Chernousov et al. 2008; Feltri and Wrabetz 2005; Rasi et al. 2010). Such synthesis of extracellular matrix components by Schwann cells organizes basal lamina as a sleeve around the Schwann cells. Interaction with axons and production of the basal lamina is interdependent, because axons are important to facilitate secretion of basal lamina components by Schwann cells, and the basal lamina is required for efficient differentiation of Schwann cells and myelination (Bunge et al. 1986; Carey et al. 1983; Eldridge et al. 1987; Fernandez-Valle et al. 1993; Podratz et al. 2001). Integrins functionally mediate interaction of Schwann cells with extracellular matrix, and impaired integrin function or ligand production perturbs their axonal radial sorting and myelination (Chernousov et al. 2008; Rasi et al. 2010). The basal lamina of Schwann cells is also an important factor affecting nerve regeneration in the PNS after injury, as its components such as laminin are substrates supporting axonal outgrowth and guidance (Bunge et al. 1989).

4.3 Axonal Signaling for Modulation of Schwann Cell Behavior

In the PNS, signals from axons control the development of Schwann cells, including proliferation of precursors derived from neural crest and differentiation for myelin formation (Jessen and Mirsky 2005). Axonal neuregulins (NRG) are a family of cell signaling molecules that regulate proliferation, differentiation, and survival of Schwann cells and interact with receptor tyrosine kinase receptor ErbB (Dong et al. 1995; Grinspan et al. 1996; Morrissey et al. 1995; Nave and Trapp 2008; Newbern and Birchmeier 2010; Trachtenberg and Thompson 1996). Deficiency of NRG isoforms in PNS or ErbB receptor complexes in Schwann cells decreases the number of Schwann cells (Morris et al. 1999; Riethmacher et al. 1997; Woldeyesus et al. 1999). NRG1 has at least 15 isoforms, and membrane-bound type III isoforms of NRG1 appear to be key regulaters of axon–Schwann cell signaling for myelination. NRG1 type III binds to ErbB2–ErbB3 receptor complexes in Schwann cells and determines the threshold triggering myelination and myelin thickness matching to axon caliber (Birchmeier and Nave 2008; Carroll et al. 1997; Cohen et al. 1992; Grinspan et al. 1996; Jin et al. 1993; Michailov et al. 2004; Taveggia et al. 2005; Vartanian et al. 1997). Levels of NRG1 type III correlate with the presence and thickness of myelin as well as the formation of Remak bundles (Michailov et al. 2004; Taveggia et al. 2005). The heterodimers of ErbB2 and ErbB3 mediate signaling through several pathways involving PI3K/Akt, Erk1/2, Ca^{2+}, FAK, and Rac/Cdc42 (Newbern and Birchmeier 2010).

Proteases have been implicated in NRG1–ErbB interactions of axons and Schwann cells. Recent studies have shown that β-amyloid-converting enzyme (BACE1), a β-secretase present in axons, is associated with myelination (Hu et al. 2006; Willem et al. 2006). BACE1-null mice have reduced PNS myelin and remyelination capacity, and consequently exhibit thinner PNS and CNS myelin with reduced levels of myelin proteins. The impaired myelination and remyelination of BACE1-null

mice were attributable to reduced rates of NRG1 cleavage, and it is suggested that BACE1 cleaves NRG1 to facilitate its binding to ErbB receptors (Hu et al. 2006). By contrast, downregulation of the axonal α-secretase, tumor necrosis factor-α-converting enzyme (TACE, ADAM17), causes hypermyelination and ectopic myelination that is similar to NRG1 type III overexpression (La Marca et al. 2011). These results indicate that the neuronal α-secretase cleaves NRG1 type III into an inactive form. Another α-secretase, ADAM10, has little effect on myelination, although it can also cleave NRG1 (Freese et al. 2009; Luo et al. 2011). Collectively, behavior of Schwann cells is regulated by NRG1, but this signaling can be modulated by activating and inactivating proteases expressed in neurons.

NRG could also mediate Schwann cell differentiation through axonal neurotrophin signaling in response to neurotrophin release from Schwann cells. It has been suggested that neurotrophins can induce Schwann cell myelination along with increase of axonal diameters (Voyvodic 1989). However, the effect of neurotrophins, such as NGF, is generally restricted to neurons expressing TrkA, and thus it suggests that myelination is facilitated by indirect mechanisms mediated by signals from axons rather than direct glial stimulation by neurotrophin (Rosenberg et al. 2006). The effect of neurotrophins may be mediated by increased NRG1 isoforms, which in turn stimulate the myelination of these DRG axons by Schwann cells (Chan et al. 2004; Esper and Loeb 2004).

Axonal signaling involving proteolytic enzymes may also be required for maintenance of myelin. Expression of prion protein PrPc in axons, but not in Schwann cells, is required for maintenance of the myelin sheath during adulthood (Bremer et al. 2010). Interestingly, a proteolytic cleavage product of PrPc is sufficient to prevent chronic demyelinating polyneuropathy caused by PrPc deficiency (Bremer et al. 2010). the molecular mechanisms of myelin maintenance by axons are still elusive but may have some important implication in the pathophysiology of adult-onset demyelinating diseases.

