters of at least a subpopulation of myelinated fibers to reduce their conduction velocity.

**Morphological analyses of claudin-19-deficient mice**

We then examined the morphology of PNS myelinated axons in Cld19−/− mice. First, in order to preserve the structure of axons as perfectly as possible, especially that of compact myelin, we fixed the saphenous nerves with chemical fixatives according to a method described previously (Tsukita and Ishikawa, 1980) and compared transverse sectional views of electron micrographs between Cld19+/+ and Cld19−/− mice (Fig. 6 A, left). At a low magnification, no difference was discerned in terms of myelination. The ratios of axonal diameter to total fi-
Sciatic nerves were immunofluorescently stained with anti–E-cadherin. Bars: (A, left) 2 μm; (A, right) 5 μm; (B) 5 μm.

Figure 5. Electrophysiological analysis of claudin-19–deficient mice. Nerves were isolated from 10-wk-old mice (n = 10 each for Cld19+/− and Cld19−/− mice), and field potential recordings were made to measure the CAPs. The distance between extracellular stimulation and recording electrodes was 2.5 cm. When nerves were stimulated with sufficiently large currents to elicit maximal CAP amplitude, CAPs obtained from all Cld19−/− mice demonstrated a single-peak, smooth waveform (open arrowheads; +/+). However, in 3 out of 10 Cld19−/− mice, CAPs showed a characteristic double-peak waveform: a peak around a normal conduction velocity (open arrowheads) and an additional delayed peak (closed arrowheads; −/−). Under the stimulus intensity to elicit half-maximal CAP amplitude, this double-peak CAP waveform was reproducibly detected in Cld19−/− mice, but never in Cld19+/− mice (not depicted). Arrows, stimulation.

Figure 6. Overall morphology of internodal segments of peripheral myelinated axons of claudin-19–deficient mice. (A) Transverse sectional views of peripheral nerves of Cld19+/− and Cld19−/− mice. Left) The saphenous nerves were examined by ultrathin section electron microscopy. No significant difference was discerned in the myelination between Cld19+/− and Cld19−/− mice (see g values in the text). (right) Transverse frozen sections were cut from the sciatic nerves and were triple stained with anti–claudin-19 pAb (green), anti–myelin basic protein mAb (red), and antineurofilament pAb (blue). In the Cld19−/− sciatic nerve, two claudin-19–positive spots were detectable in each myelinated axon, which may correspond to the outer (arrows) and inner (arrowheads) mesaxons (see Fig. 8 a). In the Cld19−/− sciatic nerve, claudin-19 appeared to be simply removed without affecting the overall morphology of myelinated axons. (B) Claudin-19 and E-cadherin in myelinated axons. Transverse frozen sections were cut from Cld19+/− and Cld19−/− sciatic nerves and were triple stained with anti-E-cadherin mAb (red), anti–claudin-19 pAb (green), and antineurofilament pAb (blue). In each Cld19+/− myelinated axon, claudin-19 was concentrated at one of the largest E-cadherin–positive puncta on the Schwann cell surface; i.e., outer mesaxon (arrows) as well as at inner mesaxon (arrowheads). In Cld19−/− axons, claudin-19 disappeared without affecting the distribution of E-cadherin. Bars: (A, left) 2 μm; (A, right) 5 μm; (B) 5 μm.

Electrophysiological analysis of claudin-19–deficient mice. Nerves were isolated from 10-wk-old mice (n = 10 each for Cld19+/− and Cld19−/− mice), and field potential recordings were made to measure the CAPs. The distance between extracellular stimulation and recording electrodes was 2.5 cm. When nerves were stimulated with sufficiently large currents to elicit maximal CAP amplitude, CAPs obtained from all Cld19−/− mice demonstrated a single-peak, smooth waveform (open arrowheads; +/+). However, in 3 out of 10 Cld19−/− mice, CAPs showed a characteristic double-peak waveform: a peak around a normal conduction velocity (open arrowheads) and an additional delayed peak (closed arrowheads; −/−). Under the stimulus intensity to elicit half-maximal CAP amplitude, this double-peak CAP waveform was reproducibly detected in Cld19−/− mice, but never in Cld19+/− mice (not depicted). Arrows, stimulation.

