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Review

Galectin-1 and galectin-3 as key molecules for peripheral nerve degeneration and regeneration

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Abstract: There is growing evidence that galectin-1 (GAL-1) and galectin-3 (GAL-3) are involved in the process of axonal regeneration after injury and the pathogenesis of peripheral neuropathies, but the precise roles of these galectins in the peripheral nervous system (PNS) remain unclear. In this paper, we summarized the distribution and possible functions of GAL-1 and GAL-3 in the PNS at mature stages under normal and pathological conditions. Predominant expression of GAL-1 and GAL-3 in isolectin B4 (IB4)-binding small non-peptidergic sensory neurons in dorsal root ganglia (DRG) accords with the involvement of these molecules in nociceptive and thermoceptive functions and GDNF-induced neurite outgrowth. Up-regulation of GAL-1 mRNA/protein in the neurons and Schwann cells upon peripheral nerve injury suggests its active participation in axonal regeneration; both GAL-1 in the reduced and oxidized forms appear to be key molecules for normal onset of Wallerian degeneration and subsequent neurite outgrowth, but the latter lacks lectin activity and may promote the process by stimulating macrophages rather than directly acting on neurons. GAL-3 deficient mice exhibited the acceleration in the process of Wallerian degeneration, but there is room for argument on its pathological roles in axonal regeneration. Up-regulation of GAL-3 protein in immortalized Schwann cells IFRS1 under diabetic conditions and its cytoprotective functions on the

cells suggest that it may act as a factor against the progression of diabetic neuropathy, but further evidence is needed to support this idea. All these findings have shed light on both physiological and pathological roles of GAL-1 and GAL-3 in the PNS, and further evidence will be helpful to develop novel strategies for functional restoration after peripheral nerve injury and therapeutic efficacy against diabetic and other neuropathies.

Keywords: galectin-1; galectin-3; dorsal root ganglion neurons; Schwann cells; macrophages; Wallerian degeneration; axonal regeneration; glial cell line-derived neurotrophic factor (GDNF); diabetic neuropathy

Abbreviations: GAL-1, galectin-1; GAL-3, galectin-3; PNS, peripheral nervous system; CRDs, carbohydrate-recognition domains; GAL-1/Ox, the oxidized form of galectin-1; DRG, dorsal root ganglia; CNS, the central nervous system; NGF, nerve growth factor; SP, substance P; GDNF, glial cell line-derived neurotrophic factor; IB4, isolectin B4; 3-DG, 3-deoxyglucosone; AGEs, advanced glycation endproducts; PI3K, phosphatidyl inositol-3'-phosphate-kinase; GAN, giant axonal neuropathy

1. Introduction

Galectins, a family of β-galactoside-binding animal lectins, are involved in a wide variety of physiological and pathological processes [1]. We've been focusing on two major galectins, galectin-1 (GAL-1) and galectin-3 (GAL-3), as modulators of axonal degeneration and regeneration in the peripheral nervous system (PNS) [2-4]. Galectins have homologous carbohydrate-recognition domains (CRDs) with about 130 amino acids; while GAL-1 has one CRD domain and exists as a monomer as well as a non-covalent homodimer, GAL-3 has chimeric structure with a N-terminal non-lectin domain associated with a C-terminal CRD [1]. Despite lacking a classical signaling sequence to enter the endoplasmic reticulum, both GAL-1 and GAL-3 are subject to externalization from various kinds of cells by a non-classical secretory pathway [5]. Following externalization, some of the galectin molecules are suggested to associate with surface or extracellular matrix glycoconjugates where lectin activity is stabilized, while the others free from glycoconjugate ligands are rapidly oxidized in the non-reducing extracellular environment [6]. Contrary to the concept that galectins are biologically active only in the reduced form, we introduced the oxidized form of GAL-1 (GAL-1/Ox) as a novel factor to promote axonal regeneration in the PNS [7]. Most of the studies on the bioactivities of GAL-1 were carried out under reducing conditions; however, GAL-1/Ox containing three intramolecular disulfide bonds lacks lectin activity and acts on the nervous tissue as a cytokine-like molecule [8]. Further studies have indicated that both reduced and oxidized forms of GAL-1 play important roles in axonal degeneration and regeneration in the PNS [9-11]. GAL-3 has also been considered as a key molecule in the neural functions [12,13]; unlike GAL-1, GAL-3 containing only one Cysteine residue in the molecule is unable to form disulfide bonds under oxidative conditions, thus it exists only as a lectin [9].

