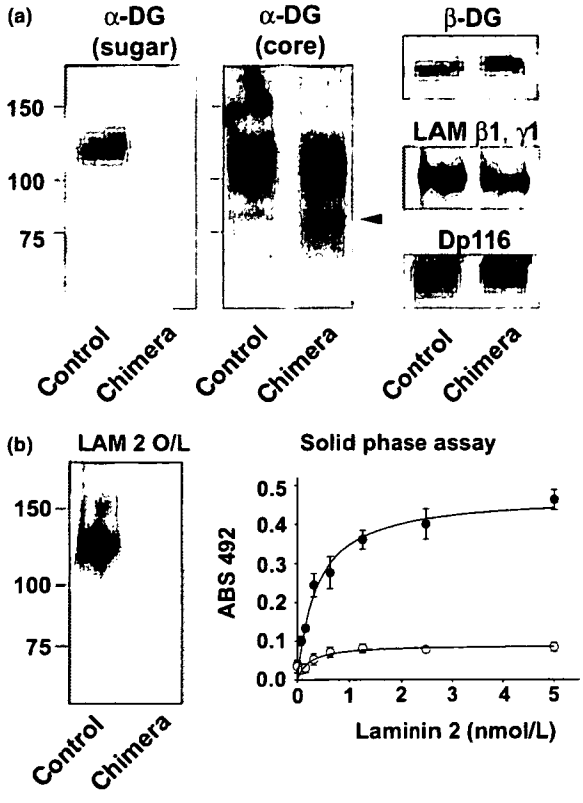

Fig. 4 Hypoglycosylation and reduced laminin-binding activity of α-DG in the peripheral nerve of fukutin-deficient chimeric mice. (a) Immunoblot analysis revealed severe reduction of immunoreactivity of 120 kD α-DG in the chimeric sciatic nerve when probed with IIH6, an antibody against sugar chain moiety of α-DG. In contrast, antibody against core protein of α-DG demonstrated only slight reduction of 120 kD α-DG in the chimeric sciatic nerve. Interestingly, an additional band of 80 kD was observed (arrowhead), suggesting that considerable fraction of α-DG was hypoglycosylated in the chimeric sciatic nerve. The expression of β-DG, laminin β1, γ1 or Dp116 was not altered. (b) Blot overlay assay demonstrated that the binding activity of α-DG to laminin-2 was greatly reduced in the chimeric sciatic nerve (left). Solid-phase binding assay demonstrated that the binding activity of α-DG to laminin-2 was quantitatively decreased in the chimeric sciatic nerve (right). LAM, laminin; O/L, overlay assay.

To see if the deficiency of fukutin affects the expression of α-DG at NMJ, we performed double immunostaining analysis using antibodies against α-DG. The immunoreactivity of α-DG with antibody against core protein of α-DG (AP1530) was localized to the NMJ as well as extrajunctional sarcolemma in both control and chimeric mice (Fig. 6a). When α-DG was probed with antibody against sugar chain moiety of α-DG (IIH6), the immunostaining of the NMJ as well as extrajunctional sarcolemma was severely reduced in the chimeric mice, indicating aberrant glycosylation of α-DG not only at the extrajunctional sarcolemma but also NMJ (Fig. 6a).

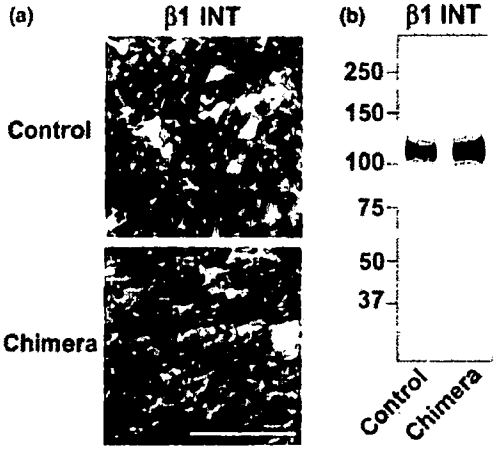

Fig. 5 Preservation of β1-integrin in the peripheral nerve of fukutin-deficient chimeric mice. Immunohistochemical analysis revealed that β1-integrin was equally localized to the outermost layer of myelin sheath in both control and chimeric mice (a). Immunoblot analysis confirmed that the expression of β1-integrin was indistinguishable between the control and chimeric mice (b). Scale bar indicates 50 μm. INT, integrin.

