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Galanin acts as a trophic factor to the central and peripheral nervous systems

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Abstract. The neuropeptide galanin is widely, but not ubiquitously, expressed in the adult nervous system. Its expression is markedly upregulated in many neuronal tissues after nerve injury or disease. Over the last 10 years we have demonstrated that the peptide plays a developmental survival role to subsets of neurons in the peripheral and central nervous systems with resulting phenotypic changes in neuropathic pain and cognition. Galanin also appears to play a trophic role to adult sensory neurons following injury, via activation of GalR2, by stimulating neurite outgrowth. Furthermore, galanin also plays a neuroprotective role to the hippocampus following excitotoxic injury, again mediated by activation of GalR2. In summary, these studies demonstrate that a GalR2 agonist might have clinical utility in a variety of human diseases that affect the nervous system. (Part of a Multi-author Review)

Keywords. Dorsal root ganglia, nociception, neuritogenesis, neuroprotection, neuronal survival.

Introduction

Since its discovery in 1983 galanin has been implicated in many diverse biological roles [1]. It has a widespread distribution throughout both the central (CNS) and peripheral nervous systems (PNS) [2–5], and following injury there is a dramatic increase in expression in many neuronal sub-populations [6–8]. This marked increase following injury has led a number of investigators to hypothesize that galanin may play a trophic role during development and/or in the adult after injury.

The physiological effects of galanin are mediated by the activation of one or more of the three known G-protein-coupled galanin receptor subtypes designated GalR1, GalR2 and GalR3. The receptors show high interspecies homology and moderate homology to each other. All three receptors couple to $G_{i/o}$ and inhibit adenylyl cyclase [9, 10] but GalR2 can in addition signal via $G_{q/11}$ to activate phospholipase C (PLC) and protein kinase C (PKC) [11, 12]. Few high-affinity galanin receptor-specific ligands exist, and the one tool that has been instrumental in delineating the functions of GalR1 and GalR2 has been the galanin fragment Gal(2–11) which acts as an agonist with 500-fold selectivity for GalR2 compared with GalR1. However, it has recently been demonstrated that Gal(2–11) can also bind and activate GalR3 in a transfected cell line with a similar affinity to GalR2 [13]. Most recently, GalR3-specific small molecule antagonists have been described [14], though these have yet to be widely studied other than in the field of depression.

This review will focus on the trophic roles played by galanin within the central and peripheral nervous systems.

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Nociceptor survival

Galanin is expressed at high levels in most cells of the developing dorsal root ganglion (DRG) from day 16 of gestation until shortly after birth [15]. In the adult, galanin is expressed at low levels in <5% of DRG neurons, which are predominantly small-diameter C-fibre nociceptors [8]. After sciatic nerve section (axotomy) galanin levels in the DRG rise by up to 120-fold [16, 17], and the peptide is abundantly expressed in ~40% of sensory neurons [18].

One of the first reports that galanin plays an important role in nociceptor survival was our finding that adult mice carrying a loss-of-function mutation in the galanin gene (Gal-KO) have a 13% reduction in the number of sensory neurons in the DRG [19] using unbiased stereological counting of L4 and L5 DRG. This finding was associated with a 24% reduction in substance P-expressing cells and a 15% reduction in calcitonin gene-related peptide (CGRP) expressing cells, both markers of small-diameter C-fibre neurons [19]. No differences were reported in the percentage of cells expressing either IB4 or neurofilament which mark non-peptidergic C-fibre and A-fibre neurons, respectively. This suggests that the absence of galanin leads to the preferential loss of small peptidergic neurons in the DRG, and is supported by the finding that there is a decrease in the percentage of the smallest diameter neurons in the Gal-KO [19]. The loss of a subset of small-diameter unmyelinated peptidergic neurons that are likely to be nociceptors appears to occur shortly after birth since there is a wave of apoptosis in the Gal-KO DRG that occurs at post-natal days 3 and 4 [19]. Most recently, we have shown that GalR2-KO mice also have a 15% reduction in the number of peptidergic neurons in the DRG [20]. Hokfelt and colleagues at the Karolinska Insitutet showed in another independently generated GalR2-KO line a 20% loss of neurons in the adult DRG measured by unbiased stereological counting [21]. Taken together, these studies further substantiate our hypothesis that the developmental survival role played by galanin in the DRG is mediated by activation of GalR2.