The aim of this paper is to summarize the recent progress in the research of distribution and function of GAL-1 and GAL-3 in the PNS at mature stages. We also introduce our new findings for the better understanding of possible roles of these molecules in the process of axonal regeneration and the pathogenesis of peripheral neuropathies.

2. Distribution of GAL-1 and GAL-3 in the peripheral nervous system

2.1. Animal tissues

GAL-1 and GAL-3 are highly expressed in peripheral nervous tissues of adult rodents, with immunoreactivity localized to cell bodies of sensory neurons, axons and Schwann cells [2,3,14,15]. The immunoreactivity for GAL-1, but not GAL-3, has been detected in spinal motoneurons [14]. Little is known about the distribution of these lectins in the autonomic nervous system; GAL-1 mRNA is not detected in sympathetic neurons of superior cervical ganglia or parasympathetic neurons of sphenopalatine ganglia [16], whereas GAL-3 immunoreactivity is found in the autonomic preganglionic neurons in the spinal cord [17].

Primary sensory neurons in dorsal root ganglia (DRG) transmit various kinds of somatosensory information from the periphery to the central nervous system (CNS). The small diameter DRG neurons possessing small myelinated and unmyelinated fibers can play an essential role in nociception and thermoreception, whereas the large diameter neurons possessing large myelinated fibers are known to be mostly sensitive mechanoreceptors [18]. Predominant expression of GAL-1 and GAL-3 in subpopulations of small DRG neurons (Figure 1) suggests that these molecules are involved in the small fiber functions [2,3,14,16,19]. Consistent with these distribution patterns, mice lacking GAL-1 showed reduced sensitivity to noxious thermal stimuli [20]. Similarly, mice deficient in GAL-3 markedly reduced herpes zoster-associated pain [21]; however, the authors in that study attributed the herpetic pain to GAL-3 expressed in macrophages/microglia in the spinal cord, rather than that expressed in DRG neurons. It should also be added that adult DRG neurons are classified into three principal subgroups by their cell size and characteristic markers; large neurons (immunoreactive for 200 kD neurofilaments), small peptidergic neurons (immunoreactive for calcitonin gene-related peptide, substance P (SP) and high-affinity nerve growth factor (NGF) receptor TrkA), and small non-peptidergic neurons (immunoreactive for glial cell line-derived neurotrophic factor (GDNF) receptors RET and GFRα and binding to isolectin B4 (IB4)) [2,22]. Whether these two groups of small neurons have distinct functions has been the subject of controversy [23]. It is generally accepted that GAL-1 mRNA/protein is predominantly expressed in small non-peptidergic neurons (Figure 2) [2,24]. Although precise distribution of GAL-3 in DRG remains unclear, our recent study revealed predominant expression of GAL-3 in small IB4-binding DRG neurons with a considerable co-localization with GAL-1 [3].

The expression of GAL-1 mRNA/protein in DRG neurons was found to be up-regulated by peripheral nerve injury. Medium- to large-sized DRG neurons, which have weak GAL-1-immunoreactivity when uninjured, were found to increase the immunoreactivity 1–2 weeks after sciatic nerve axotomy [25]. GAL-1 mRNA/protein in spinal motoneurons similarly increased 7 and 14 days after spinal nerve axotomy [26]. These findings suggest that axtomy can trigger the synthesis of GAL-1 in cell bodies of DRG neurons and motoneurons. GAL-1 up-regulated in neuronal cell bodies may be transported to a damaged region and secreted [9,19]. We failed to find papers that described the expression of GAL-3 in DRG neurons or motoneurons after axotomy. However, GAL-3 (*aka* MAC-2) up-regulated in Schwann cells and macrophages after peripheral nerve injury may play a role in phagocytosis during Wallerian degeneration [27,28].

