CMT1X phenotypes represent loss of GJB1 gene function

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Charcot–Marie–Tooth disease type 1 (CMT1) is caused by mutations in different genes expressed by myelinating Schwann cells. Mutations in gap junction beta 1 (GJB1) cause CMT1X, the second most frequent form of CMT1, so named because GJB1 is located on the X chromosome. GJB1 encodes the gap junction protein connexin 32 (Cx32), one of approximately 20 mammalian connexins. In myelinating Schwann cells, Cx32 probably forms so-called “reflexive” gap junctions (between layers of the same cell) in noncompact myelin, found in paranodal loops and Schmidt–Lanterman incisures. Cx32 mutants may disrupt the diffusion of small molecules and ions across the peripheral nervous system (PNS) myelin sheath.

Patients with missense mutations (amino acid substitutions) in other forms of CMT1 have variable phenotypes, depending on the particular mutation in the causal gene. By comparison, in CMT1X, the genotype–phenotype correlations are uncertain. Generally, women are less disabled than men with corresponding GJB1 mutations because of “ionization” effects. Some GJB1 mutations are associated with a variety of “CNS phenotypes,” but the neuropathy caused by these mutations has appeared to be similar to other cases of CMT1X. Nevertheless, there are reports that particular GJB1 mutations are associated with severe neuropathy. In this current study, we analyze 73 male patients with CMT1X with 28 different GJB1 mutations and find that they all had a similar phenotype to that caused by a complete deletion of the GJB1 gene. Length-dependent axonal degeneration, but not demyelination, appears to best account for the clinical disability of CMT1X.

Methods. Patient ascertainment and evaluation. Patients were evaluated at the University of Western Ontario and Wayne State University CMT programs. Evaluations consisted of a neurologic history and examination and nerve conduction studies. Genetic testing through Canadian molecular diagnostic laboratory labs or Athena Laboratories was performed before or after the visit to document GJB1 mutations. All studies were approved by institutional review boards, and appropriate consents were obtained by the treating physicians.

Evaluation of CMT. The severity of the peripheral neuropathy was evaluated in all patients by the CMT Neuropathy Score (CMTNS), a validated measurement of disability in CMT pa-
patients.¹⁶ The CMTNS is a composite score based on the history of symptoms (total possible points = 12), the neurologic examination (total possible points = 16), and clinical neurophysiology (total possible points = 8); the maximum score is 36. Patients with mild, intermediate, and severe disability typically have a CMTNS between 1 and 10, 11 to 20, and 21 or greater.¹⁶ To quantify disability in patients who had been followed for years but had not undergone repeat nerve conduction studies (NCSs), we also devised and performed the CMT Symptom Score (CMTSS), which represents the first component of the CMTNS and includes the sensory symptoms and the motor symptoms in the legs and arms; therefore, the CMTSS reflects the patient’s own perception of his/her sensory disturbance and impairment of motor functions. The patients’ symptoms were evaluated retrospectively and were scored as the CMTSS for each decade (e.g., age 10, 20, 30, 40 years, etc.).

Clinical electrophysiology. NCSs were performed by standard techniques utilizing either Nicolet Viking or Synergy (Oxford Medical Systems) electromyography (EMG) systems. Temperature was maintained at 34 °C. Surface electrodes were used in all studies. Sensory conduction studies were performed using antidromic techniques. Nerve conduction velocities were calculated by standard techniques.

Motor unit number estimates (MUNE) were obtained for ulnar-innervated hypothenar muscles and musculocutaneous-innervated biceps brachii/brachialis muscles using the decomposition-enhanced spike-triggered averaging (STA) method. This method allows multiple motor units (MUs) to be collected from a single contraction by using EMG decomposition algorithms.¹⁷ As previously described,¹⁸ a compound motor action potential (CMPA) was obtained for each muscle by supramaximal stimulation of the appropriate nerve using standard electrode placement for motor studies. With the surface electrodes remaining in place and connected to one channel of the amplifier, a concentric needle electrode connected to a second amplifier channel was placed in the muscle. Subjects then performed a mild to moderate contraction with the needle was maintained in a stable position, and both the needle- and surface-detected EMG signal were collected and saved. The decomposition algorithm then extracted the individual needle detected MU potentials (MUPs) and their corresponding firing times from the composite EMG signal. These firing times served as triggering sources to extract the corresponding surface-detected MUPs (S-MUPs) using STA.¹² Only MU trains with adequate numbers of detected needle MUPs were accepted and used to derive the CMTNS. The average of the negative peak amplitude of the sample of S-MUPs was determined and divided into the negative peak amplitude of CMPA to determine the MUNE. The decomposition technique has been compared to the conventional STA technique and has been shown to reliably identify multiple MUs within a single contraction.¹² It allows for sampling of many more units than conventional STA.¹²