4.4 Metabolic Link Between Schwann Cells and Axons

Axon-ensheathing cells in the vertebrate nervous system, including Schwann cells, have specific roles for myelination and rapid saltatory conduction, but previous studies have also revealed further roles of these glia in axonal support, in particular, such as survival of the axons that they ensheath (Nave 2010a). This concept is supported by observations that axons are predisposed to degeneration in primary diseases of myelin. For example, recent studies have revealed frequent axonal transections and progressive axon loss in the inflammatory demyelinating disease of CNS, multiple sclerosis (Ferguson et al. 1997; Trapp and Nave 2008; Trapp et al. 1998). Progressive axonal degeneration is also found in human neurological diseases that affect oligodendrocytes, such as leukodystrophies (Nave and Trapp 2008). Inherited peripheral neuropathies, Charcot-Marie-Tooth disease (CMT) type 1, which are caused by Schwann cell dysfunction, also exhibit axon degeneration

and loss, which are common among all CMT diseases (Nave et al. 2007). CMT1 is caused by mutations in molecules expressed in Schwann cells, including peripheral myelin protein 22 (*PMP22*) and myelin protein zero (*MPZ*; P_0), which are characterized by demyelination (Nave et al. 2007; Scherer and Wrabetz 2008). Although these diseases exhibit demyelination along with axonal degeneration, it was suggested that myelinating glia support axonal functions independently of myelin. In the CNS, mouse mutants with specific oligodendrocyte defects, such as absence of PLP or CNP, show normal myelin formation but display pathology of progressive axonal loss in the CNS (Griffiths et al. 1998; Lappe-Siefke et al. 2003). In the PNS, some mutations in *MPZ* can cause an axonal form of CMT disease, CMT type 2, where conduction velocity and myelination are not affected but sensory defects and hearing loss are caused by loss of axons (Laura et al. 2007). Typically, the genes causing CMT type 2 are expressed in neurons, but P_0 abundant in PNS myelin is also related to CMT type 2, which is characterized by axonal loss with relatively spared myelin. In mice lacking myelin-associated glycoprotein (MAG), which is expressed by myelinating glia, myelination is normal, but some axons degenerate and axonal diameters are reduced in PNS as well as CNS (Nguyen et al. 2009; Yin et al. 1998).

Axonal degeneration contributes to permanent neurological disability in primary diseases of myelin (Nave et al. 2007; Nave and Trapp 2008; Trapp and Nave 2008). Although the mechanisms of axonal pathology and degeneration after demyelination or dysmyelination are not yet fully understood, they may be associated with the influence of myelin, which alters the structure and metabolism of the axon (de Waegh et al. 1992; Sanchez et al. 1996). One of the influences of myelinating glia is the increase of axonal caliber mediated by posttranslational modifications of axonal cytoskeletons (Colello et al. 1994; Kirkpatrick et al. 2001; Windebank et al. 1985). Signal transduction pathways between axons and myelinating glia would affect posttranslational modification of axonal cytoskeletal proteins including neurofilaments, microtubules, and their associated proteins, which controls axonal caliber and transport (Sousa and Bhat 2007). In *shiverer* mice, where oligodendrocytes form only a few layers of noncompacted myelin around axons without any signs of oligodendrocyte degeneration, the axonal cytoskeletons fail to fully mature and axon diameters remain small (Brady et al. 1999; Griffiths et al. 1998; Inoue et al. 1981; Rosenbluth 1980; Shine et al. 1992). The small axonal diameter in the *shiverer* mutants is caused by narrowly spaced nonphosphorylated neurofilaments and microtubules, which are reminiscent axons in MAG-deficient mice (Nguyen et al. 2009; Yin et al. 1998). However, these axons in *shiverer* mutants do not degenerate. Axons without normal myelin in *shiverer* mice more easily degenerate when oligodendrocytes are further compromised by the absence of PLP1. These results indicate that perturbed maturation of axonal structures itself does not cause axonal degeneration or loss.

The progressive and distally pronounced axonal degeneration in myelin deficit may be related to adaptation and impairment of metabolic homeostasis in axons, given that cell bodies and distal segments of long axons could be distinct biochemical compartments, with respect to metabolic reactions (Nave 2010a).

