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Nerve conduction velocity measurements reveal the functional deficit in ceramide galactosyl transferase-deficient (cgt−/−) mice

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Abstract

Biochemical and ultrastructural studies of ceramide galactosyltransferase (CGT) in a CGT-deficient mouse line (cgt − / −) were complemented by nerve conduction velocity (NCV) measurements in motor nerves (sciatic nerve in the hind limbs) of wild type (wt) and cgt − / − mice. Stimulation and recording electrodes were adapted to the small size of developing mice during their myelination period. Motor NCVs in wt mice ranged between 16 and 26 m/s but in cgt − / − mice between 6 and 13 m/s, which corresponds to the conductance of unmyelinated peripheral nerves.

These electrophysiologic data provide additional functional parameters to the neuropathology of a new form of a dysmyelinosis.
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Keywords: Gene targeting; Galactocerebroside and sulfatide (cgt−/−) deficient mouse model, Myelin membrane lipid bilayer; Nerve conduction velocity; Dysmyelinosis

1. Introduction

Axons in the central nervous system (CNS) and the peripheral nervous system (PNS) are ensheathed by oligodendrocyte and Schwann cell plasma membrane processes which form the multilayered myelin membrane. Saltatory nerve conduction in central and peripheral axons is largely determined by the integrity of its myelin sheaths. The protein and lipids constituents of the highly ordered myelin membranes are synthesized and assembled by oligodendrocytes and Schwann cells, respectively, during the short myelination period, e.g. between p10 and p30 in mice. The lipid bilayer lends myelin the insulator function in the internodes of axons. It contains as major glycosphingolipid classes the myelin-specific galactocerebrosides (GalCs) and GalC-derived sulfatides (sGalCs). The key enzyme in their synthesis is ceramide galactosyltransferase (CGT) [1]. We have studied the function of GalC and sGalC in a CGT-deficient mouse line completely lacking GalC and sGalC in CNS and PNS myelin lipid bilayer. The CGT-deficient mouse mutant was generated by gene targeting by homologous recombination.

Biochemical analyses proved the complete loss of the CGT activity and consequently of cerebrosides and derived sulfatides [2]. Comparative complementary biophysical studies with lipid extracts of CNS and PNS myelin of wild type (wt) and cgt − / − mice revealed the perturbation of the tight packing of the lipid bilayer due to the loss of the two glycolipid classes, which is not sufficiently compensated [3].

Ultrastructurally, CNS myelin shows several abnormalities regarding the interaction between inner loop and axolemma, e.g. vacuolation, wrapping of two independent oligodendrocyte processes around the same axon. The multilayer myelin sheath, however, reveals the compacted appearance like those wrapping PNS axons in wt mice. Also, the periodicity of main and intermediate dense lines appears unconspicuous.

Cgt − / − mice developed a severe neurological phenotype with whole body tremor and frequent seizures and early

Abbreviations: CNS, central nervous system; PNS, peripheral nervous system; GalC, galactocerebroside; sGalC, GalC-derived sulfatides; CGT, ceramide galactosyltransferase; MBP, myelin basic protein; wt, wild type; MAG, myelin-associated glycoprotein; ES cell, embryonic stem cell; OMgp, oligodendrocyte myelin glycoprotein; PLP, proteolipid protein.

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death at the end of the myelination period (approx. 4–6 weeks).

These unexpected dichotomy of the severe functional neurological impairment on one hand and the regular morphology of peripheral myelin sheaths and the regular periodicity of CNS myelin led us to examine the electrophysiological properties of peripheral motor neuron axons. Nerve conduction velocity (NCV) of motor nerves in the hind limbs was measured.

Electrophysiological examination of peripheral nerves is able to assess the velocity of propagation of electrical stimuli through the peripheral nerve and thereby renders information on the functional integrity of the myelin sheath. The application of NCV measurements in humans was first demonstrated by Helmholtz in 1867 [4]. The first application in a clinical study was described by Hodes et al. in 1948 [5]. Since then, motor and later sensory NCV measurements have become a standard method of clinical electrophysiology in neurological diagnostics.