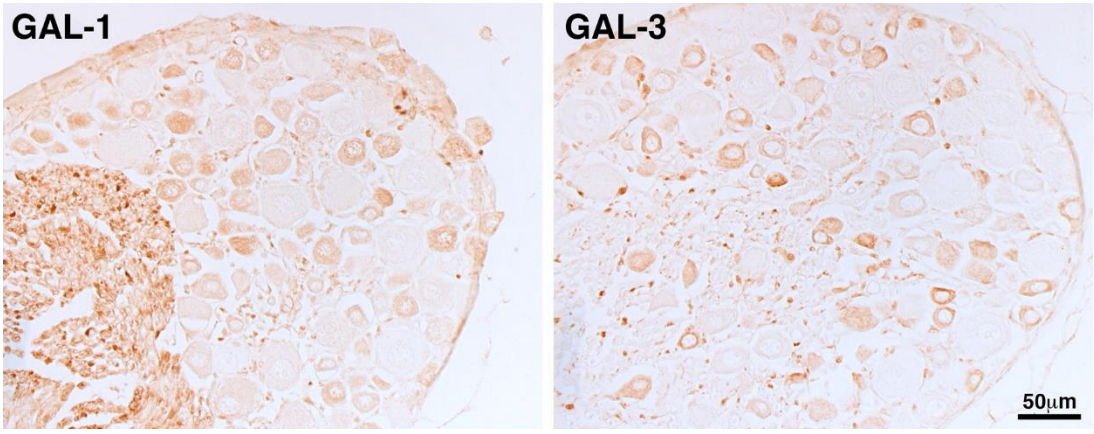

Figure 1. Immunohistochemical localization of GAL-1 and GAL-3 in the sections of adult rat DRG. The immunoreaction was visualized using 0.01% diaminobenzidine tetrahydro-chloride and 0.01% hydrogen chloride. The small neuron-dominant expression patterns of these galectins suggest their functional roles in nociception and thermoreception [2,3,14,16,19].