We therefore hypothesised that the loss of a subset of nociceptors shortly after birth in the Gal-KO and GalR2-KO animals might alter the development of injury-related pain behaviours in the adult animal. Our studies have shown that the Gal-KO animals demonstrate markedly diminished nociceptive behaviours in a number of different neuropathic and inflammatory pain models [19, 22, 23], associated with significantly reduced electrophysiological responses in the spinal cord after nerve injury or inflammation [24, 25]. Most recently, we have shown that GalR2-KO mice also have markedly diminished nociceptive behaviours in neuropathic and inflammatory pain models [20]. These data imply that the loss of a subset of sensory neurons that are likely to be nociceptors underlies the absence of allodynia observed in the Gal-KO and GalR2-KO animals. It also explains the apparent discrepancy between the absence of neuropathic pain behaviour in the Gal-KO and GalR2-KO animals and the previous body of published data demonstrating an inhibitory role for galanin in pain processing in the intact animal and in particular after nerve injury [22, 26, 27].

An analogous survival role in the adult DRG is also evident after nerve injury. Studies have shown that 7 days following axotomy approximately 24% of the DRG neurons in adult wildtype (WT) animals are lost [28]. However, studies using a galanin over-expressing transgenic mouse line show that the total number of DRG neurons lost following axotomy is less than in the WT controls, indicating a rescue effect of the high levels of galanin [29].

Neurite outgrowth

The trophic role of galanin in the DRG during development is recapitulated in the adult following peripheral nerve injury. Following a crush injury to the sciatic nerve regeneration is reduced by 35% in adult Gal-KO animals compared to the WT controls and this is associated with long-term sensorimotor functional deficits [19]. This impaired regenerative capacity *in vivo* is paralleled by a reduction in neuritogenesis *in vitro* of adult mouse-dispersed DRG neurons [19]. The number of sensory neurons from Gal-KO mice that produce neurites is reduced by a third, and neurite length is almost halved compared to the WT controls [19]. These deficits in neurite outgrowth can be rescued by the addition of galanin peptide (Fig. 1) [30]. These results are supported by the finding that both the mean axonal length and the number of branch points is increased in the neurites of dissociated rat DRG following treatment with galanin [31].

GalR1-KO mice have no reduction in regenerative capacity following a nerve crush injury [32] nor any impairment in neuritogenesis *in vitro* [30], suggesting that GalR1 is not responsible for mediating the regenerative role played by galanin. The deficits seen in neuritogenesis in GalKO mice can also be rescued by the addition of Gal(2–11) and inhibition of PKC reduced neurite outgrowth from WT cultures to levels observed in GalKO cultures [30]. These results suggest that GalR2 may be responsible for mediating the neuritogenic role of galanin. Most recently, this