Quadriceps muscle was double-stained with α-BTX and fasciculin 2, which specifically labels acetylcholinesterase (AChE) (Peng *et al.* 1999). In the control mice, the labeling with fasciculin 2 was co-localized with α-BTX at the NMJ (Fig. 7a). In the chimeric mice, in contrast, the fasciculin 2 staining of NMJ was much smaller than control, although it was co-localized with α-BTX (Fig. 7a). Quadriceps muscle was also double-stained with α-BTX and antibody against synaptophysin, a marker for pre-synaptic nerve terminal. In the control mice, the immunoreactivity of synaptophysin was co-localized with α-BTX at the NMJ (Fig. 7b). In the chimeric mice, in contrast, the synaptophysin staining of NMJ was much smaller than control, although it was co-localized with α-BTX (Fig. 7b).

Agrin is not stably expressed and agrin-binding activity of α-DG is decreased at the neuromuscular junction in fukutin-deficient chimeric mice

To investigate the mechanism of defective NMJ formation in the fukutin-deficient chimeric mice, we assessed the expression of agrin, a key molecule involved in the clustering of AChR. Immunofluorescent analysis demonstrated that agrin was expressed at the sarcolemma in the control mice, whereas its expression was significantly reduced in the chimeric mice (Fig. 8). Furthermore, agrin was enriched at the NMJ in the control mice, where it was co-localized with AChR as probed by α-BTX (Fig. 8). In sharp contrast, agrin was not clearly detected at the NMJ in the chimeric mice (Fig. 8). Apart from agrin, laminin has also been implicated in the clustering of AChR at the NMJ (Sugiyama *et al.* 1997). Therefore, we studied the expression of laminin at the

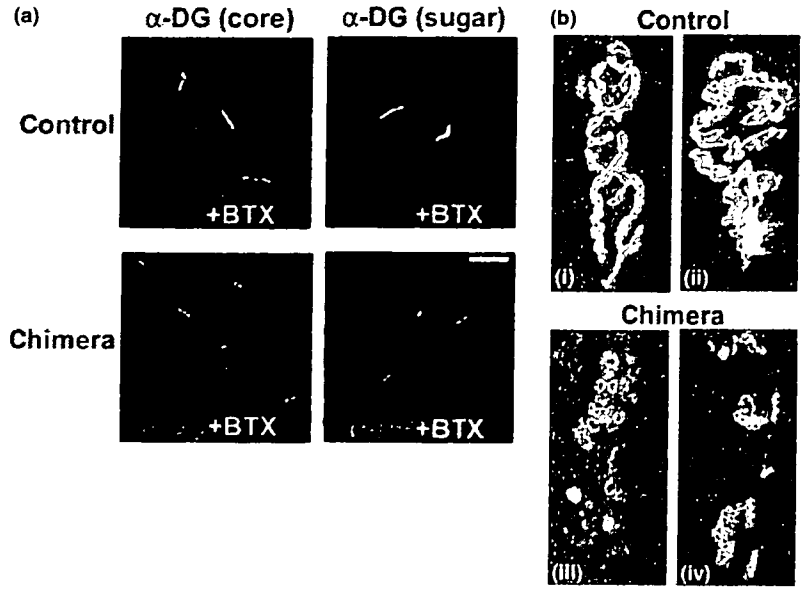

Fig. 6 Small and fragmented neuromuscular junctions (NMJ) in fukutin-deficient chimeric mice. (a) Double staining of quadriceps muscle with anti-α-DG core protein (red) and Alexa fluor 488-labeled α-BTX (green). Chimeric NMJs were small and fragmented in appearance compared with control. When α-DG was probed with antibody against sugar chain moiety of α-DG (IIH6), immunostaining of the NMJ as well as extrajunctional sarcolemma was severely reduced in the chimeric mice. Scale bar indicates 50 μm. (b) Longitudinal sections of sternocleidomastoid muscle stained by α-BTX. In the control mice, the pattern of AChR staining was smooth and continuous in appearance. In contrast, NMJs of the chimeric mice showed a discontinuous and fragmented pattern of AChR staining. Scale bar indicates 10 μm.

sarcolemma and NMJ. Although laminin was localized to the NMJ as well as extrajunctional sarcolemma in the control mice, its expression was severely reduced in these locations in the chimeric mice.