The claudin-19 deficiency did not appear to affect the overall organization of the node of Ranvier (Fig. 7). In longitudinal sections of myelinated nerves in both Cld19+/− and Cld19−/− axons, the nodal region was clearly discernible between the terminal loops of Schwann cells. Although whole-mount immunostaining revealed that in Cld19−/− myelinated axons, claudin-19
was completely undetectable in paranodal regions (unpublished data), the terminal loops appeared to be normal. Unfortunately, however, it was technically difficult to conclusively demonstrate the existence and/or absence of TJ-like structures between these loops of Cld19+/+ and Cld19−/− axons by ultrathin section and by freeze-fracture replica electron microscopy. Importantly, also in the paranodal region of Cld19−/− axons, electron-dense transverse bands between terminal loops and axonal membranes were observed with a normal appearance.

Next, we closely compared the transverse sectional images of the internodal portion between Cld19+/+ and Cld19−/− myelinated axons. The overall structure of the internodal portion of Cld19−/− myelinated axons appeared to be indistinguishable from that of Cld19+/+ axons (Fig. 8 a). However, close inspection identified a clear difference in the outer and inner mesaxons between Cld19+/+ and Cld19−/− axons. At the outer mesaxon of Cld19+/+ axons, without exception, one to three “kissing points” of TJs, where the extracellular space was completely obliterated, were observed (Fig. 8 b). In contrast, the outer mesaxon of Cld19−/− axons lacked these kissing points completely, leaving widened intercellular gaps between apposed Schwann cell membranes (Fig. 8 b’). Consistent with this finding, TJ strand–like structures were frequently observed at the outer mesaxon of Cld19+/+ axons, but were never observed in Cld19−/− axons (Fig. 8, c and c’). At the inner mesaxon of Cld19+/+ axons, one kissing point of TJs was usually observed, though in some axons this kissing point was difficult to distinguish from the compact myelin (Fig. 8 d). At the inner mesaxon of Cld19−/− axons, typical kissing points were never detectable (Fig. 8 d’). Therefore, we concluded that in the Cld19−/− PNS, TJs themselves completely disappeared from myelinated Schwann cells (at least from their outer/inner mesaxons) without affecting their overall, peculiar cellular morphology.

Discussion

In multicellular organisms, the internal environment must be divided into various, compositionally distinct fluid compartments. This compartmentalization is usually established by cellular sheets of epithelia/endothelia that function as diffusion barriers to maintain the internal environment of each compartment. Multiple cells constitute these cellular sheets; thus, for these sheets to function as barriers, there must be some seal to the diffusion of solutes through the paracellular pathway. TJs have been shown to be responsible for this intercellular sealing in vertebrates (Anderson and Van Itallie, 1995; Balda and Matter, 1998; Tsukita et al., 2001; Anderson et al., 2004; Schneeberger and Lynch, 2004). The myelin sheath in the CNS/PNS constitutes a very peculiar compartment; each compartment is established by a single cell, an oligodendrocyte in the CNS, and a Schwann cell in the PNS together with the axon. To establish this unique compartment, the paracellular pathways within individual oligodendrocytes/Schwann cells are mostly sealed by compact myelin, whereas in the region of paranodal loops and Schmidt-Lanterman incisures, where compact myelin is absent, they must be sealed by other types of structures (Arroyo and Scherer, 2000; Poliak et al., 2002; Spiegel and Pelles, 2002; Salzer, 2003). Indeed, to date, electron microscopy has revealed the occurrence of TJ-like structures at these loops and incisures in addition to the outer and inner mesaxons (Fig. 2 A). However, a lack of information on TJ-specific cell adhesion molecules in the epithelia/endothelia has long hampered clarification of whether these structures can really be regarded as a variant of TJs both structurally and functionally. Set against this background, the claudin family, which was initially thought to consist of 16 members (Morita et al., 1999a; Simon et al., 1999; Tsukita and Furuse, 1999), was identified in the epithelia as major constituents of TJ strands. Among the claudins, it was claudin-11, which had been identified as an OSP, that constituted the TJ-like structures of oligodendrocytes (Morita et al., 1999b), and analyses with knockout mice suggested that these TJs were functionally indispensable for CNS myelinated axons (Gow et al., 1999).