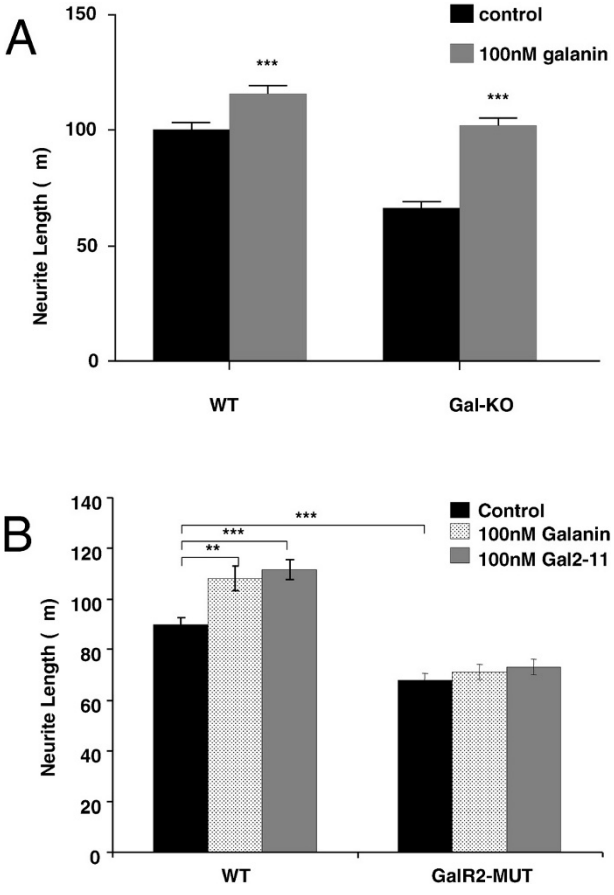

Figure 1. (*A*) Neurite length from dissociated DRG cultures isolated from WT and Gal-KO animals in the presence and absence of 100 nM galanin peptide. Addition of 100 nM galanin to WT cultures and Gal-KO significantly increased neurite length. Data are presented as mean length ± SEM (t-test *** $p < 0.001$). Figure reproduced from [30], with permission (copyright 2003 by Society for Neuroscience). (*B*) Neurite length from dissociated DRG cultures isolated from WT and GalR2-MUT mice in the presence and absence of 100 nM galanin or 100 nM Gal(2–11). GalR2-MUT animals demonstrated significant deficits in the neurite length compared with WT controls. Addition of galanin or Gal(2–11) to WT cultures significantly increased neurite length, whereas no significant differences were observed in the GalR2-MUT cultures (t-test ** $p < 0.01$, *** $p < 0.001$). Figure reproduced from [20], with permission.

hypothesis has been confirmed by the finding that GalR2-KO animals have a one-third reduction in neurite outgrowth from cultured adult DRG neurons which cannot be rescued by the addition of galanin or Gal(2–11) (Fig. 1) [20].

Neuronal survival in the CNS

In addition to galanin playing an important survival role to a subset of DRG neurons, the peptide also appears to be an important developmental survival factor for subpopulations of neurons within the brain. Galanin colocalises with choline acetyltransferase (ChAT) in approximately 30–35% of cholinergic neurons in the medial septum and the vertical limb diagonal band (VLDB) of the basal forebrain [3, 33], many of which project to the hippocampus [33, 34]. Following nerve injury both the mRNA and peptide levels of galanin have been shown to increase in many brain regions such as the medial septum and VLDB following a fibria fornix bundle transaction [6], the molecular layer of the hippocampus after entorhinal cortex lesion [35], the magnocellular secretory neurons of the hypothalamus following hypophysectomy [36], and the dorsal raphe and thalamus after removal of the frontoparietal cortex [7].

Studies in Gal-KO mice demonstrate that the chronic absence of galanin during development results in the loss of a third of the total number of cholinergic septo-hippocampal neurons [37]. Furthermore, this developmental cell loss appears to be associated with a wave of apoptosis in the forebrain at postnatal day 7 [37]. This loss is associated with marked age-dependent deficits in the Morris water maze and the induction of long-term potentiation in the CA1 region of the hippocampus [37].

Galanin may also play a trophic role to other CNS neuronal phenotypes. High levels of galanin and GalR1 and GalR2 are present in the subventricular and subgranular zones of the hippocampus both during development and in the adult [38, 39]. These areas are known to be sites of adult neurogenesis [40]. Recent findings have shown that neurospheres derived from adult hippocampal progenitor cells displayed increased neurite outgrowth following treatment with galanin [41], indicating a growth-promoting role for galanin in neurogenesis.