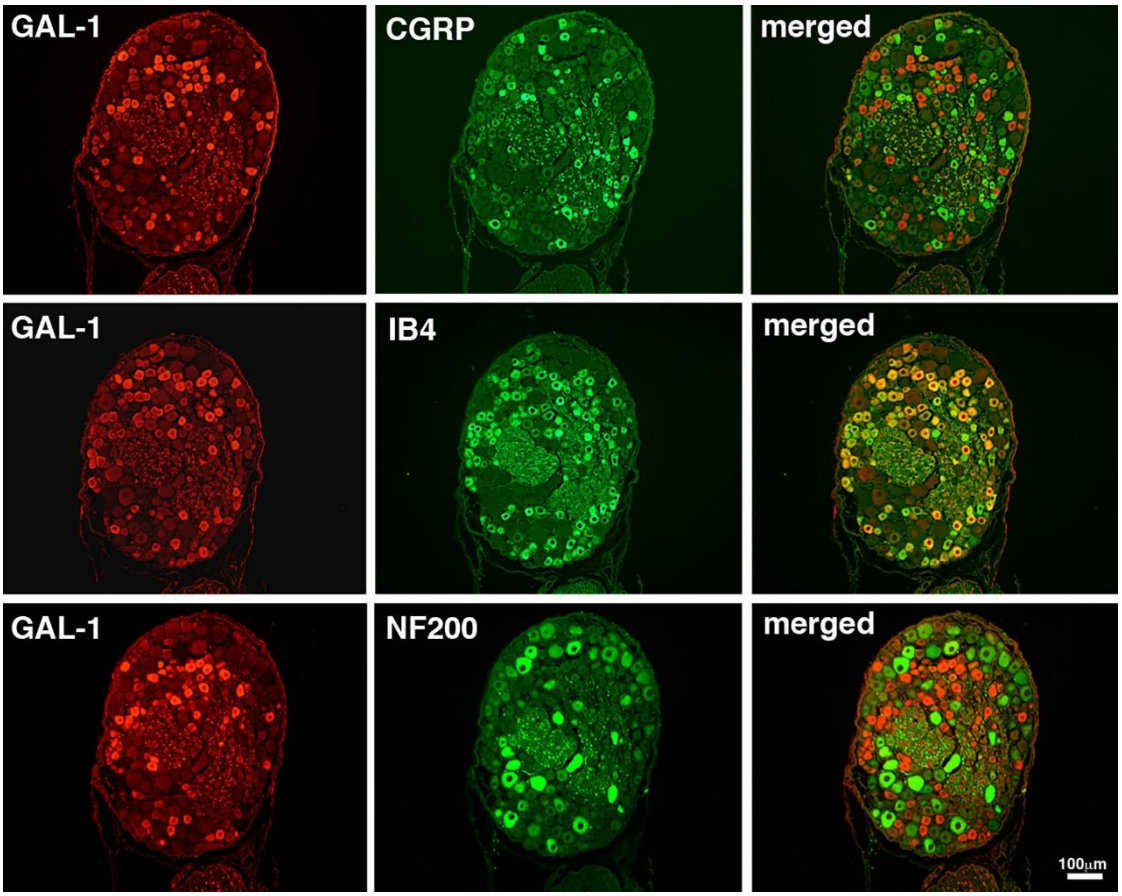

Figure 2. Immunofluorescence micrographs of adult rat DRG sections, stained with antibodies to GAL-1 (red) and CGRP, IB4, or NF200 (green). The merged pictures are on the right. GAL-1 is predominantly expressed in IB4-binding small non-peptidergic neurons [2,24] (reproduced from Sango et al., *Basic Principles of Peripheral Nerve Disorders* 2012 [2]).

2.2. Cultured neurons and Schwann cells

Intense GAL-1 immunoreactivity was detected in almost all DRG neurons in culture (Figure 3). Enzymatic and mechanical dissociation of DRGs to isolate neurons from non-neuronal cells can be a more potent inducer for the synthesis of GAL-1 than axotomy *in vivo* [19]. In contrast, we observed predominant expression of GAL-3 in small DRG neurons *in vitro* as well as *in vivo* (Figure 3). Double immunofluorescent staining showed a tendency that GAL-3 was co-localized with non-peptidergic neuron markers IB4 and $P2X_3$ purinoreceptor [29], but not with a peptidergic neuron marker SP (Figure 4). These findings suggest that the small non-peptidergic neuron-predominant expression pattern of GAL-3 is persistent after *in vivo/in vitro* replacement.

Because GDNF is likely to exert its major effects on small non-peptidergic neurons through RET and GFRα1 receptors [23,30], it seems plausible that synthesis and/or distribution of GAL-1 and GAL-3 in these neurons can be modulated by GDNF. We observed that recombinant GDNF applied to culture medium enhanced neurite outgrowth as well as protein expression of GAL-1 and GAL-3 in cultured adult rat DRG cells [3]. These findings suggest that both GAL-1 and GAL-3 are downstream target molecules for the GDNF signaling in DRG neurons. The possible functional roles of these galectins in GDNF-induced neurite outgrowth will be discussed in the 3rd section.

Both GAL-1 and GAL-3 are expressed in primary cultured Schwann cells, as well as immortalized adult rodent Schwann cells IMS32 and IFRS1 [3,4,19,31]. In contrast to DRG neurons, treatment with GDNF failed to up-regulate GAL-1 expression in IFRS1 cells [3], which implies that GDNF/GAL-1 signaling axis is more associated with neurite outgrowth from DRG neurons, rather than Schwann cell migration and myelination. Exposure to high glucose (30 mM) and 3-deoxyglucosone (3-DG), a precursor of advanced glycation endproducts (AGEs), significantly up-regulated GAL-3 expression in IFRS1 cells [4]. The high glucose-induced up-regulation of GAL-3 was abolished by co-treatment with an anti-glycated agent aminoguanidine or an anti-oxidant trans-resveratrol. These findings suggest the involvement of GAL-3 in the pathogenesis of diabetic neuropathy, such as glycation and oxidative stress. The roles of GAL-3 in Schwann cells under diabetic conditions will be described in the 4th section.