Interestingly, however, claudin-11 was not expressed in the PNS. Therefore, for a better understanding of the physiology of Schwann cells in the PNS, the question of whether the...
TJ-like structures reported in Schwann cells can be regarded as TJs both structurally and functionally, and if so, what species of claudins constitute them, needs to be answered. Along this line, claudins expressed in Schwann cells were searched for by RT-PCR using mouse sciatic nerve RNA with primers for claudin-1 to -16 (Poliak et al., 2002). As a result, claudin-1, -2, -5, -10, and -15 were identified, and immunofluorescence microscopy showed that claudin-1 and -5 were concentrated at paranodal loops/mesaxons and Schmidt-Lanterman incisures, respectively. However, our anti–claudin-1 pAbs did not give any specific signals from the myelinated nerves of the PNS, and claudin-5 was detected very clearly in blood vessel endothelial cells, but not in the incisures of myelinated axons in peripheral nerves. Therefore, with the expectation that, similar to claudin-11...
in oligodendrocytes, some specific claudin species are primarily expressed in Schwann cells, we examined the expression levels of newly identified claudin species (claudin-17 to -24) by using Northern blotting, and found that claudin-19 was expressed in large amounts in the PNS. Expectedly, immunofluorescence microscopy with anti–claudin-19 pAb revealed that claudin-19 was highly and characteristically abundant in paranodal loops, in outer/inner mesaxons, and in incisures where TJ-like structures have been detected by electron microscopy (Arroyo and Scherer, 2000; Poliak et al., 2002; Spiegel and Peles, 2002). Considering that in Cld19<sup>-/-</sup> mice, TJ-like structures completely disappeared (at least from the outer/inner mesaxons), it is safe to say that, similar to oligodendrocytes, Schwann cells also bear real TJs, and a single species of claudin (claudin-19) primarily constitutes them.

From the viewpoint of compartmentalization in myelinated axons, the intercellular sealing by the axo-glial paranodal junction is also important. This junction resembles the septate junction of invertebrates not only in appearance (Rosenbluth, 1976; Pedraza et al., 2001) but also in molecular organization. For example, Caspr/Paranodin (a single membrane-spanning protein) and protein 4.1B (a membrane skeleton protein) reportedly form a molecular complex at the vertebrate paranodal junction (Einheber et al., 1997; Menegoz et al., 1997; Peles et al., 1997; Arroyo and Scherer, 2000; Salzer, 2003), and at the intervertebrate septate junctions, neurexin IV and coracle, which are homologous to Caspr/Paranodin and protein 4.1B, respectively, form a similar complex (Fehon et al., 1994; Baumgartner et al., 1996; Bellen et al., 1998). On the other hand, recent genetic analyses of Drosophila melanogaster mutants with a malformation of the trachea tube identified two D. melanogaster claudins, megatrachea and sinuous, which are localized at septate junctions, as being directly involved in the barrier function of tracheal epithelial cells (Behr et al., 2003; Wu et al., 2004). These findings naturally lead to speculation that also at the paranodal junctions of vertebrate PNS, some claudin or claudinlike membrane protein occurs to seal the axo-glial intercellular space. However, claudin-19 does not seem to be the putative paranodal claudin. Immunofluorescence microscopy revealed that in the paranodal region, claudin-19 was distributed more outwardly from Caspr-positive paranodal junctions themselves, and the septalike structures of paranodal junctions were not affected at the electron microscopic level in Cld19<sup>-/-</sup> mice. The possible relationship between paranodal junctions and claudins (or claudinlike molecules) should be examined in detail in the future.

In general, it is now believed that TJs play a key role not only in paracellular sealing but also in the establishment of epithelial cell polarity (Balda and Matter, 1998; Tsukita et al., 2001; Anderson et al., 2004). Through detailed genetical analyses with Caenorhabditis elegans and D. melanogaster, several protein complexes were identified as being directly involved in the cellular polarity (Gibson and Perrimon, 2003; Nelson, 2003; Roh and Margolis, 2003; Schneeberger and Lynch, 2004). These complexes were highly conserved throughout evolution, and among them, in vertebrates, PAR-3/aPKC/PAR-6, Crumbs3/PALS1/PATJ, and Scrib/mDlg/mlgl were shown to participate in the establishment of epithelial cell polarity by interacting with TJs. However, quite unexpectedly, Cld19<sup>-/-</sup> Schwann cells appeared to normally wrap their plasma membranes concentrically around the axon to form layers of compact myelin and the complicated structures of the node of Ranvier, though they lacked TJs. Furthermore, once-established myelin sheaths remained unaffected without showing any signs of demyelination up to at least 2 yr after birth. Therefore, although Schwann cells exhibited a very unique cellular morphogenesis, it would be reasonable to conclude that claudin-19–based TJs are not required for this polarized morphogenesis.