Next, we evaluated the agrin and laminin-binding activity of α-DG at the NMJ. First, we overlaid the blots of the total homogenate of skeletal muscle with agrin or laminin. The binding of both agrin and laminin to α-DG was greatly reduced in the chimeric mice compared with control (Figs 9a and b, left panels). Then, to assess the agrin and laminin-binding activity of α-DG at the NMJ and extrajunctional sarcolemma separately, we performed *in situ* ligand overlay assay. The cross sections of skeletal muscle were overlaid with extrinsic agrin or laminin labeled by FITC. The FITC-labeled agrin bound to both NMJ and extrajunctional sarcolemma in the control skeletal muscle (Fig. 9a, right panel). The binding of agrin was stronger at the NMJ than extrajunctional sarcolemma as revealed by double staining with α-BTX (Fig. 9a, right panel). In sharp contrast, agrin bound only weakly to the NMJ and did not significantly bind to the extrajunctional sarcolemma in the chimeric mice (Fig. 9a, right panel). We also performed *in situ* laminin overlay assay. When the skeletal muscle was overlaid with FITC-labeled extrinsic laminin, laminin bound strongly to the NMJ as well as extrajunctional sarcolemma in the control mice (Fig. 9b, right panel). However, laminin did not significantly bound to the NMJ or extrajunctional sarcolemma in the chimeric mice (Fig. 9b, right panel).

Discussion

In the present study, we have shown the decreased density of myelinated nerve fibers in the peripheral nerve of fukutin-deficient chimeric mice. This was observed from P15 to 23 months of age. By electron microscopy, numerous non-myelinated axons with abnormally large caliber were observed. These large non-myelinated axons were surrounded by small calibered non-myelinated axons and ensheathed by the same Schwann cell. During the normal development of peripheral nerve, individual Schwann cells either ensheath multiple small non-myelinated axons or sort larger axons into 1 : 1 relationship, forming a myelinated axon. Typically, axons with a diameter of 1 μm or greater are myelinated (Peters *et al.* 1991). In our chimeric mice, however, many axons larger than 1 μm in diameter were left unmyelinated and ensheathed together with much smaller axons by the same Schwann cell. These findings indicate that the radial sorting mechanism is defective in the chimeric mice. In support of this notion, Schwann cells that appeared to ensheath multiple myelinated axons were also observed. In the spinal roots and sciatic nerves of young chimeric mice (P30), furthermore, large clusters of non-myelinating Schwann cells were observed.

These findings resembled those reported in *dystrophic mice* (dy/dy, dy^{2j}/dy^{2j}), which have mutations in the gene encoding laminin α2 chain (Sunada *et al.* 1995). The defective myelin formation in these mice is characterized by the clusters of juxtaposed non-myelinated axons, which are most prominent in the nerve roots (Bradley and Jenkison 1973). Similar clusters of non-myelinated axons were also observed in the mice with targeted disruption of laminin γ1 or laminin α4 chain (Chen and Strickland 2003; Wallquist *et al.* 2005; Yang *et al.* 2005). Interestingly, myodystrophic mice (*myd*) and enervated mice (*enr*), in which mutations of the *Large* gene result in the reduction of laminin-binding activity of skeletal muscle α–DG, exhibit similar defects in

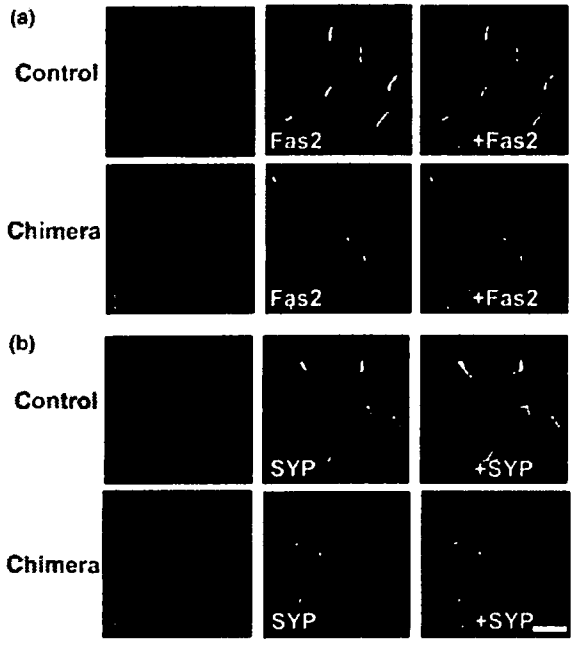

Fig. 7 Defective expression of acetylcholinesterase and synaptophysin at the neuromuscular junction (NMJ) of fukutin-deficient chimeric mice. (a) Quadriceps muscle was double-stained with rhodamine-labeled α-BTX (red) and FITC-labeled fasciculin 2 (green), a marker for AChE. In the control mice, the labeling with fasciculin 2 was co-localized with AChR. In the chimeric mice, the labeling with fasciculin 2 was much smaller than control, although it was co-localized with α-BTX staining. (b) Quadriceps muscle was double-stained with rhodamine-labeled α-BTX (red) and antibody against synaptophysin (green), a marker for presynaptic nerve terminal. Although the immunoreactivity of synaptophysin was co-localized with AChR in both control and chimeric mice, the staining was much smaller in the chimeric than control mice. BTX, bungarotoxin; Fas2, fasciculin 2; SYP, synaptophysin. Scale bar indicates 50 μm.