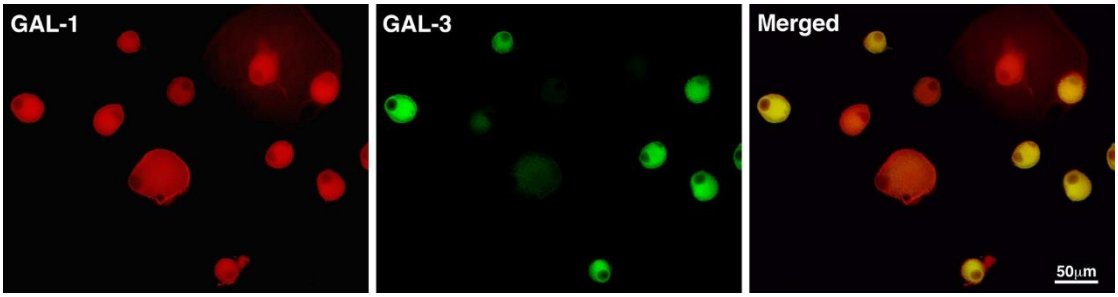

Figure 3. Immunohistochemical localization of GAL-1 (red) and GAL-3 (green) in adult rat DRG neurons after 2 days in culture. GAL-1 is distributed to almost all neurons [19], whereas GAL-3 is predominantly expressed in small neurons.

3. Roles of GAL-1 and GAL-3 in axonal regeneration

Successful nerve regeneration with functional repair in the PNS can be attributed to both intrinsic and extrinsic factors, such as the ability of neurons to regenerate axons, the distal