2. Material and methods

2.1. Generation of cgt−/− mouse line

The construction of the targeting vector has been described [6]. The SpeI linearized targeting vector was used for transfection by electroporation and G418 (300 mg/ml) and gancyclovir (0.2 mM) double selection of R1 cells (129sv-cp) F1 background. C57BL/6 blastocysts were injected with homologously recombined embryonic stem (ES) cell clones [7,8]. Chimeric male offsprings were intercrossed with C57BL/6 mice. Heterozygous offsprings were intercrossed and homozygous cgt − / − mice were obtained in the F2 generation.

2.2. Conduction velocity measurement

Nerve conduction velocity (NCV) of motor nerves in the hind limbs was measured in cohorts of five wild type and eight cgt − / − mice all aged 25 to 30 days. We stimulated the sciatic nerve distally at the foot joint and proximally close to the hip, using conventional neurophysiological testing equipment, a Medelec electromyograph, model MS92. The stimulation and recording electrodes were miniaturized and adapted to the small size of the wild type and cgt − / − mice.

Both wild type and cgt − / − mice were anaestheisized by intraperitoneal injection. Hind limbs were fixed to a cork plate by thin steel (insect) needles. A platinum wire was wrapped around the distal foot joint and connected to a grounding lead. Two sterilized pins were placed into the intrinsic foot muscles at a distance of about 5 mm, connected to different and non-different leads and the sciatic nerve stimulated using anatomical tweezers which were attached to a bipolar stimulating cable. All leads were than connected to the standard electrophysiological testing system. For stimulation, we used 0.2 ms rectangular electrical impulses with an intensity above the motor threshold of the peripheral (i.e. sciatic) nerve which was confirmed by observing the twitch of intrinsic foot muscles. The repetition rate was set at 1 Hz. Stimulation was performed distally at the proximal foot joint and as far proximally as possible on the free extremity. The distance between distal and proximal stimulation sites was measured with a ruler. We determined the distal motor latency as well as the motor NCV over the extremity as distance divided by latency. The amplitude of the compound muscle action potential (CMAP) was measured peak-to-peak. The measurements were performed in warm ambient room temperature (22 °C), no further temperature correction of NCV values was performed. The literature contains only sparse information on normal ranges of NCV values in the sciatic nerve of mice aged 25 to 30 days. Nagakawa et al. [9] found a value of 27.6 ± 6 m/s in the age group 4 to 8 weeks (28 to 56 days). Toyoshima et al. [10] quote a value of 18.1 ± 2.6 m/s in wt mice aged 15 to 19 days. Other authors examined much older wt mice demonstrating higher NCV values. Weiss et al. [11] found a value of 53 ± 8 m/s in wt mice aged 12 to 14 months, and Huizar et al. [12] published a value of 53 ± 9 m/s for wt mice aged 9 to 28 weeks.

3. Results

We have recently generated a CGT-deficient (cgt − / −) mouse line by gene targeting. The disruption of the gene led to a complete lack of oligodendrocyte- and Schwann cell-specific expression of the CGT gene as proven by Northern blot hybridization analysis and the complete loss of enzymatic activity in the galactosyl transferase assay in cgt − / − mutant mice. These data also assessed the presence of only one CGT gene copy responsible for galactosphingolipid synthesis [6,13].

Oligodendrocytes and Schwann cells of this cgt − / − null mutant mouse assemble a myelin membrane, the lipid bilayer of which completely lacks GalC and sGalC. This dramatic change in the plasmamembrane (myelin) lipid bilayer composition of the nullizygous mutant mice is not associated with alterations in the expression of mRNA of the predominant myelin proteins MBP, proteolipid protein (PLP), myelin-associated glycoprotein (MAG), and oligodendrocyte myelin glycoprotein (OMgp) of oligodendrocytes, and MBP and P0 of Schwann cells. The number of oligodendrocytes of wt and cgt − / − mice labeled with the MBP antisense RNA probe in situ hybridization was similar, indicating a normal development of the oligodendrocyte lineage in the cgt − / − mutant mouse.