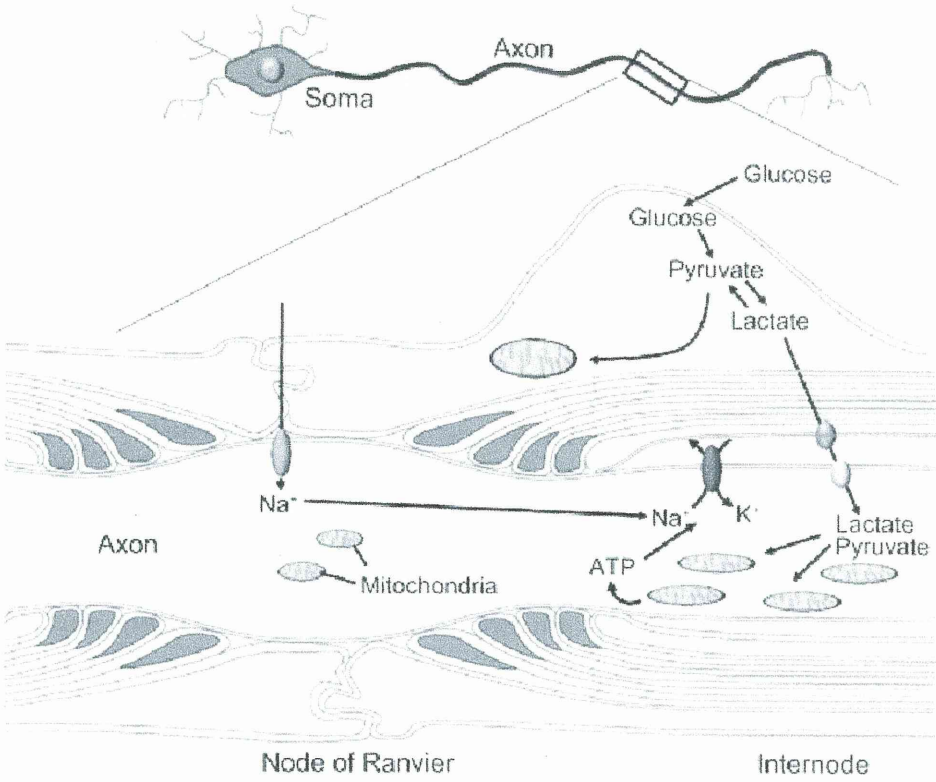

Fig. 4.3 Schematic drawing of metabolic flow upon nerve conduction in myelinated peripheral nerves. Nerve conduction causes Na^+ influx through voltage-gated Na^+ channels at the nodal axolemma. Na^+ is required to be excluded through internodal Na^+/K^+-ATPase in an energy-dependent manner. Axonal mitochondria are enriched in internodal axoplasm, and energy substrates for these mitochondria are likely to be provided through myelinating Schwann cells. Current evidence indicates that lactate generated by Schwann cells is transferred to axons through unidentified transporters

Neuronal Na^+/K^+-ATPases, which use most axonal ATP to exchange axoplasmic Na^+ with extracellular K^+, are present along the entire internodal axolemma (McGrail et al. 1991; Young et al. 2008), suggesting that axonal energy demands on nerve conduction are not restricted around the nodes of Ranvier (Fig. 4.3). This concept is supported by previous observation that the bulk of mitochondrial volume resides in internodes, and the mitochondrial distribution also suggests that most ATP within axons is generated there (Fig. 4.3) (Ohno et al. 2011; Perge et al. 2009). Because mitochondria are the major source of ATP, the internodal enrichment of mitochondria also helps facilitate axonal transport, which is also energy dependent.

Impaired mitochondrial distribution and function have been implicated in the pathogenesis of myelin diseases (Coleman 2005; Trapp and Stys 2009). Axonal conduction/depolarization depends on activation of voltage-gated Na^+ channels. For repetitive conduction, the axolemma must exchange axonal Na^+ for extracellular K^+ in an energy-dependent manner by Na^+/K^+ ATPases. By concentrating voltage-gated Na^+ channels in the nodal axolemma, myelin not only increases the speed of nerve conduction but also conserves energy. Disruption of normal myelin would

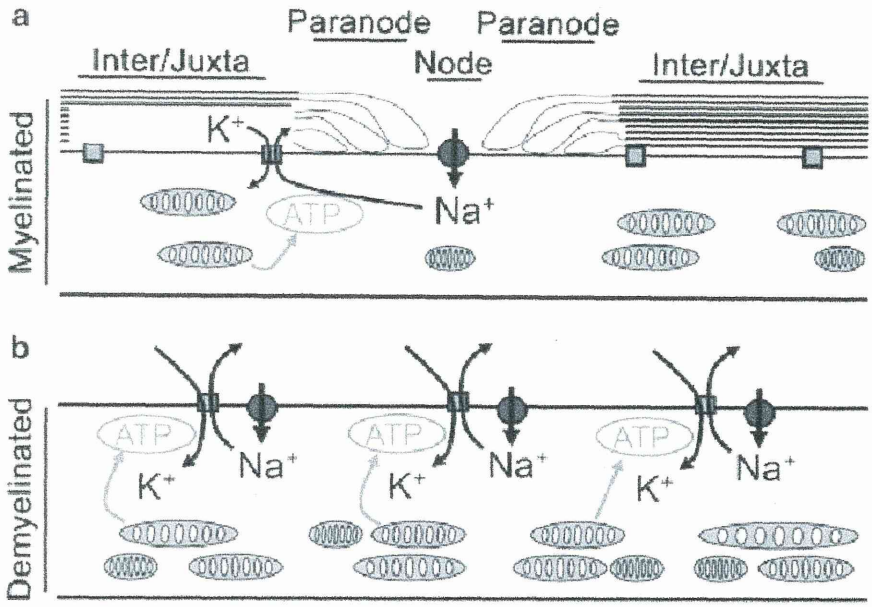

Fig. 4.4 Alterations of energy demand and mitochondrial distribution in myelinated and demyelinated axons. In myelinated axons (**a**), voltage-gated Na^+ channels are concentrated in nodal axolemma. Upon nerve conduction, a limited amount of Na^+ enters the axons, which is excluded from internodal/juxtaparanodal Na^+/K^+-ATPases consuming ATP. These ATP are likely to be originated from axonal mitochondria enriched in internodal/juxtaparanodal (*Inter/Juxta*) regions. In demyelinated axons (**b**), Na^+ channels redistribute diffusely along the entire axolemma to restore conduction. Upon nerve conduction, more Na^+ gets into the axons, and more ATP is required for Na^+ exclusion. The demyelinated axons have increased volume of axonal mitochondria, presumably to produce ATP sufficient for the Na^+ exclusion