Neuroprotection

In light of the above data sets we hypothesised that galanin may also play a survival role following injury, by reducing cell death in hippocampal models of apoptosis and excitotoxicity. Our findings over the last 3 years demonstrate that following a peripheral injection of kainic acid, Gal-KO animals displayed a significantly higher degree of cell death in both the CA1 and CA3 regions of the hippocampus than WT controls [42]. In addition *in vitro* treatment with staurosporine or glutamate to both organotypic and dispersed primary hippocampal neurons induced significantly more cell death in Gal-KO animals than WT controls. In contrast, Gal-OE animals demonstrated less hippocampal cell death following kainic acid injection *in vivo* and staurosporine treatment in organotypic cultures, than their WT controls (Fig, 2). Furthermore, the addition of exogenous galanin

peptide or Gal(2–11) reduced the amount of cell death when co-administered with either glutamate or staurosporine in WT organotypic and dispersed primary hippocampal cultures (Fig. 2) [42]. These results demonstrate that galanin acts as a neuroprotective factor to the hippocampus. Most recently, we have extended these findings using GalR2-KO animals, demonstrating that organotypic cultures from GalR2-KO mice treated with glutamate show more cell death than the WT controls and this cannot be rescued with the addition of either exogenous galanin or Gal(2–11) (Fig. 2) [43]. In support of these findings studies have shown that glutamate-induced upregulation of both *c-fos* mRNA and c-Fos protein can be reversed using Gal(2–11) and the nuclear alterations, which are observed 24 h after glutamate exposure, are antagonised by Gal(2–11) in a dose-dependent manner [44]. Similarly, *in vivo* studies using an adeno-associated virus expressing the coding sequence for the galanin peptide fused to the fibronectin secretory signal sequence (AAV-FIB-GAL) reduces kainic acid-induced hippocampal cell death in rats [45]. In summary, these results suggest that the neuroprotective effects of galanin in the hippocampus are mediated via activation of GalR2.

The finding that one of the important physiological roles of the upregulation of galanin following injury may be its neuroprotective action has led people to study its potential trophic role in Alzheimer's disease (AD). In the basal forebrain of AD patients there is a significant loss of cholinergic neurons [46], an accumulation of β-amyloid [47, 48] and galanin immunoreactivity is significantly increased compared to normal controls [49–51]. Studies using cultured rat basal forebrain neurons show that pre-treatment with either galanin or Gal(2–11) can protect against β-amyloid-induced cell death [52], implying that a GalR2 agonist might reduce neuronal damage and/or slow down disease progression in AD.