Statistical analysis. Analyses of variance (ANOVs) were calculated to determine relationships between patient age and disability as measured by CMTNS, CMTES, or CMTSS. Decades of life formed the independent measure, and because a patient could contribute more than once, (partial) blocking on ID was used to take this into account. Significant ANOVAs were followed up with Tukey tests. To examine the impact of specific mutations on the CMTNS, CMTES, and CMTSS at each decade of life, we calculated means at each decade (with the samples split according to the different components in each of the scores) and tested them with ANOVAs, adjusted using Bonferroni correction to account for the substantial increase in type I error rate. These tests were repeated on the medians (as opposed to means) with nonparametric tests. Patients with adequate numbers of detected needle MUPs were included in the analysis. Significant ANOVAs were followed up with Bonferroni correction to account for the increase in type I error due to multiple tests. Finally, correlations were calculated between disability and specific mutation and between motor unit numbers and specific disability and then tested for significance.

Results. Description of cohort. Although more than 260 GJB1 mutations have been reported to cause CMT1X (http://www.molgen.ua.ac.be/CMTMutations/DataSource/MutByGene.cfm), it is not known whether the severity of neuropathy is related to the particular GJB1 mutation. To address this issue, we evaluated 73 male patients with CMT1X in whom the GJB1 mutation had been determined by sequencing of leukocyte-derived genomic DNA. Affected women were not considered because varying degrees of X inactivation generate variable penetrance, and hence their inclusion would confound the analysis. The patients were from 38 different pedigrees, had 28 distinct mutations, and ranged from ages 9 to 76. Two patients had a complete deletion of the GJB1 gene. The remaining patients had assorted missense or nonsense (resulting in the premature truncation of protein translation) mutations in the GJB1 gene that encode each of the intracellular, extracellular, and transmembrane domains of the Cx32 protein, with the exception of the third transmembrane domain. The patients and their mutations are summarized in table E-1 on the Neurology Web site (www.neurology.org), and the locations of the mutations in Cx32 are illustrated in figure E-1. At the time of the first visit, we measured disability in each patient using the CMTNS. CMTNS values ranged from 1 (minimal disability) to 29 (severe disability requiring the use of a wheelchair). The mean CMTNS of all patients was 15.5 (SD 7.1), representing an intermediate severity.¹⁶

CMTNS, CMTES, and CMTSS increase with age in all mutations. It has long been recognized that clinical disability increases with age in people who have CMT.¹¹,¹² To analyze disability in our patients with CMT1X quantitatively, we correlated the CMTNS scores from their initial study with their age at that time: a cross-sectional evaluation of multiple patients with different mutations at single time points, grouped per decade. As shown in figure 1, the CMTNS increased an average of 2.89 points/decade (F₇,₂₃ = 10.26, p < 0.01); the mean CMTNS was 3, 10, 11, 16, 22, 20, 20, and 23 for the first through the eighth decades. To determine whether weakness or sensory loss was particularly responsible for increases in the CMTNS, we correlated the individual motor and sensory...
components of the CMTNS, taken from the neurology examination, with the patients' age. All motor and sensory examination components increased proportionally with age, suggesting that the progressive loss of strength and sensation contributed equally to the higher CMTNS in older patients (table).

To permit the longitudinal analysis of individual patients, we used both the CMTES and the CMTSS to assess their disability retrospectively, from examinations antedating the prospective study. The CMTSS was used for all patients as it was based on their recollection of symptoms. The CMTES were used only if one of the investigators had personally evaluated and examined the patient in prior visits. In individual patients, both the CMTES (2.61 points/decade; \( F_{7,51} = 17.51, p < 0.01 \)) and the CMTSS (0.95 points/decade; \( F_{7,39} = 57.42, p < 0.01 \)) increased with age in a progressive fashion. The mean CMTES and mean CMTSS also demonstrate the increase in scores per decade (figure 2).