therefore require an adaptive response of energy metabolism from axons. Upon demyelination, mitochondrial volume is increased in the demyelinated axons of human brain and animal models (Mahad et al. 2009; Mutsaers and Carroll 1998; Sathornsumetee et al. 2000; Witte et al. 2009). After demyelination, Na^+ channels diffusely redistribute along the demyelinated axolemma to restore nerve conduction at the expense of increased ATP consumption to drive the Na^+/K^+ ATPases (Craner et al. 2004; Waxman 2008). It is reasonable, therefore, that increases in axonal mitochondrial sizes parallel the increased energy demands of nerve conduction after demyelination in PNS and CNS axons (Fig. 4.4) (Kiryu-Seo et al. 2010; Zambonin et al. 2011). It was also demonstrated that perturbed paranodal junctions in the PNS result in the accumulation of axonal mitochondria around the nodal regions (Einheber et al. 2006; Sun et al. 2009). Dysmyelination increased densities of axonal mitochondria in CNS, as shown in *shiverer* mice and PLP1 mutants (Andrews et al. 2006; Hogan et al. 2009). Collectively, these results support the concept that metabolic relationship between axons and Schwann cells is critically dependent on axo–glial interactions and associated with adaptive alterations in mitochondrial functions.

Molecular mechanisms regulating metabolic adaptation of axonal mitochondria against demyelination or dysmyelination still remain to be elucidated but are likely to

be involved in regulation of two populations of axonal mitochondria. The majority of axonal mitochondria are present at stationary sites, which can be composed of single or multiple stationary mitochondria and enriched in axonal areas with high ATP consumption such as growth cones (Kiryu-Seo et al. 2010; Misgeld et al. 2007; Saxton and Hollenbeck 2012; Sheng and Cai 2012). Motile mitochondria are generally smaller and are transported throughout the axon in anterograde and retrograde directions. Demyelination increases the size of the stationary site as well as the transport velocity of axonal mitochondria (Kiryu-Seo et al. 2010). After remyelination, stationary site size and transport velocity were similar to those in the myelinated axons. It was indicated that these mitochondrial alterations were mediated by adaptive responses, at least partly, involving a stress-induced transcription factor, activating transcription factor 3 (ATF3) (Kiryu-Seo et al. 2010). It is also possible that key aspects of this regulation would include increased axoplasmic Ca^{2+} and posttranslational modifications of axoplasmic proteins to halt motile mitochondira, because local inhibition of mitochondrial movement can increase the sizes of stationary mitochondria in axons (Chada and Hollenbeck 2004; Macaskill et al. 2009; Morris and Hollenbeck 1993; Wang and Schwarz 2009). As already described, demyelination increases axoplasmic Na^+ as a result of insufficient ATP production, and increased axoplasmic Na^+ in turn increases axoplasmic Ca^{2+} through reverse operation of Na^+/Ca^{2+} exchangers (Trapp and Stys 2009). Axonal survival would be impaired by the generation of nitric oxide by inflammatory cells, which diffuses into demyelinated axons and contributes to perturbation of mitochondrial ATP generation (Smith and Lassmann 2002; Trapp and Stys 2009). Apart from the acute axonal transection and loss mediated by toxic substances from inflammatory cells, axonal degeneration following demyelination or as a result of dysmyelination is also a chronic process taking months or years to develop (Trapp and Nave 2008). The initial axonal response to demyelination, therefore, reestablishes axonal function and is likely to include changes in mitochondrial distribution, behavior, and life cycles.

Axonal mitochondria have limited lifespans that are dependent on their dynamics and presumably modulated by their overall activity, and thus abnormal mitochondrial dynamics results in impaired axonal integrity (Saxton and Hollenbeck 2012; Sheng and Cai 2012). In neuropathies of PNS, this concept is supported by evidence that molecules regulating mitochondrial fusion/fission and transport, such as mitofusin 2 (Mfn2) and ganglioside-induced differentiation associated protein 1 (GDAP1), are responsible for some forms of CMT (Baxter et al. 2002; Cuesta et al. 2002; Niemann et al. 2005; Zuchner et al. 2004). Newly synthesized mitochondria are largely generated in the neuronal cell body, transported down along the axon, and delivered to stationary sites, where they become fused with stationary mitochondria. Dysfunctional mitochondrial segments are removed from stationary mitochondria through a process called fission, and then transported to the neuronal perikarya, where they are degraded (Saxton and Hollenbeck 2012; Twig et al. 2008). This entire life cycle of axonal mitochondria relying on transcription and translation in cell bodies may render distal axonal segments vulnerable to disruption of energetic homeostasis. The degeneration of axons associated with relevant symptoms such as distally pronounced motor and sensory deficits is progressive and length dependent

in primary myelin diseases (Marrosu et al. 1998; Zhou and Griffin 2003); this may explain why a progressive length-dependent loss of axons is commonly observed in peripheral neuropathies and leukodystrophies, first affecting fibers innervating distal regions of extremities in the PNS or the longest spinal tracts in the CNS (Griffin and Watson 1988; Suter and Scherer 2003).