Finally, the question has naturally arisen as to what the real physiological function of claudin-19–based TJs in Schwann cells is. Cld19<sup>-/-</sup> mice exhibited significant behavioral abnormalities that were caused by PNS deficits. Based on an accumulated knowledge of TJs in the epithelia, it would be reasonable to speculate that these behavioral abnormalities are attributable to the defects in the electrical sealing by TJs in Schwann cells. However, compact myelin and the paranodal axo-glial junctions are also directly involved in the electrical sealing in Schwann cells, and it remains unclear how these structures are synergistically coordinated in the Schwann cell–based compartmentalization. Indeed, electrophysiological analyses of isolated peripheral nerves favored the notion that the saltatory conduction of myelinated axons itself was affected in Cld19<sup>-/-</sup> mice, showing peculiar double-peak CAP waveforms (Fig. 5). As it was technically difficult to clarify the molecular mechanism behind the generation of such peculiar CAP waveforms in Cld19<sup>-/-</sup> mice in more detail (at least in our hands) mainly because of the short length of isolated mouse sciatic nerves, it is still premature to further discuss the relationship between the behavioral abnormalities and double-peak CAP waveforms in Cld19<sup>-/-</sup> mice. Therefore, it is safe to say that these TJs were functionally indispensable for PNS myelinated axons.

The behavioral abnormalities of Cld19<sup>-/-</sup> mice were relatively mild, so that these mice grew normally and were fertile. Therefore, it is tempting to speculate about the possible existence of a human hereditary peripheral neuropathy; i.e., Charcot-Marie-Tooth (CMT) neuropathy caused by mutations in the claudin-19 gene. CMT is now known to show extensive genetic heterogeneity, and disease-causing mutations have been identified in different genes with a wide range of biological functions (Bertorini et al., 2004; Shy, 2004). The human claudin-19 gene is located at 1p34-1, and, interestingly, a recent analysis of two unrelated families with dominant, intermediate CMT narrowed down the responsible locus at 1p34-p35 (Jordanova et al., 2003). The possible involvement of claudin-19 mutations in this type of CMT should be examined in the future.

In this study, we identified claudin-19 as a major constituent of the TJ strands of Schwann cells and generated Cld19<sup>-/-</sup> mice. Schwann cells are unique in terms of cell morphogenesis, cell–cell adhesion, cell motility, etc. Therefore, the Cld19<sup>-/-</sup> mice will provide a valuable resource for studying not only the molecular events governing saltatory conduction but also the molecular mechanism underlying these general cellular events.
Materials and methods

Antibodies

Rabbit anti-mouse claudin-11 pAb was raised and characterized previously (Morita et al., 1999b). Mouse anti-pig neuronfilament 200 pAb and rat anti-human myelin basic protein mAb were purchased from Sigma-Aldrich and Serotec Ltd., respectively. Rat anti–mouse E-cadherin mAb (ECD2) was provided by M. Takeichi (Center for Developmental Biology, Kobe University, Japan).

Anti–claudin-19 pAbs were raised in rabbits using keyhole limpet hemocyanin–conjugated peptide (MCL19 peptide) corresponding to the COOH-Terminal 19 amino acids of mouse claudin-19. Antiseras were affinity purified using beads coupled with MCL19 peptide before use. The specificity of these pAbs was confirmed by immunoblotting for GST fusion proteins with the COOH-Terminal cytoplasmic tails of mouse claudin-1 to -16 (Morita et al., 1999b; Kuchi-Sashin et al., 2002) and claudin-19.

Isolation of mouse claudin-19 cDNA and transfaction

Mouse claudin-19 cDNA was isolated by RTR-PCR. The first strand of cDNA was synthesized with the total RNA of a mouse kidney. PCR was performed with a primer set comprising a 5′ noncoding region primer (MCL19F, 5′-AGCTCCAGCTCGGCTACTTC-3′) and a 3′ coding region primer including a stop codon (MCL19R, 5′-TCAGACGGTACCTCTG-GCACGACATTGTA-3′). The nucleotide sequence of mouse claudin-19 cDNA that was obtained by RTR-PCR was confirmed by directly sequencing the amplified fragments (sequence data are available from GenBank/EMBL/DDB) under accession no. AF486651.