Disability does not correlate with particular mutations. To determine whether particular mutations caused a more severe neuropathy, we correlated the CMTNS from each patient’s initial visit, independent of age, with the various mutations causing their neuropathy. We found no correlation between disease severity and specific mutations (\( r = 0.07; \text{NS} \)). There was considerable variability of the CMTNS among many patients carrying the same mutation. For example, the CMTNS for patients with Ser26Leu mutations ranged from 7 to 27, Trp33Ser from 5 to 15, and Met34Thr from 8 to 19. Thus, disability at initial visit, independent of the patient’s age, did not correlate with specific mutations.

Given that CMT1X worsens with age, we next wished to determine whether patients of comparable age had similar scores regardless of their mutation. To address this issue, we compared the CMTNS, CMTES, and CMTSS for the various mutations in patients of the same or similar age and investigated whether any of the scores progressed more rapidly with particular mutations. Because of the very large number of comparisons (e.g., \( 8 \times 3 \times 2 = 48 \) contrasts), a Bonferroni correction was used for each set of tests, resulting in only a sixfold increase in type I error rate. As shown in figure 3, virtually all patients of a given age had similar scores regardless of their mutation. Scores were similar, although not identical for all patients of a given phenotype and age (see error bars in figure 3), consistent with the fact that there remain certain epigenetic factors operative in individual patients that remain to be identified. Nevertheless, there were no mutations associated with markedly more severe neuropathy. Patients with two mutations—Glu208Lys and Ser26Leu—appeared to reach their maximum disability level approximately a decade earlier than patients with other mutations, but did not exceed them at older ages (figure 3). However, disability caused by these mutations was not statistically different from patients with other mutations whether measured by the CMTNS, CMTSS, or CMTES.

Deletions, missense, and nonsense mutations cause similar phenotypes. Missense and nonsense mutations in MPZ and PMP22 often cause more severe disability than do deletions, presumably because the resulting mutant protein gains an abnormal function.\(^1,2\)\(^1\) To determine whether missense or nonsense GJB1 mutations cause a “toxic” gain of function, we compared the CMTNS scores of patients with missense and nonsense mutations with those of patients with a GJB1 deletion. As is shown in figure 3, the differences between scores of patients with deletion were not significantly different from those of patients with either missense or nonsense mutations. Thus, we infer that in male patients with CMT1X, missense or nonsense mutations cause a simple loss of function, at least in terms of clinical impairment.

Length-dependent axonal loss correlates with disability. Previous studies in mice have suggested that motor dysfunction in CMT1X is caused by length-dependent axonal degeneration,\(^2\)\(^2\) even though Cx32 gap junction protein is expressed in myelinating Schwann cells and not in neurons.\(^2\)\(^3\) To address this question, we further analyzed results from neurophysiologic studies performed on the upper extremities of our patients. Ulnar motor nerve conduction velocities (MNCVs) between the wrist and elbow were predominantly in the “intermediate range” between 30 and 50 m/s (figure 4A). Occasional patients had forearm ulnar MNCV of <30 m/s; these were always associated...
with markedly reduced CMAP amplitudes. Ulnar MNCVs around the elbow were even more strongly restricted to values between 30 and 50 m/s. There was only a mild correlation between the ulnar MNCV below the elbow and the age of the patient ($r = 0.21$) but not between the ulnar MNCV around the elbow and age. Similarly, the median nerve MNCV also did not correlate with age. However, a strong correlation was observed between the ulnar ($r = 0.405$) and median ($r = 0.414$) CMAP amplitudes and age. There were no correlations observed between particular mutations and either ulnar or median MNCV or CMAP amplitudes. These results suggest that in CMT1X, as was shown in CMT1A,$^{24}$ conduction velocity slowing does not clearly progress with advancing age, but axonal loss does, as measured by CMAP amplitude. To further address the question of distal axonal loss, we performed MUNE in a distal (hypothenar) and a proximal (biceps brachii/brachialis) upper limb muscle group and correlated these findings with the corresponding CMTNS. Normal values for both the adductor digiti minimi (ADM) and biceps muscles are $200.19$ The MUNE in CMT1X patients demonstrated greater loss of MUs in hypothenar (mean $20.2$ as compared with the biceps (mean $173.4$). The lower MUNE values for the ADM correlated with higher CMTNS values ($r = 0.60; p < 0.01$) (figure 5A). Correlation between the biceps MUNE and the CMTNS was much weaker ($r = 0.165; NS$) (figure 5B). Although average surface-detected MU potential sizes (AS-MUP) were markedly increased (ADM mean $357.5$ $250.3$ $V$; musculocutaneous mean $85.92$ $48.24$ $V$) compared with normal (ADM mean $100$ $V$; musculocutaneous $60$ $V$), neither AS-MUP significantly increased with age (data not shown). These data demonstrates that in
CMT1X, similar to that seen in CMT1A, functional disability (as measured by CMTNS) correlates with motor axonal loss (as measured by MUNE). The data also imply that collateral reinnervation is unable to adequately compensate for axonal loss (i.e., MUNEs reduce with age but AS-MUP does not).