Recent studies suggested that myelin-forming glia provide energy substances such as lactate for the axonal energy production (Nave 2010b). This concept is supported by observations that disruption of monocarboxylic acid transporter 1 (MCT1), which mediates lactate transport from oligodendrocytes to axons for the local energy supply to axons, leads to axonal degeneration in CNS (Funfschilling et al. 2012; Lee et al. 2012). Aberrant axonal degeneration under disruption of MCT1 is likely to be caused by reduced lactate export out of the oligodendroglia. The notion that energy substrates of axonal mitochondria are provided by myelinating glia is consistent with the internodal enrichment of axonal mitochondria, which means that mitochondria are more abundant in axonal regions covered by myelin (Ohno et al. 2011). These observations are also supported by previous findings that trophic support provided by myelin and myelin-forming cells is regulated at the level of individual internodes (Griffiths et al. 1998; Yin et al. 2006). Recent studies suggested that glycogen of Schwann cells in myelinated peripheral nerve fibers provides energy substrates for ensheathed axons during impaired supply of glucose (Brown et al. 2012). It was indicated that lactate is a primary substrate that is generated from glycogen and then shuttled from Schwann cells to axons to maintain axonal ATP stores and excitability. Although the beneficial support of Schwann cell glycogen for unmyelinated axons of Remak bundles was not observed under hypoglycemia (Brown et al. 2012), it is possible that glucose is uptaken largely by Schwann cells in Remak fibers, and the Schwann cells in turn provide lactate as energy substrates to the unmyelinated axons under normal conditions (Vega et al. 1998, 2003). Although it remains to be established if MCT1 is expressed and serves as a shuttling molecule in Schwann cells, these studies provided molecular evidences that energy substrates such as lactate are exported to the extracellular space and taken up by ensheathed axons for energy production by axonal mitochondria (Fig. 4.3).

Glycolysis is assumed to occur throughout the axoplasm. However, if the movement of glycolytic enzymes synthesized in the soma is driven by slow axonal transport, the efficiency of axonal glycolysis could be limited in a length-dependent fashion (Spencer et al. 1979). This possibility suggests that long distal axons may require more metabolic support for mitochondrial energy production than short proximal axons, and this metabolic support could be a trophic function of glia (pyruvate, lactate, or its derivates) for axonal mitochondria in long fiber tracts (Nave 2010b). However, it was recently reported that glyceraldehyde-3-phosphate dehydrogenase (GAPDH) localizes on fast-moving vesicles within axons, and that the glycolytic enzymes located on these vesicles are critical to maintain the high velocities characteristic of fast axonal transport (Zala et al. 2013). It remains to be elucidated if the attachment of glycolytic enzymes to vesicles, mediated by huntingtin, Rab2, and posttranslational modifications of the enzymes themselves, is affected

in neurological diseases (Tisdale et al. 2004; Yang et al. 2005; Zala et al. 2013). These studies raised a possibility that modulation of glycolytic energy production in long distal axons is involved in axonal degeneration caused by impaired axonal energy metabolism.

Previous studies support the notion that, apart from lactate, exchange of small metabolites between axons and myelinating glia is extensive and bidirectional. In mice with disrupted mitochondrial metabolism, exclusively seen in Schwann cells by selective depletion of mitochondrial transcription factor A (Tfam-SCKO), pathological features similar to peripheral neuropathies were observed, indicating that peripheral neuropathy occurs secondary to mitochondrial dysfunction of Schwann cells (Viader et al. 2011). Disruption of mitochondria in Schwann cells activates an abnormal integrated stress response, and the actions of heme-regulated inhibitor kinase alter lipid metabolism from fatty acid synthesis toward oxidation (Viader et al. 2013). These changes in the lipid metabolism of Schwann cells deplete myelin lipid components and accumulate acylcarnitines, an intermediate of fatty acid β-oxidation, which is released from Schwann cells and induces axonal degeneration.

Schmidt–Lanterman incisures are a series of funnel-shaped clefts among the compact myelin in the PNS and appear as a series of cytoplasmic openings (Hall and Williams 1970; Peters et al. 1991). Incisures contain connexin 32 (Cx32) to form gap junctions and may have important roles in trafficking of ions and small molecules between inner and outer Schwann cell compartments (Balice-Gordon et al. 1998). This concept is supported by the observation that mutations which impair Cx32 functions cause an X-linked form of CMT, and genetic ablation of Cx32 in mice induces similar pathological phenotypes (Anzini et al. 1997; Bergoffen et al. 1993; Scherer et al. 1998). However, axonal loss or degeneration is mild in these knockout mice (Anzini et al. 1997), indicating that Cx32-dependent gap junctions are redundant for the transport of metabolites to axons.