Mouse claudin-19 cDNA containing the entire coding region was subcloned into pcAG-neo expression vector (Furuse et al., 1998b) or pCI-neo expression vector (Promega). These expression vectors were transplanted into mouse L fibroblasts with lipofectamine plus (GIBCO-BRL). Cells were plated on 10-cm dishes in DME medium supplemented with 10% FCS for 48 h and selected by adding G418 at a final concentration of 500 mg/ml. At day 14 of culture, the G418-resistant colonies were removed, and L cells stably expressing mouse claudin-19 were screened by immunofluorescent staining with claudin-19 pAb.

Generation of Clδ19/- mice

Three overlapping clones encoding mouse claudin-19 were obtained by screening a 129/Sv genomic library. Using one of them, the targeting vector was constructed as shown in Fig. 3 A. The diethyliythiophenol A expression cassette (MC1pDT-A) was placed outside the 5′-arm of homology for negative selection. As shown in Fig. 3 A, four exons covered the whole ORF of claudin-19. Thus, this targeting vector was designed to delete all of these exons by replacing them with the pgk-neo cassette. J1 ES cells were electroporated with the targeting vector and selected for ~9 d in the presence of G418. The G418-resistant colonies were removed, and L cells stably expressing mouse claudin-19 were screened by immunoblotting.

Northern blotting

The total RNA was isolated from mouse brain and sciatic nerves according to a method developed previously (Chomczynski and Sacchi, 1987), and aliquots of total RNA (1 μg) were separated with 1.0% agarose-formaldehyde gel electrophoresis and were transferred onto nylon membranes (Roche Diagnostics). For other tissues, mouse multiple tissue membranes (Schleicher and Schuell) was used. Hybridization with a digoxigenin-labeled RNA probe, which was prepared from a PCR fragment amplified with primers MCL19F and MCL19R, was performed according to the manufacturer’s protocol (Roche Diagnostics). After extensive washing, the membranes were incubated with CDPD (TROPIX) and exposed to X-ray films.

Electron microscopy

For ultrathin section electron microscopy, the saphenous nerve, which runs just beneath the skin along the thigh, was used in order to preserve the ultrastructural integrity of axons covered well (Tsuchita and Ishikawa, 1980). Under ether anesthesia, the saphenous nerve was carefully exposed in 10-wk-old mice and was fixed in situ for 20 min with 2.5% glutaraldehyde and 2% formaldehyde in 0.1 M sodium cacodylate buffer, pH 7.2. Then, the nerve was removed together with the underlying muscle, and fixation was continued overnight at 4°C. In some samples, tannic acid was added to the fixative at a concentration of 0.1% to clearly visualize the kissing points of TJ. After being washed thoroughly with 0.1 M sodium cacodylate buffer, the nerve was cut into 1-mm-long segments, which were then postfixed in 1% OsO4 in the same buffer for 2 h on ice. The segments were washed with distilled water and stained en bloc with 0.5% uranyl acetate for 2 h. They were dehydrated in ethanol and embedded in Epon 812. Thin sections were cut, double stained with uranyl acetate and lead citrate, and then examined under an electron microscope (model 1200EX; JEOL) at an acceleration voltage of 100 kV.

For freeze-fracture electron microscopy, sciatic nerves from 10-wk-old mice were fixed in 2% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.2, for 3 h at RT, washed with 0.1 M sodium cacodylate buffer three times, immersed in 30% glycerol in 0.1 M sodium cacodylate buffer for 2 h, and then frozen in liquid nitrogen. Frozen samples were fractured at ~100°C and platinum shadowed unidirectionally at an angle of 45° in Balzers Freeze Etching System (model BAF060; Bal-Tec). The samples were then immersed in household bleach, and replicas floating off the samples were washed with distilled water. Replicas were picked up on formvar-filmed grids and examined with an electron microscope (model 1200EX, JEOL) at an acceleration voltage of 100 kV.

Behavioral tests

All behavioral tests were performed with male mice that were ~10–13 wk old at the start of the testing. Mice were housed in a room with a 12-h light/dark cycle (lights on at 7:00 a.m.) with access to food and water ad libitum. Behavioral testing was performed between 9:00 a.m. and 6:00 p.m. After the tests, all of the apparatus were cleaned with super hypochlorous water to deodorize the smell of mice.