Discussion. We evaluated 73 male patients with 28 distinct GJB1 mutations and found a similar age-related phenotype in all of them. Several of the mutations have not been previously reported: Trp24Arg, Glu119X, Cys173Phe, Glu186Val, Cys201Gly, and Pro267fs (frameshift mutation) (table E-1; figure E-1). Disability was relatively mild (CMTNS of 10) during the first two decades but continued to progress, so that it was severe (CMTNS typically 21 to 23) after age 60, independent of the particular mutation. A similar phenotype was identified in patients in whom GJB1 was deleted. Thus, these data indicate that at least as far as the neuropathy is concerned, most GJB1 mutations cause a simple loss of function.

Our results contrast with preliminary analyses carried out by both ourselves and others. Deletions, frameshifts, and premature truncations appeared to be more deleterious than missense mutations in one series of 29 families with 20 different GJB1 mutations.23,24 A second group25 examined 53 male patients from 13 families and reported that missense mutations in the second transmembrane domain or adjacent cytoplasmic loop may have caused milder CMT1X. In our current study, however, we evaluated five different mutations within the second transmembrane domain or subsequent cytoplasmic loop and found no significant difference from other mutations. It was only by scoring patients at each decade of life through the use of the CMTNS and its variants that we were able to determine that no particular mutation appeared more severe than a deletion of the entire protein. An additional group14 evaluated 41 men with 27 different mutations and postulated that patients with an onset of symptoms prior to age 10 were more likely to have mutations that disrupted Cx32 function in transfected cells. Previously, these authors suggested that the R22X mutation in particular caused a more severe phenotype.13 However, both articles reported that functional disability was mild or moderate in all their male patients, suggesting that any differences in severity in their patients must have been relatively small.13,14 Although we cannot exclude some phenotypic variability between our patients and those previously reported with the same or similar mutations, we interpret our data to suggest that most, if not all, CMT1X mutations cause disability by a loss of normal Cx32 function. Whether Phe235Cys is an exception remains to be determined, as one person with this mutation developed a severe phenotype as a young girl,26,27 whereas our patients with this same mutation have typical CMT1X.

Initially, it had appeared that there may be a relationship between clinical severity and the trafficking of Cx32 mutant protein, as mutants that do not reach the cell surface seemed to be associated with a more severe phenotype.28 However, this correlation does not hold true for the 27 Cx32 mutants reported here, as 4 of them are intracellularly retained in transfected cells (Met34Thr, Glu186Lys, Glu208Lys, Try211X), and 11 appear to reach the cell membrane (Trp3Ser, Arg15Gln, Arg22Gln, Ser26Leu, Gln80Arg, Val95Met, Glu102Gly, Arg107Trp, Leu156Arg, Pro168Ser, Phe235Cys).27-33 Moreover, eight of these mutations (Arg15Gln, Arg22Gln, Ser26Leu, Met34Thr, Gln80Arg, Glu102Gly, Pro172Ser, Cys235Phe) make functional channels in oocytes or transfected mammalian cells, although most have abnormal electrophysiologic characteristics.27,29,33-36 Thus, the ability of Cx32 mutants to form functional channels by in vitro analysis does not correlate with a lesser degree of neuropathy.