The same metabolites can play distinct roles in differentiation of myelinating glia in PNS and CNS. In the CNS, blocking of Na^+-dependent action potentials inhibits proliferation of oligodendrocytes and myelination (Barres and Raff 1993; Demerens et al. 1996). It was proposed that axonal electrical activity stimulates ATP release from axons and facilitates oligodendrocyte myelination through cytokines released from astrocytes (Ishibashi et al. 2006). By contrast, in the PNS, the axonal release of ATP perturbs Schwann cell differentiation and myelination through the purinergic P2 receptor (Stevens and Fields 2000).

4.5 Conclusions and Perspectives

Recent advances in genetic techniques, transgenic models, and myelinating cultures during the past decades have already begun to elucidate the molecular and cellular mechanisms by which Schwann cells and axons modulate the behaviors and fates of each other. The list of molecules that are associated with defects of Schwann cells

and axons in human PNS diseases is rapidly increasing, and improved biological techniques for tailoring rodent models for in vivo and in vitro manipulation or bioimaging have provided various approaches to further studies of the underlying mechanisms. Future studies are necessary to clarify common features and distinct pathways where ensheathing glia support axonal functions and integrity. Further understanding of the cellular mechanisms for Schwann cell support for axons and axonal signals determining Schwann cell behavior continues to be critical to reveal the physiology of the PNS and also to develop new therapies in peripheral nerve diseases.

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総　説

生体内凍結技法による肺組織切片標本上での生体物質分布と血行動態の可視化法

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要　旨：近年，蛍光標識蛋白などを用いた生体内イメージング法による生きた動物臓器の機能的形態像や生体物質の動態解析が注目されている。しかし一方，通常の組織切片標本作製法のための灌流固定法や浸漬固定法と，その後のアルコール脱水による光顕用組織試料では，人工産物としての細胞組織の収縮や血液・間質液等の可溶性成分の流失が起こるため，正常血行動態を維持した生きた動物の機能的形態像を観察することは困難である。当教室では，すでに「生体内凍結技法」という，生きた麻酔下動物の各種臓器をできるだけ速やかに凍結する方法を開発し，凍結置換固定法などと併用することにより，生きた動物臓器の機能分子形態学的解析を進展させてきた。本稿では，この生体内凍結技法により作製した，正常血行動態下と実験的肺水腫モデルでのマウス肺組織標本を用いて，肺の呼吸運動に伴うダイナミックな肺組織形態像と，その血行動態や病理学的変化を検討したので報告する。

キーワード　生体内凍結技法，凍結置換固定法，血行動態の可視化法，可溶性血清蛋白

I. はじめに

一般的に生命体を構成する細胞や組織の機能は，動物生体内臓器の構造に依存しているので，それをターゲットとする形態学的アプローチは常に必要である。まず，以前より行われているが，これらの形態観察法として，最初に生体内標的臓器から切除した組織塊をホルマリン等の水溶性固定剤に浸漬，あるいは血管内灌流することによって十分に化学固定する。その後，低濃度より徐々に上げて完全にアルコール脱水してからパラフィン等に包埋後，薄切する。さらにヘマトキシリン・エオジン（HE）染色などによる組織や細胞の形態像，あるいは免疫染色によって，その組織・細胞内の特定物質の局在を可視化する（図 1a）。この古典的な光顕用試料作製法の歴史は古く，現在でも多くの組織学・病理学の標本作製に用いられている。一方では，近年のデジタル顕微鏡や撮影装置開発と蛍光標識法などの進歩により，生体内イメージングが高解像度で可能となり，血行動態が維持されて，細胞間質液に満たされた微小環境を保ったまま，生きた動物臓器内での生体物質の動的変化が経時的に可視化されるようになった[1-6]。しかし，古典的な試料作製法による光顕像と比較しながら，生体内イメージング法による画像が議論されていることがある。この際に，一見して対応しているように見られる二つの方法による形態像は，実際には虚血・酸欠のために多くは解離しているはずである。以前より，生きた動物臓器を観察するために発展してきた生体内イメージング法であるのに対して，従来の古典的な切片試料作製法では，灌流固定したり，生体内臓器から組織を切除するために，血流が遮断され虚血・酸欠が起こり，さらに固定・ア

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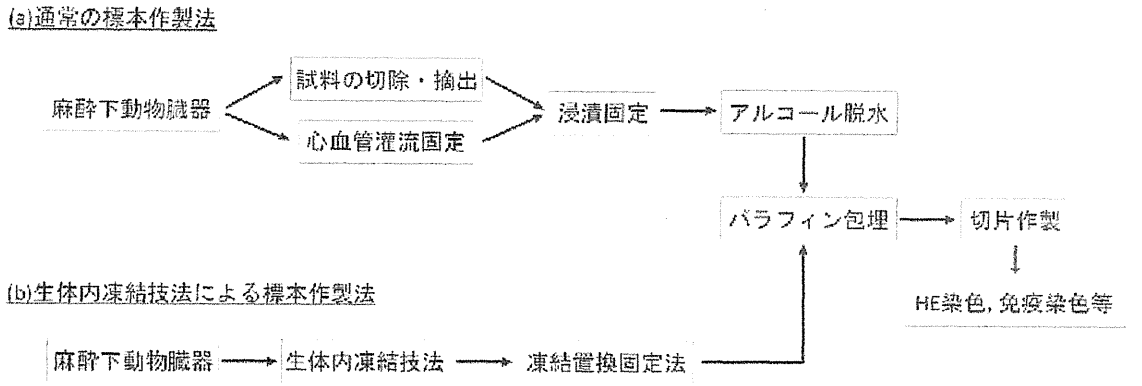