The lipid extract of myelin from CNS and PNS of cgt − / − mice clearly showed the complete absence of GalC and

Table 1
Electrophysiological data of cgt − / − and wt mice

cgt − / −				wt			
DML [ms]	PML [ms]	NCV [m/s]	Ampl [mV]	DML [ms]	PML [ms]	NCV [m/s]	Ampl [mV]
1.8	2.9	13	1.5	1.2	2.1	16	3.0
1.5	2.8	12	1.5	1.1	2.2	16	6.0
1.2	2.7	12	1.0	1.0	1.8	26	5.5
1.3	2.9	11	2.0	1.2	2.3	20	4.5
2.0	4.9	6	1.5	2.4	2.8	23	4.0
2.7	4.9	7	2.0				
3.5	5.9	6	0.4				
3.2	5.0	8	3.0				

sGalC. Two new glycolipid bands were identified as glucocerebrosides. These glucocerebrosides and their sulfate esters form however minor components in the lipid pattern unable to compensate the loss of the bulk galactosphingolipids. Although a minor part of the ceramide component specific for GalC is utilized for this reduced synthesis of glucocerebrosides, the hydrophobic core of the bilayer of the myelin membrane and the carbohydrate polar head group and charge pattern of the membrane surface contributed by GalC and sGalC in the cgt − / − mouse remain seriously altered [2,6].

Homozygous mice had reached only about half the size of the wt siblings. cgt − / − mice developed a whole body tremor after day 10–12 accompanied by an increasing loss of locomotor activity and a very conspicuous gait pattern. Their neurological phenotype is furthermore characterized by severe behavioral deficits: minute motor activity in the open field test, failure to perform the horizontal wire test due to an increasing weakness of their front and hind legs, which develops to a severe paralysis around day 21. Most of the homozygous mice die shortly after the myelination period (>p30). Heterozygous mice show no neurological symptoms [2,6].

Electrophysiology was carried out with wt and cgt − / − mice between p25 and p30, which is toward the end of the myelination period. In wt mice ($n=5$) we measured motor NCV values between 16 and 26 m/s (median 20 m/s). These values were within normal limits indicating normal functions of motor nerves and its myelin sheath. The NCV values for the wt mice of this age group correspond well to the data of other authors [9,10]. However, in cgt − / − mice ($n=8$), motor NCV values between 6 and 13 m/s (median 9.5 m/s) were measured. Table 1 gives the values for distal motor latency, NCV, and amplitude of the reaction potential for all measurements (Table 1). The statistical comparison of the two groups (Mann–Whitney rank sum test) revealed a significant group difference ($P=0.002$) (Fig. 1).

Likewise, the amplitude of the CMAP ranged from 3.0 to 6.0 mV (median 4.5 mV) in the wt mice and from 0.4 mV to 3.0 mV (median 1.5 mV) in the cgt − / − mice. These values also were statistically significantly different ($P=0.002$, Mann–Whitney rank sum test) (Fig. 2).

The results obtained from the cgt − / − group correspond well to those of dysfunctional motor fibers in severely demyelinating diseases of peripheral nerves, e.g. severe

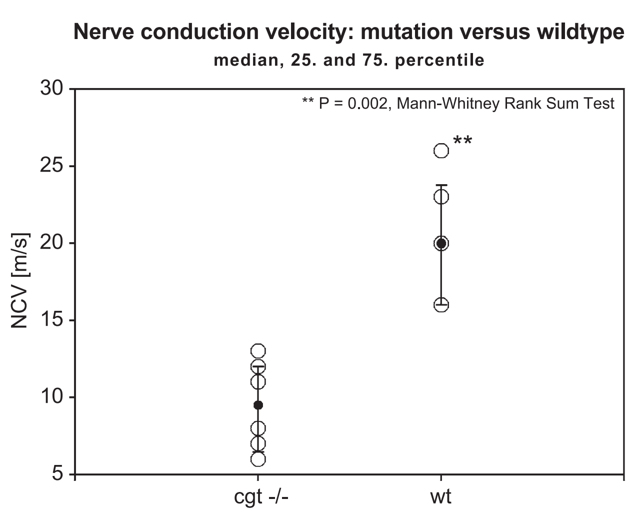

Fig. 1. Group comparison of nerve conduction velocities (in m/s) of wild type (wt) and cgt − / − mice. Median, 25th, and 75th percentile.