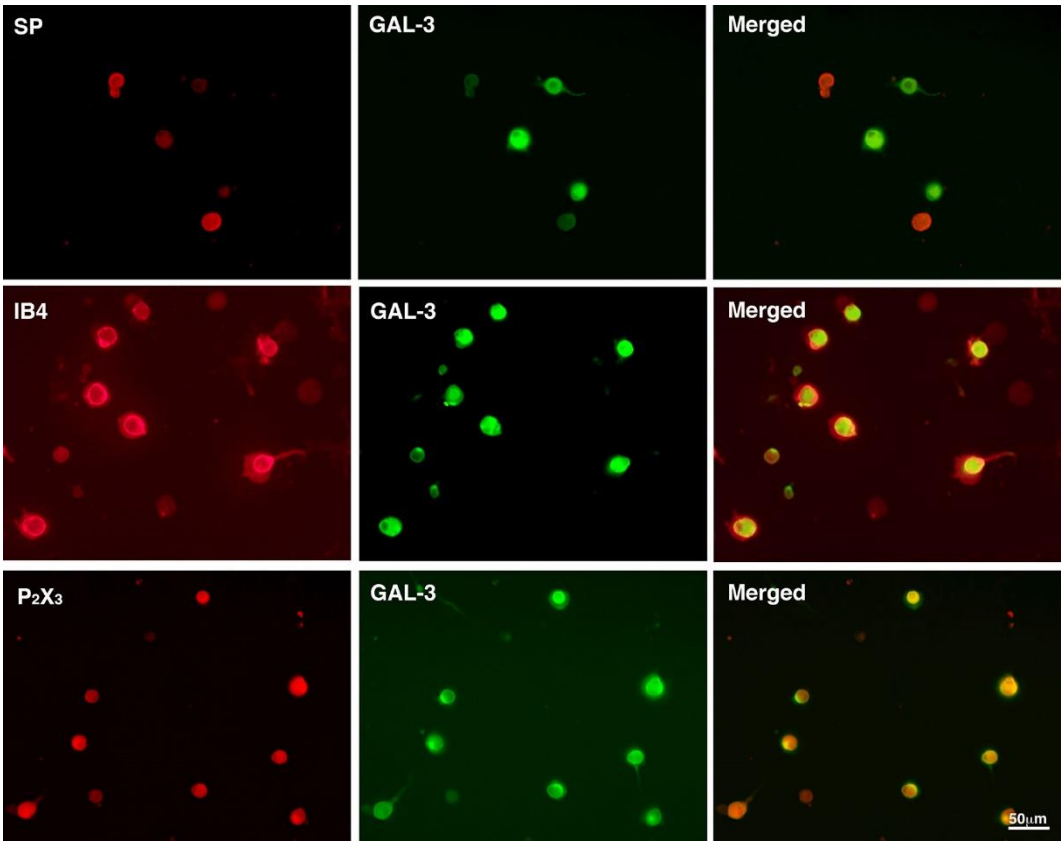

Figure 4. Immunohistochemical localization of GAL-3 (green) and substance P (SP), IB4, or P_2X_3 (red) in adult rat DRG neurons after 2 days in culture. SP is a peptidergic neuron marker [23], whereas IB4 and $P2X_3$ are non-peptidergic neuron markers [29]. The pictures indicate that GAL-3 is predominantly expressed in non-peptidergic neurons.

environment supportive of axonal growth, and the target tissues receptive to re-innervation [32]. The growing evidence suggest that both GAL-1 and GAL-3 are involved in the process of Wallerian degeneration distal to the injury and subsequent axonal regrowth and functional re-innervation [13]; both reduced and oxidized forms of GAL-1 have been shown to promote axonal regeneration *in vivo* and *in vitro*, whereas GAL-3 appears to be a key molecule in phagocytosis during Wallerian degeneration, as described above.

3.1. GAL-1 in the reduced form

When used as a coating substratum, recombinant reduced GAL-1 promoted adhesion and neurite fasciculation of newborn rat DRG neurons [33] and olfactory neurons in culture [34,35]. In contrast, however, we saw no significant effects of GAL-1 on attachment or neurite extension of adult rat DRG neurons [3]. Plachta et al. [11] identified GAL-1 in the reduced form as an inducible factor for the degeneration of neuronal processes growing from embryonic stem cell–derived neurons. They also observed a delay in the process of elimination of peripheral nerve endings after sciatic nerve injury in GAL-1 deficient mice. These finding suggest that GAL-1 is a key molecule for the normal onset of Wallerian degeneration.

3.2. GAL-1 in the oxidized form (GAL-1/Ox)

The bioactivity of recombinant GAL-1/Ox was extensively evaluated both *in vivo* and *in vitro* [2,7-9,15]. GAL-1/Ox dose-dependently (pg/mL range) increased the number and length of neurites emerging from transected nerve terminals of DRG explants embedded in collagen gel. A similarly low concentration of recombinant GAL-1/Ox also promoted the elongation rate of regenerating axons with migrating Schwann cells after peripheral nerve transection and crush *in vivo*. In contrast, treatment with recombinant GAL-3, which has a typical lectin activity under non-reducing conditions, failed to enhance axonal regeneration either in the *in vitro* or *in vivo* experiments [7]. Moreover, a GAL-1 mutant CSGAL-1, in which all six cysteine residues were replaced by serine, possessed the lectin activity even under non-reducing conditions but lacked the neurite outgrowth-promoting activity [8]. These findings suggest that the lectin properties are not involved in the function of GAL-1/Ox as a stimulant of axonal regeneration. A N-terminally processed from of GAL-1 (GAL-1β) exists as a monomer regardless of redox conditions, and displayed the neurite outgrowth-promoting activity equivalent to the full-length form of GAL-1/Ox [36]. Unlike the neurotrophic factors such as NGF and GDNF, recombinant GAL-1/Ox failed to enhance neurite outgrowth from isolated DRG neurons [2,3]. Although the receptors that specifically recognize GAL-1/Ox have not been identified, it is more likely that GAL-1/Ox stimulates non-neuronal cells to promote axonal regeneration rather than directly acts on neurons. Subsequent analyses by others and us have indicated that macrophages can be a target cell of GAL-1/Ox [37-41]. On the basis of these findings, we have proposed a possible action mechanism of GAL-1/Ox as follows; 1) GAL-1 in the reduced form is released from neurons and Schwann cells upon axonal injury, 2) some of GAL-1 molecule is converted to GAL-1/Ox in the extracellular space, and 3) GAL-1/Ox stimulates macrophages to secrete some neurotrophic molecules, which in turn enhances neurite regeneration and Schwann cell migration.