図1．通常（a）および生体内凍結技法（b）による組織標本作製法
生体内凍結技法を用いて作製されたパラフィン包埋試料は，通常の標本作製法を用いて作製されたパラフィン包埋試料と同様に扱うことができる．

ルコール脱水中に血液や細胞間質液といった液性成分は常に流出してしまっている。このことは，生きた動物生体内の状態とは大きく変化していることになる。特に，アルコール脱水によっても，脂質成分溶出や試料収縮が容易に起こってしまう。そこで，生きた動物臓器の生体内機能状態を反映した光顕用切片試料作製のためには，生体内凍結技法が必要となる[7-12]（図1b）。

II. 生体内凍結技法の手技と特徴

すでに1996年頃に開発したこの生体内凍結技法によって，麻酔下実験動物における虚血・酸欠の影響が全く無い細胞組織の形態像が得られる[7]。さらに動物生体内臓器の可溶性物質が，この生体内凍結技法により，瞬時に氷中に閉じ込められるために，全ての物質の移動と流失が起こらずに臓器組織の局所に保存される。本稿では，最近発表した呼気と吸気時の肺胞構造と肺胞中隔壁内毛細血管が，ダイナミックに変化するという生きたマウス肺組織について概説する。

通常の化学固定法による光顕用試料作製法では，ダイナミックな機能的肺組織の肺胞中隔壁内毛細血管構造の保持は困難である（図2）。一方，生体内凍結技法で試料を作製すると，生きた動物の呼吸状態を反映した組織像が得られる。具体的な生体内凍結技法としては，麻酔下マウスに人工呼吸器を接続して開胸後，可及的速やかに，あらかじめ液体窒素中（-196℃）で作製した液性イソペンタン・プロパン混合寒剤（-193℃）を，露出した肺表面に直接かけて生体内凍結する（図3a）。その後，発泡スチロール箱の液体窒素中（-196℃）で，肺を小型歯科用電気ドリルで摘出し保存する。

さらに血行動態解析のためには，生体内凍結施行前に，右心室などから量子ドット（Qdot）を注入し，その後の任意の時間で生体内凍結を行うこともできる（図3b）。このQdotとは，カドミウムーセレンを芯としたナノ粒子で，様々な粒子径のものが人工合成できるが，その粒子径によって紫外線励起で特定波長の蛍光が，半永久的に放たれる特徴を持っている[13-18]。本研究では，このナノ粒子周囲をグルタチオンで被覆したもので，血管外へ漏出しない赤色蛍光を放つ約10 nm粒子のQdotを使用した[19-21]。さらに病態解析モデルとして，上行大動脈結紮により，実験的肺高血圧症の肺浮腫を引き起こして生体内凍結した[22]。これらの生体内凍結試料は，2%パラホルムアルデヒド含有アセトン中で，-80℃から徐々に温度を室温まで上げて凍結置換固定し，型のごとくパラフィン包埋した。この凍結置換固定では，低温下で氷晶を有機溶剤（アセトン等）に置換

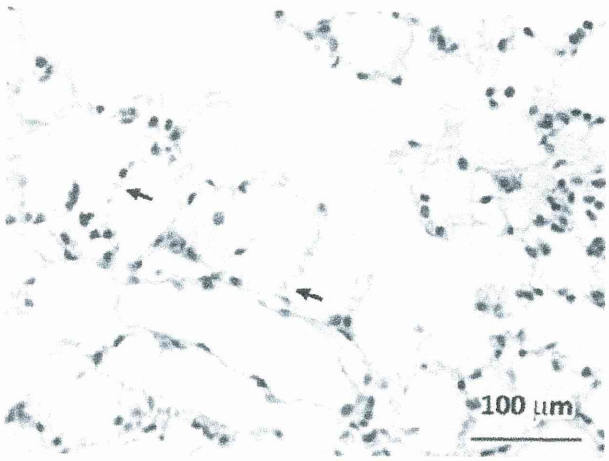

図2. 灌流固定法により作製した肺組織標本：矢印は肺胞毛細血管内に残った赤血球を示す.

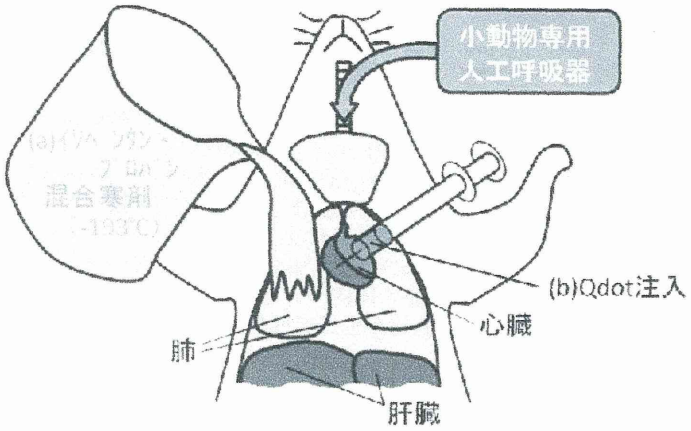

図3. Qdotの右心室内注入と肺の生体内凍結の模式図
(a) イソペンタン・プロパン混合寒剤（-193℃）を直接動物臓器にかけることにより生体内凍結する.
(b) 生体内凍結する前に，心臓（右心室）よりQdotを注入することもできる.