Our finding that disability in CMT1X correlates with a decrease in MUNE in the upper extremity...
provides further support for the concept that clinical disability in CMT1 is more strongly related to axonal loss than to demyelination. The cause of axonal loss in CMT1X, and indeed in all kinds of demyelinating diseases, remains to be determined. Although demyelination is less prominent in CMT1X than in other kinds of CMT1, it is the first pathologic finding in GJB1/Cx32-null mice. If loss of Cx32 function in myelinating Schwann cells causes disability associated with the neuropathy, then CMT1X in male patients is theoretically amenable to gene replacement approaches. In support of this concept is the observation that the development of demyelination in GJB1-null mice is largely prevented by expressing the human GJB1 gene exclusively in myelinating Schwann cells.

Our results have implications for treatment strategies in CMT1X. The cross-sectional analysis in our cohort suggests that disability progresses at a rate of about 3 CMTNS points/decade in males, with similar rates of progression in the CMTES and CMTSS components. Although we recognize that these calculations need to be confirmed in prospective studies, they suggest that CMT1X patient disability in men progresses at a rate of about 0.3 CMTNS point/year. Given that interinvestigator variability in the CMTNS is about 1 point, clinical trials in CMT1X will require at least 5 years of follow-up to provide significant results (assuming that the therapeutic agent halts progression of the neuropathy).

In contrast to GJB1 mutations, there is abundant evidence that some PMP22 and MPZ mutations cause a more severe neuropathy (called Dejerine Sottas neuropathy or congenital hypomyelinating neuropathy) than do other mutations in the same genes, especially those that are thought to cause loss of function. Cx32, MPZ, and PMP22 are all integral membrane proteins so that their synthesis and trafficking are predicted to be similar. Some Cx32 mutants are as well as the majority of dominant PMP22 mutants and at least some dominant MPZ mutants are retained in the endoplasmic reticulum and are likely degraded by the endoplasmic reticulum-associated degradation system.

Why, then, should MPZ and PMP22 mutants, and not Cx32 mutants, cause an abnormal gain of function in myelinating Schwann cells? There are several, nonmutually exclusive, considerations. First, in contrast to MPZ and PMP22, Cx32 is not glycosylated and hence should not interact with the endoplasmic reticulum machinery that retains improperly glycosylated proteins. In addition, unlike some MPZ mutants and most PMP22 mutants, Cx32 mutants do not generate protein aggregates. It remains to be determined whether endoplasmic reticulum–retained Cx32 mutants induce an unfolded protein response, as do some MPZ mutants. Another factor may be that Schwann cells express much more MPZ and PMP22 than Cx32, which is undetectable on Coomassie blue–stained polyacrylamide gels of PNS myelin (S.S. Scherer, personal observation). Thus, myelinating Schwann cells may be able to handle a low level of misfolded Cx32. Proteolipid protein (PLP) provides a precedent for this possibility, as PLP constitutes more than 50% of CNS myelin protein but <1% of PNS myelin protein. Many PLP1 mutations cause severe CNS dysmyelination; these PLP mutants are retained in the endoplasmic reticulum and activate the unfolded protein response. However, these same mutations do not cause peripheral neuropathy even though they are retained in the endoplasmic reticulum of transfected Schwann cells. An additional consideration is that Schwann cells may be resistant to the toxic effects of mutant Cx32 and PLP protein, at least more resistant than oligodendrocytes.

A final possibility would be that 1 of the other 20 mammalian connexins might replace mutant Cx32 in PNS myelin gap junctions, mitigating the effects of at least some Cx32 mutations. However, this hypothesis is not supported by current data. Connexin 29 (Cx29) is the only other connexin that has been identified in PNS myelin. Although Cx29 is expressed by myelinating Schwann cells and localizes with Cx32 by immunohistochemistry, attempts have failed to demonstrate that the two directly interact to form heterotypic gap junctions (S.S. Scherer, unpublished observations). Therefore, there is no current evidence to support a role for an additional connexin in the pathogenesis of CMT1X.

References


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