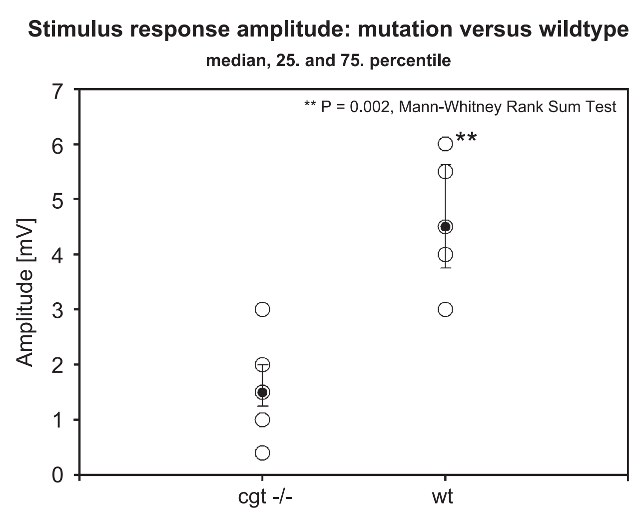

Fig. 2. Group comparison of compound action potentials (CMAP) (in mV) of wild type (wt) and cgt − / − mice. Median, 25th and 75th percentile.

Guillain–Barré syndrome (GBS), chronic inflammatory demyelinating polyneuropathy (CIDP) or hereditary polyneuropathies (HMSN type I). We attributed the significant amplitude reduction in the cgt − / − mice to the atrophy of the target muscles.

4. Discussion

Spontaneous or genetically modified mouse mutants with deficiencies of specific components of the myelin sheath are powerful tools in studies of the structure–function relationship of the constituents of myelin sheath of axons of the CNS and PNS. Most genetic studies focused so far on the structure–function relation of the main myelin proteins: the peripheral membrane protein MBP, the integral PLP and MAG, synthesized by oligodendrocytes and P0 by Schwann cells (Fig. 3).

Our knowledge about the structure–function relationship of myelin proteins has rapidly emerged particularly from the natural mutants, e.g. the MBP mutant shiverer [14–16], the myelin deficient rat (md-rat) [17], and genetically manipulated null-allelic mouse mutants, e.g. of PLP (plp − / −) [17,18], the MAG (mag − / −) [19], P0 deficient mouse [14,20] and the double mutantmouse lines (plp − / − , mbp − / −) [20] and (plp − / − mag − / −) [22], generated by homologous recombination and intercrossing of the single gene deletion mutants.

The relevance of the molecular assembly of the myelin lipid bilayer, however, particularly that of the oligodendrocyte specific complex glycosphingolipids, has escaped attention for a long time, until the key enzyme CGT in the biosynthesis of galactocerebrosides and sulfatides was isolated and characterized [1,21], its cDNA cloned and its gene structure elucidated [13,23].

This in vivo analysis of the function of the GalC and sGalC constituents of the lipid bilayer of the myelin membrane of mammalian CNS and PNS is, to our knowledge, the first example which demonstrates the great potential of gene targeting by homologous recombination in studies addressing functional aspects of lipid structures in eukaryotic biological membranes.

The results reported here demonstrate that gene ablation of CGT in the mouse affects only the assembly of the myelin sheath but not the axon. Our electromyographic measurements of the nerve conductance convincingly complement the genetic, biochemical and morphological parameters of the new phenotype arising from the loss of structure and loss of function of the ceramide galactosyltransferase (CGT). As demonstrated here, CGT-deficiency is a new type of a severe and early lethal dysmyelinosis. Its molecular basis is a perturbed lipid bilayer structure of myelin, the integrity of which is pivotal for the insulator function of the myelin membrane structure of CNS and PNS for saltatory conduction of axons although the periodicity, thickness and compaction of the myelin sheath of peripheral nerves are indiscriminant from wild type myelin sheaths, as documented in Fig. 3a. Myelin of the CNS shows several abnormal features which have been discussed earlier in a comprehensive electromicroscopic study in this laboratory (Fig. 3b) [24].