して固定する方法で，生体内凍結時の機能的形態像や物質局在を保存できることが知られている。

III. 正常肺組織の機能的形態像および血清蛋白局在の解析

1. 呼気と吸気時肺組織の可視化

麻酔下マウスを人工呼吸器で管理して呼気時と吸気時にタイミングを合わせて生体内凍結すると，肉眼的にも肺が縮小した状態と拡大した状態で凍結保存される。その光顕切片試料のHE染色により，肺胞中隔壁内毛細血管構造や流動赤血球が明瞭に観察できる。なお，血行動態によって様々に変形した流動赤血球と，進行方向に向かって矢じり型や勾玉型といった形状が同定できるのは，この生体内凍結組織標本の特徴と言える。さらに，肺胞中隔壁の機能的形態像は呼吸状態を反映して異なり，呼気では肥厚して蛇行し，吸気では肺胞腔の拡大とともに

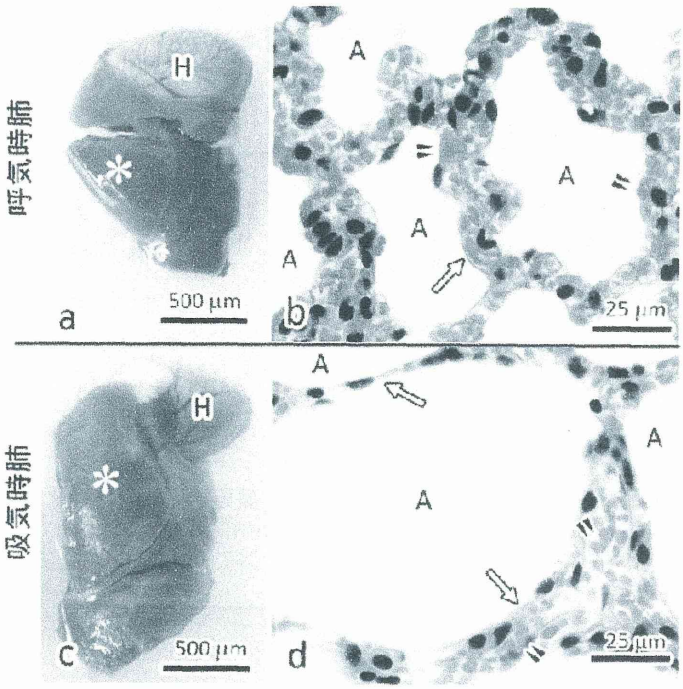

図 4. 生体内凍結－凍結置換固定後の呼気 (a, b) および吸気肺 (c, d) の肉眼像 (a, c) と HE 染色した肺組織像
(b, d)：肺組織は，肉眼の＊印部位から得た．生体内凍結－凍結置換固定により，呼気および吸気時の肺が縮小・拡大する肺葉がそのまま維持される．
白矢印 (⇒)：(b) 呼気時に厚く屈曲した肺胞中隔壁と (d) 吸気時の伸展した肺胞中隔壁
二重矢頭 (b, d)：流速や進行方向によって変化した様々な形状の赤血球
A：肺胞腔，H：心臓．

伸展して薄くなっているのが観察できる(図 4)。

2. Qdot による肺胞中隔壁内血管配置と血行動態の可視化法

麻酔下マウスを人工呼吸器で管理して，Qdot を右心室から注入し，1 秒後に生体内凍結した肺組織切片を検討すると，肺内流入直後の肺胞中隔壁組織部位が判別できる（図 5a, b)。その Qdot 分布は，太い血管とそれに続く毛細血管に見られ，したがってこの太い血管は，細動脈であることがわかる（図 5b)。一方，Qdot が未だ見られない肺胞中隔壁内毛細血管に続く太い血管も見られ，これらは細静脈であると同定できる。次に Qdot 注入 2 秒後に生体内凍結した肺を検討すると，Qdot に完全に満たされた太い血管，それに続く毛細血管，更にその先に血管壁に沿ってのみ Qdot が見られる太い血管が観察できる（図 5c-e)。これらは細動脈から肺胞中隔壁内毛細血管を経て，細静脈に流れ込む瞬間を捉えていると考えられる。このような機能的形態像は，血行動態を考えると一見当然のように思えるが，これまでは光顕用組織切片では観察することができなかった。また，この Qdot 局在の経時的変化を考えて細動脈と細静脈を区別すると，肺組織切片上で小葉間細動脈と細静脈が，交互に並ぶ肺胞中隔壁内血管構築が明らかとなり，生きた動物肺の機能状態を反映した血行動態が，組織像と同時に光