In normal mice, the axotomy-induced accumulation of macrophages in sciatic nerve distal to ligation was inhibited by injection of anti-GAL-1 antibody. In contrast, injection of GAL-1/Ox into uninjured sciatic nerve promoted the accumulation of macrophages. In agreement with these findings, GAL-1 deficient mice displayed delayed and diminished macrophage accumulation by sciatic nerve injury [40]. These findings suggest the involvement of GAL-1/Ox, as well as GAL-1 in the reduced from [11], in the prompt response of macrophages to axonal injury, which is essential for the normal onset of Wallerian degeneration.

3.3. Involvement of GAL-3 in Wallerian degeneration

As mentioned above, we failed to observe the significant bioactivity of recombinant GAL-3 on axonal regeneration *in vivo* (nerve transection/crush models) or *in vitro* (neurite elongation from DRG explants) [7]. However, the endogenous function of GAL-3 in the PNS after injury is well characterized by the striking phenotypes displayed by the genetic manipulation in mice [13,42,43]. Narcico et al. [42] reported that GAL-3 deficient mice displayed faster axonal regeneration with earlier functional recovery after sciatic nerve crush than their wild-type littermates. The following study by the same group [43] revealed enhanced phagocytic potential of macrophages and Schwann cells in GAL-3 deficient mice than wild-type mice. They also observed more robust up-regulation of RNA/protein expression of the pro-inflammatory cytokines IL-1β and TNF-α, as well as Toll-like

receptor-2 and -4, in injured nerves from GAL-3 deficient mice than those from WT mice. These findings suggest that the absence of GAL-3 augments the inflammatory responses and the phagocytic activity of macrophages and Schwann cells after axonal injury, thereby being a cause of the acceleration in the process of Wallerian degeneration. Although these studies have shed light on the pathological roles of GAL-3 in axonal regeneration, it is controversial whether GAL-3 inhibits the function of macrophages and Schwann cells after injury or disease in the PNS [3,4,12,13,27,28,44,45].

3.4. Involvement of GAL-1 and GAL-3 in GDNF-induced neurite outgrowth

Predominant expression of GAL-1 and GAL-3 in GDNF-responsive small non-peptidergic DRG neurons as described above led us to speculate that these lectins might be involved in the signaling pathways in GDNF-induced neurite outgrowth [46]. In our study [3], treatment of cultured DRG neurons with recombinant GDNF significantly increased the neurite length, as well as the population of IB4-binding neurons and the protein expression of GAL-1. The GDNF-induced DRG neurite outgrowth and GAL-1 up-regulation were abolished by anti-RET antibody and phosphatidyl inositol-3'-phosphate-kinase (PI3K) inhibitor LY294002. These findings suggest that the neurite-outgrowth promoting activity of GDNF may be attributable, at least partly, to the up-regulation of GAL-1 through RET-PI3K pathway. However, we observed no significant differences between GAL-1 deficient mice and wild-type mice in DRG neurite outgrowth in the presence or absence of GDNF. Some studies imply functional redundancies between GAL-1 and other lectin molecules, including GAL-3 [47]. Considerable colocalization of GAL-3 with GAL-1 in DRG sections and GDNF-induced up-regulation of GAL-3 as well as GAL-1 in DRG cultures suggest that the both galectins are downstream target molecules for the GDNF signaling in DRG neurons. In addition, recombinant GAL-3 used as a coating substratum slightly but significantly promoted neurite outgrowth from cultured DRG neurons, whereas recombinant GAL-1 had no significant effects. Although further analyses such as the selective knock down of GAL-3 and double knock down of GAL-1 and GAL-3 are required, it seems reasonable to suppose that GAL-3 might play a more crucial role than GAL-1 and/or compensate for GAL-1 deficiency in GDNF-induced neurite outgrowth.