From a molecular view, the following structural features render in a concerted cooperativity the lipid bilayer of myelin impermeable for ions. Galactosphingolipids GalC

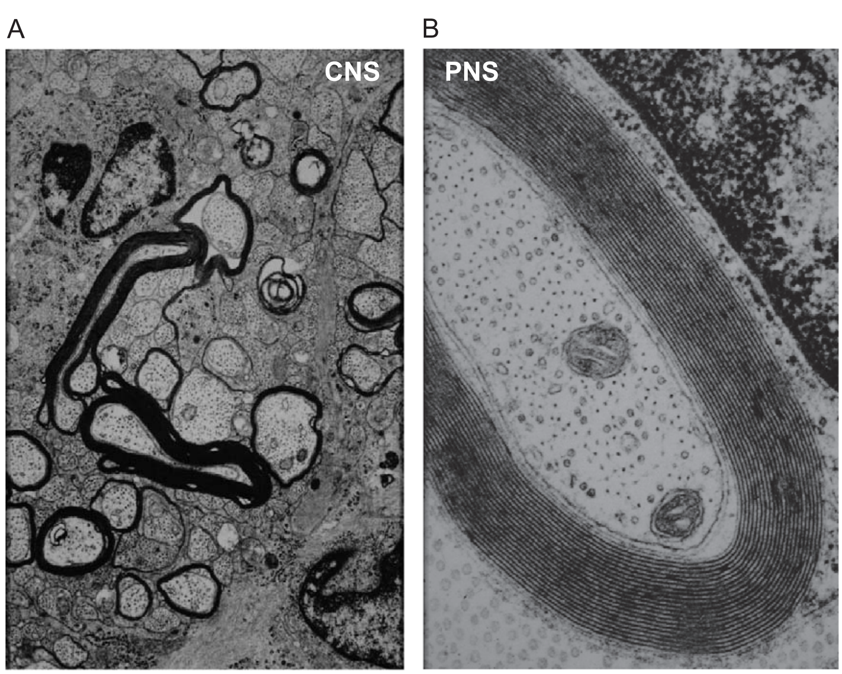

Fig. 3. Electronmicroscopy of (A) of central nervous system (CNS), (B) myelinated axon of peripheral nerve (PNS).

and sGalC contribute to the structure and stability of the myelin lipid bilayer by

(i) The all-trans configuration of their long chain "(C18–C26) mostly saturated and α-hydroxylated fatty acyl residues cause the tight packing of the hydrophobic core domain.
(ii) The polar head groups of GalC and sGalC contribute significantly to the inner and outer membrane surfaces. They provide a polyanionic membrane surface array for strong ionic interactions at the extracytoplasmic surface which stabilizes the tight compaction of the multilayer membrane system.
(iii) At the interphase of the membrane, the abundant galactosphingolipids form a dense network of H-bonding, enhancing the tight packing of sphingolipids.

The cgt −/− mouse lacks GalC and sGalC in the lipid bilayer of myelin. Cholesterol and highly unsaturated phospholipids become the main constituents for the assembly of the lipid bilayer. The dramatically altered electrophysiology following the structural perturbation in the lipid bilayer of CNS and PNS myelin membrane results from the loss of insulating properties of the internodes and blocked saltatory conduction. The saltatory conduction of myelinated axons in homozygous cgt −/− mice is turned into the propagation of the action potential by continuous depolarization of the axon, due to the complete loss of the insulating properties of the multilayered internodal membrane system. Ectopic discharges along the axons of CNS and PNS block saltatory conduction cause whole body tremor and seizures which become fatal at the end of the myelination period around p30 in the mouse. This dysmyelinating phenotype is comparable with that observed in chronic peripheral neuropathies in humans.

In summary, our results underline the high performance of NCV-measurements adapted in a miniaturized configuration to young wt and cgt −/− mice.

The facile and reproducible method applied here for quantifying the conduction velocity of axons of wt and cgt −/− mice reveals that NCV of axons in the mutant no longer represents saltatory conductance. We conclude that this is due to the impaired insulator function of the GalC- and sGalC-deficient myelin membrane. Their absence in the lipid bilayer induces the structural perturbation of the highly ordered myelin membrane lipid bilayer as described before [3]. The method enables us to assess the functional deficits of the myelin sheath in CGT-deficient mice, a new form of a dysmyelinosis that affects both CNS and PNS.

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