radial sorting (Rayburn and Peterson 1978; Kelly *et al.* 1994; Levedakou *et al.* 2005).

In the present study, we have found that the sugar chain moiety and laminin-binding activity of α–DG were severely reduced in the peripheral nerve of fukutin-deficient chimeric mice, suggesting that hypoglycosylation of α–DG might play a role in the pathogenesis of the aforementioned phenotype of the chimeric mice. We have previously demonstrated that the selective disruption of the DG gene in Schwann cells results in defective myelination in mice (P0-DG null mice). However, the hallmarks of the pathological findings of these mice were somewhat different from those of the fukutin-deficient chimeric mice, and irregular thickening and extensive folding of myelin sheath of peripheral nerve fibers were the major findings (Saito *et al.* 2003). Interestingly, in this respect, it was proposed that substrates of Large in addition to α–DG might be involved in the pathogenesis of the phenotype of *myd* and *enr* mice (Levedakou and Popko 2006). At present, target glycoproteins of fukutin, Large and related glycosyltransferases remain unidentified except α-DG. Under these circumstances, we analyzed the expression of β1-integrin in the chimeric sciatic nerve, because (1) its ablation has been shown to result in the formation of bundles of non-myelinated axons (Feltri *et al.* 2002) and (2) there is a possibility that β1-integrin, which is a glycoprotein, could be a target of fukutin and related glycosyltransferases. However, we did not find a significant abnormality in its expression, suggesting that dysfunction of β1-integrin may not play a significant role in the pathogenesis of impaired radial sorting of axons in the fukutin-deficient chimeric mice.

Recently, mutations of the gene encoding L-periaxin, a Schwann cell cytoplasmic protein that interacts with β-DG via DRP2, were identified in Charcot-Marie-Tooth disease type 4F (Boerkoel *et al.* 2001; Sherman *et al.* 2001).

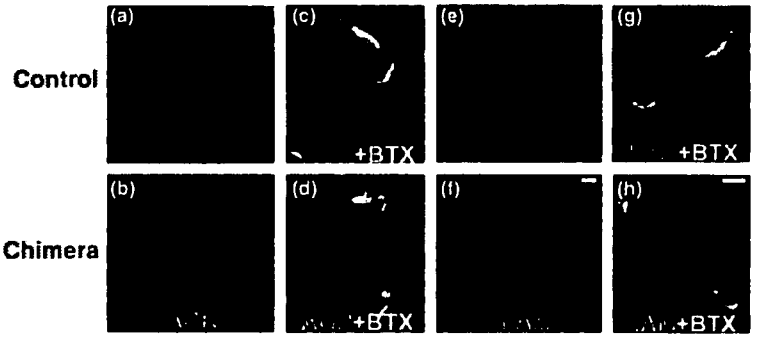

Fig. 8 Decreased expression of agrin and laminin at the neuromuscular junction (NMJ) in fukutin-deficient chimeric mice. Immunohistochemical analysis demonstrated that agrin (red) was expressed in the sarcolemma in the control mice (a), whereas its expression was significantly reduced in the chimeric mice (b). Agrin was enriched at the NMJ of the control mice where it was co-localized with AChR probed by FITC-labeled α-BTX (green) (c). In contrast, agrin was severely reduced in the sarcolemma and not clearly detected at the NMJ in the chimeric mice (d). Laminin α2 (red) was localized to the NMJ as well as extrajunctional sarcolemma in the control mice (e, g). However, its expression was severely reduced at both NMJ and extrajunctional sarcolemma in the chimeric mice (f, h). AGR, agrin; BTX, bungarotoxin; LAM, laminin α2. Scale bar indicates 20 μm.