Motor coordination and balance were assessed with the beam test and rotorod test. The beam (walking) test was adapted from Carter et al. (1999) by measuring the ability of mice to traverse a narrow beam to reach a dark box. The beams, with a rough painted surface, consisted of two different strips of iron (each measuring 100 cm long; one was 2.8 cm [thick bar] and the other was 1.1 cm [thin bar] in diameter) placed horizontally 50 cm above the bench surface. One session of five trials was performed using the 2.8-cm beam. Mice were then tested using the 1.1-cm beam. Mice were allowed up to 60 s to traverse each beam. The number of sideslips was recorded for each trial by the Image OF program (see the last paragraph of this section). The rotorod test using an accelerating rotorod (UGO Basile) was performed by placing a mouse on a rotating drum (3-cm diam) and by measuring the time each animal was able to maintain its balance on the rod. The speed of the rotorod was accelerated from 4 to 40 rpm over a 5-min period.

Locomotor activity was evaluated in the open field test. Each subject was placed in the center of the open field apparatus (40 × 40 × 30 cm;
Accuscan Instruments). Total distance traveled (cm), vertical activity (measuring by counting the number of photobeam interruptions), time spent in the center, and the break-beam counts for stereotyped behavior were recorded. Data were collected for 30 min. To obtain an index of sensorimotor gating [the percent prepulse inhibition], the prepulse inhibition test was performed as described previously (Mirzabekov et al. 2000). For this test, a startle reflex measurement system [O’Hara & Co.] was used. A test session began by placing a mouse in a plexiglas cylinder, where it was left undisturbed for 10 min. The duration of white noise that was used as the startle stimulus was 40 ms for all trial types. The startle response was recorded for 140 ms (measuring the response every 1 ms) starting with the onset of the prepulse stimulus. The background noise level in each chamber was 70 dB. The peak startle amplitude recorded during the 140-ms sampling window was used as the dependent variable. A test session consisted of six trial types [i.e., two types for startle stimulus-only trials, and four types for prepulse inhibition trials]. The intensity of the startle stimulus was 110 or 120 dB. The prepulse sound was presented 100 ms before the startle stimulus, and its intensity was 74 or 78 dB. Four combinations of prepulse and startle stimuli were used (74–110, 78–110, 74–120, and 78–120). Six blocks of the six trial types were presented in pseudorandom order such that each trial type was presented once within a block. The mean intertrial interval was 15 s (range, 10–20 s).

The application used for the beam test [Image OF] was based on the public domain National Institutes of Health’s Image program (developed by Wayne Rasband at the National Institute of Mental Health and available at http://rsb.info.nih.gov/nih-image/) and was modified for each test by Tsuyoshi Miyakawa [O’ Hara & Co.]. Statistical analysis was conducted using StatView [SAS Institute]. Data were analyzed using the two-tailed t test, two-way analysis of variance by between groups [ANOVA], or two-way repeated measures ANOVA, unless noted otherwise. Values in tables and graphs were expressed as the mean ± SEM.

Electrophysiological analyses
Conduction properties of myelinated axons were examined in sciatic-tibial nerves acutely isolated from 10-wk-old mice. Mice were anesthetized with sodium pentobarbital (Nembutal; 0.05 mg/g body weight, i.p.), and the nerves were connected to a stimulus electrode of the proximal end of the nerve were connected to a stimulus CAPs were monitored in real time on an oscilloscope and stored on the Ag-AgCl electrodes arranged in the moist recording chamber. The electrodes of the proximal end of the nerve were connected to a stimulus isolator [model a55301]; Nihon Kohden], and those of the distal end were connected to an AC-coupled amplifier [gain, 1 k; band-pass filter, 1.5 Hz-1 kHz; model AB610J; Nihon Kohden] to record the CAP. The conduction distance of the nerve [the distance from the negative stimulating to recording electrode] was 25 mm in all experiments. All recordings were performed at RT (26–28°C). Each electrical stimulus was applied for a 100-μs duration, and an interval of 1 s was maintained between each stimulus. CAPs were monitored in real time on an oscilloscope and stored on a PC computer using a digitizer [model DIGIDATA 1322A; Axon Instruments, Inc.] for offline analysis. The stored data were analyzed using pClamp9 [Axon Instruments, Inc.].

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