4. Roles of GAL-1 and GAL-3 in the pathogenesis of peripheral neuropathies

4.1. Diabetic neuropathy

Metabolic disorders due to hyperglycemia, such as increased flux of the polyol and hexosamine pathways, formation of AGEs, alteration of protein kinase C activities, and increased oxidative stress, are believed to play a major role in the development and progression of diabetic neuropathy [48]. There is sufficient evidence to show that Schwann cells are highly vulnerable to these disorders [49], and IMS32 and IFRS1 cells have been utilized as useful tools for the study of diabetic neuropathy [31]. GAL-3 is recognized as an AGE-binding protein, but its significance in the pathogenesis of diabetic complications remains unclear [50]. As described above, we observed the up-regulation of GAL-3 in IFRS1 cells under diabetic conditions. In addition, recombinant GAL-3 applied to culture medium resulted in the significant up-regulation of an anti-apoptotic marker Bcl-2 and the downregulation of an oxidative stress marker 4-hydroxynonenal [4]. Furthermore, knockdown of GAL-3 expression in

IFRS1 cells by RNA interference resulted in the impaired cell viability after 7 days exposure to 3-DG (Niimi et al., unpublished data). These findings suggest cytoprotective properties of GAL-3 in Schwann cells under diabetic conditions. In agreement with our studies, GAL-3 deficient mice rendered diabetic by streptozotocin displayed more severe nephropathy with increased accumulation of renal glomerular AGEs than their normal littermates [51]. GAL-3 may play a role in the prevention of diabetic complications by removing the increased amounts of AGEs, but further evidence is needed to support this idea.

So far, no papers have documented the involvement of GAL-1 in the pathogenesis of diabetic neuropathy. However, recent studies have implicated GAL-1 as an angiogenic factor associated with proliferative diabetic retinopathy [52] and as a marker protein for the progression of diabetic nephropathy [53]. Our ongoing studies to establish immortalized Schwann cells from GAL-1 deficient mice will be helpful to indicate possible roles of GAL-1 in diabetic neuropathy.

4.2. Other neuropathies

Contrary to our expectations, surprisingly few studies have so far been made at the implications of GAL-1 and GAL-3 in peripheral neuropathies other than diabetic neuropathy. Giant axonal neuropathy (GAN) is hereditary neurodegenerative disease affecting both the CNS and PNS, and characterized by aggregates of intermediate filaments in different tissues. Proteomic analysis in cultured fibroblasts from patients with GAN and age- and gender-matched healthy people revealed the up-regulation of GAL-1 in the GAN samples [54]. Because GAL-1 plays a role in cytoskeletal regulation [10], its dysregulation might be involved in the formation of aggregates of intermediate filaments. Several studies have indicated the roles of GAL-1 and GAL-3 in regulation of myelination in the CNS and their involvement in demyelinating disorders such as multiple sclerosis [13,45,55]; however, little is known about their participation in demyelinating neuropathies [56].

5. Conclusion

We summarized the progress of research for nearly 3 decades on the distribution and function of GAL-1 and GAL-3 in the PNS. Although there is room for further investigation, it is no doubt that both galectins are key molecules for peripheral nerve degeneration and regeneration. The future direction of the research in this field will be to develop novel strategies for functional restoration after peripheral nerve injury and therapeutic efficacy against diabetic and other neuropathies. One of the critical issues for the therapeutic applications of GAL-1 and GAL-3 is establishing the effective systems to deliver these molecules toward the pathologically relevant sites [57]. Although our previous approaches for the therapeutic use of GAL-1/Ox against peripheral nerve injury [58] were unsuccessful, the progress of research on characterization of human GAL-1/Ox [59,60] would pave the way for its exploitation as a pharmaceutical product. In addition, recent studies indicate the beneficial effects of GAL-1 on wound healing [61] and GAL-3 against diabetes and other metabolic disorders [62], which may also be applicable to the prevention and treatment of diabetic neuropathy.

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Conflict of interest

All authors declare no conflict of interest in this paper.

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