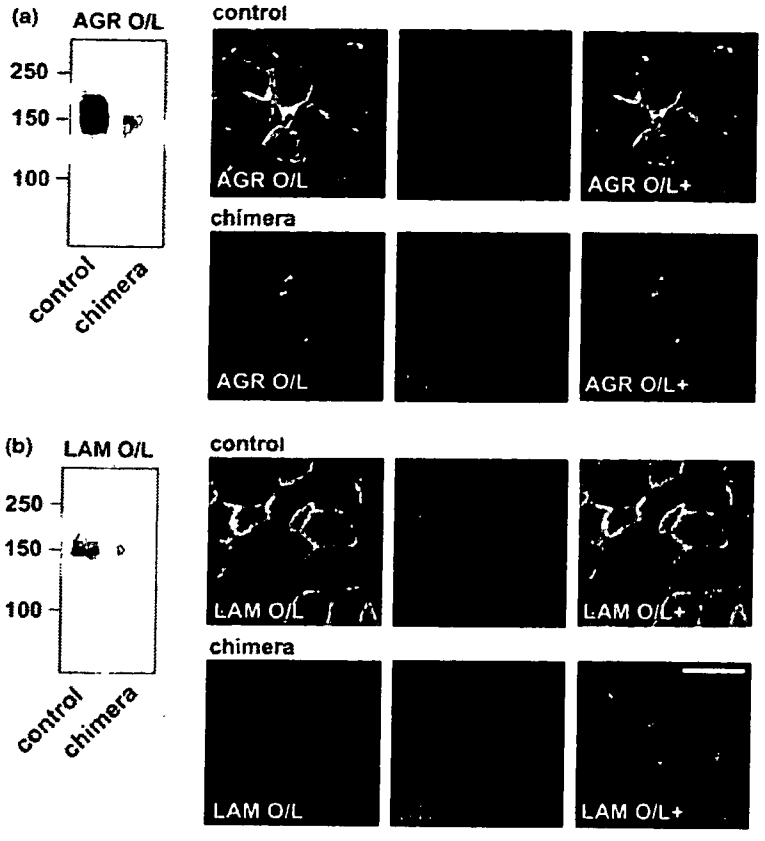

Fig. 9 Decreased ligand-binding activity of α-DG at the neuromuscular junction (NMJ) of fukutin-deficient chimeric mice. (a) Blot overlay assay demonstrated that the binding activity of α-DG to agrin was reduced in the skeletal muscle of chimeric mice (left). *In situ* ligand binding assay showed that FITC-labeled agrin (green) bound to both NMJ and extrajunctional sarcolemma in the control mice. The binding of agrin was stronger at the NMJ than extrajunctional sarcolemma as revealed by double staining with rhodamine-labeled α-BTX (red). In sharp contrast, agrin bound only weakly to the NMJ and did not bind significantly to the extrajunctional sarcolemma in the chimeric mice (right). (b) Blot overlay assay revealed that the binding activity of α-DG to laminin was reduced in the skeletal muscle of chimeric mice (left). FITC-labeled laminin bound strongly to the NMJ as well as extrajunctional sarcolemma in the control mice. However, laminin did not bind to the NMJ or extrajunctional sarcolemma in the chimeric mice (right). AGR, agrin; BTX, bungarotoxin; LAM, laminin; O/L, overlay assay. Scale bar indicates 50 μm.

Peripheral nerve of the patients and its mouse model exhibited hypermyelination and in/out-folding of myelin sheath as well as demyelination/remyelination, which were very similar to those observed in the P0-DG null mice (Gillespie *et al.* 2000; Boerkoel *et al.* 2001; Guilbot *et al.* 2001). Taken together, it would be intriguing to postulate that disruption of the extracellular linkage of DG may lead to the impaired radial sorting of axons, while disruption of the cytoplasmic linkage of DG may result in extensive myelin folding or hypermyelination. The other intriguing possibility would be that glycoconjugates other than α-DG and β1-integrin might be hypoglycosylated in the fukutin-deficient chimeric mice and that this might lead to the impaired radial sorting of axons.

In addition to the impaired radial sorting, degradation of cytoskeletal structure and cell organelles was observed in the axon and Schwann cell cytoplasm in the older chimeric mice. These observations suggest that impairment of radial sorting which occurred during the late embryonic to early post-natal stages may eventually lead to degeneration of these cells and result in the formation of abnormal nerve bundles seen in the old chimeric mice over a long period of time.

In the fukutin-deficient chimeric mice, the architecture of NMJ was abnormal. The NMJs were fragmented in appearance and the normal continuous pattern of AChR staining was lost. These observations are consistent with our recent report of aberrant NMJ in the patients with FCMD and *myd* mice (Taniguchi *et al.* 2006). In the formation of NMJ, agrin derived from motoneurons plays a crucial role in the clustering of AChR at the post-synaptic membrane. The neural agrin activates MuSK, a muscle-specific receptor tyrosin kinase and leads to the clustering of AChR (Sanes *et al.* 1998; Borges and Ferns 2001). α-DG was suggested to be involved in this agrin induced clustering of AChR (Jacobson *et al.* 1998; Kahl and Campanelli 2003). Apart from agrin, laminin also induces the clustering of AChR through a MuSK-independent pathway and DG has been implicated in this process (Sugiyama *et al.* 1997; Montanaro *et al.* 1998; Jacobson *et al.* 2001). Furthermore, DG is also known to mediate the localization of AChE at synaptic basal lamina by interacting with perlecan (Peng *et al.* 1998, 1999). Altogether, these findings suggest that the NMJ fragmentation in the chimeric mice may be caused by disruption of the linkage between α-DG and its ligands, agrin and laminin, because of aberrant glycosylation of α-DG.

To confirm this, we assessed the agrin and laminin-binding activity of α-DG and the expression of agrin and

laminin at the NMJ of the chimeric mice. We found that α-DG was hypoglycosylated at the NMJ of the chimeric mice as revealed by immunohistochemical analysis using antibodies against the core protein (AP1530) and the sugar chain moiety (IIH6) of α-DG. Because several distinct α-DG glycoforms exist in the skeletal muscle and glycosylation of skeletal muscle α-DG is controlled by innervation (Leschziner *et al.* 2000; McDearmon *et al.* 2001), it is also possible that the glycan structures of α-DG at the NMJ may differ from those of extrajunctional sarcolemma. Under these circumstances, it was important to assess the binding activity of α-DG to agrin or laminin at the NMJ exclusively. For this purpose, we performed *in situ* ligand overlay assay using FITC-labeled agrin or laminin. In the control mice, the FITC-labeled extrinsic agrin and laminin bound to the sarcolemma clearly, indicating that the agrin/laminin-binding sugar chain moieties on α-DG are not fully occupied or saturated by the intrinsic agrin/laminin in the basal lamina *in vivo*. Interestingly, the binding of these fluorescent extrinsic ligands to the NMJ and extrajunctional sarcolemma was greatly reduced in the chimeric mice, indicating that the ligand-binding activity of α-DG was defective at the NMJ as well as extrajunctional sarcolemma in the chimeric mice. We also found that the expression of agrin and laninin was decreased at the NMJ and extrajunctional sarcolemma of the chimeric mice. Defective ligand-binding activity of α-DG may explain for the decreased expression of agrin and laninin at the NMJ and extrajunctional sarcolemma of the chimeric mice, because it may destabilize the proper localization of these ligands in the basal lamina. Taken together, our results indicate that disruption of the linkage between α-DG and its ligands leads to the aberrant clustering of AChR at the NMJ. In support of this notion, it was reported recently that the extracellular domain of α-DG modulates agrin-induced AChR aggregation in myotubes (Tremblay and Carbonetto 2006).

We have also found that the expression of synaptophysin, a marker for pre-synaptic nerve terminal, is disturbed at the NMJ in the chimeric mice. At present, we do not have definite explanations for this finding. One possibility is that it may simply reflect the disturbed NMJ formation in the chimeric mice. Another intriguing possibility is that it may be secondary to the defective myelination of peripherall motor nerve fibers in the chimeric mice.

In conclusion, we have demonstrated that fukutin plays a crucial role in the myelination of peripheral nerve and formation of NMJ. We propose that these effects of fukutin are at least partially exerted by regulating the sugar chain structure of α-DG involved in the interaction with laminin and agrin. It would be interesting, in the future, to evaluate the status of peripheral nerve and NMJ in the patients with α-dystroglycanopathies and to clarify the roles of their defects in the expression of the phenotype of theses disorders.

Acknowledgments

We thank Miki Yamanaka and Yuka Sasayama for their expert technical assistance. This work was supported by (1) Research Grants 16B-1 and 17A-10 for Nervous and Mental Disorders (Ministry of Health, Labor and Welfare), (2) Research on Psychiatric and Neurological Diseases and Mental Health (Ministry of Health, Labor and Welfare), and (3) Research Grant 16390256, 40286993, 17590898 and 'Open Research Center' Project for Private Universities: matching fund subsidy from MEXT (Ministry of Education, Culture, Sports, Science and Technology), 2004-2008, (4) The 21st Century COE program from MEXT.

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