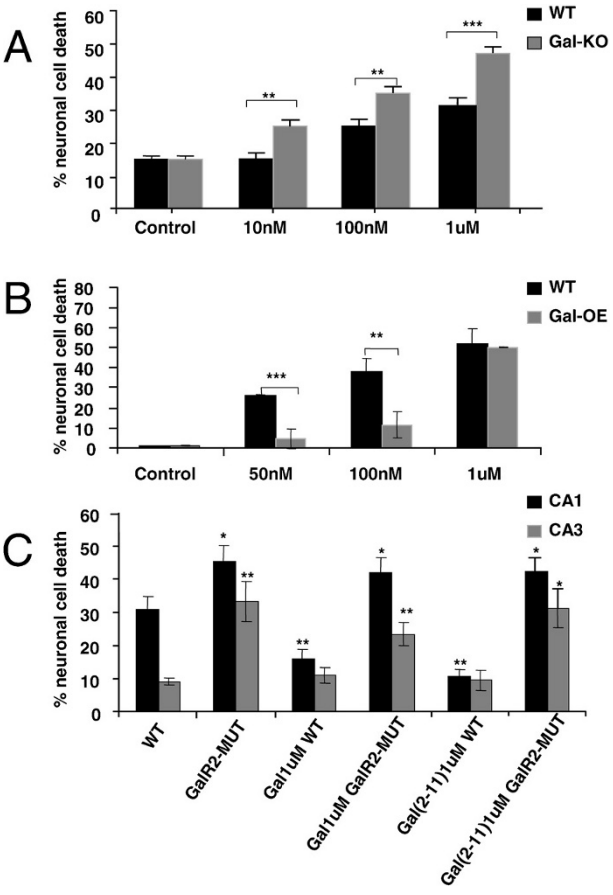

Figure 2. (*A*) Organotypic cultures from Gal-KO cultures after incubation with 10 nM–1 μM staurosporine had significantly more neuronal cell death than WT controls at each concentration of staurosporine in the CA1 subfield. Cell death was visualised by propidium iodide staining. Each point represents the mean ± SEM (** $p < 0.01$, *** $p < 0.001$). Figure reproduced from [42] with permission. (*B*) Organotypic cultures from galanin overexpressing (Gal-OE) mice were incubated with 50 nM–1 μM staurosporine. Gal-OE cultures had significantly less neuronal cell death than WT cultures at 100 and 50 nM staurosporine. Each point represents the mean ± SEM (** $p < 0.01$, *** $p < 0.001$). Figure reproduced from [42] with permission. (*C*) Effect of treatment with 2 mM glutamate on GalR2-MUT and WT hippocampal organotypic cultures, in the presence or absence of 1 μM galanin or Gal(2–11). Results demonstrate significantly greater cell death in the CA1 and CA3 regions of the hippocampus in GalR2-MUT than WT cultures. The protective effects of galanin or Gal(2–11) are abolished in GalR2-MUT cultures. Bars represent mean ± SEM (* $p < 0.05$, ** $p < 0.01$). Figure reproduced from [43] with permission.

Analysis of the enhancer regions of the galanin gene that confer responsiveness to axotomy

Many of the trophic roles played by galanin in the adult appear to be of particular physiological importance after nerve injury, when levels of the endogenous peptide are high. The upstream molecular cascades and transcription factors which mediate the upregulation in galanin gene transcription after neuronal injury have yet to be fully elucidated. Data shows that nerve growth factor (NGF) plays an inhibitory role on galanin expression [53–55], whilst LIF and IL-6 have been shown to positively regulate galanin gene expression [56, 57]. Both LIF and IL-6 bind to the gp130 coreceptor and are thought to principally regulate target gene activation by phosphorylation of Jak2 and Stat3 [57]. There appears to be crosstalk between the NGF and IL-6 pathways since IL-6-mediated Stat activation is inhibited in superior cervical explant cultures by NGF [58]. Most recently, we identified two regions upstream of the transcriptional start site of the galanin gene as being crucial to the upregulation of the peptide following axotomy. The deletion of an 18-bp region, approx-

imately 2.5 kb upstream of the start site which has overlapping putative Ets, Stat and Smad binding sites, and a 23-bp region, approximately 4.3 kb upsteam of the start site with adjacent Stat and Smad binding sites, abolishes the upregulation of galanin in the adult DRG after axotomy but has no effect on galanin expression during development or in the intact adult DRG (Fig. 3) [59]. Further, a bioinformatic analysis of the upstream regions of a number of other axotomy-responsive genes demonstrated that the close proximity of putative Ets, Stat and Smad binding sites appears to be a common motif in injury-induced upregulation in gene expression. Taken together, these findings imply that Ets, Stat and/or Smad proteins not only regulate galanin expression, but may also control the transcription of other axotomy-responsive genes in the DRG. If these nuclear protein complexes and the genes they regulate can be further defined and dissected, then that may lead to the identification of new therapeutic targets for the treatment of neuropathy and associated neuropathic pain.

Galanin- and GalR2-dependent signalling cascades

Many of the trophic effects discussed in this review appear to be mediated via activation of GalR2, but the molecular cascades downstream of the receptor subtype are as yet largely unknown. There is now an increasing body of evidence to suggest that DRG activation of extracellular signal-related kinase (ERK) and the serine/threonine kinase Akt occurs in nociceptors after injury [60] and that both of these kinases have been shown to play survival roles in sensory neurons [61, 62]. Studies using Chinese hamster ovary (CHO) cell lines which stably express either GalR1 or GalR2 show that galanin can elevate the levels of mitogen-activated protein kinase (MAPK and hence ERK) by 2–3-fold [11]. Galanin has also been shown to activate ERK by 1.6-fold in a PKC-dependent manner in a GalR2-expressing small-cell lung cancer clonal cell line [11; 63]. We recently demonstrated that galanin can activate both ERK and Akt in WT mouse DRG and that intact adult DRG from GalR2-KO animals has lower levels of pERK and higher levels of pAKT than the WT controls [20]. Similarly, the addition of galanin rapidly stimulates the levels of both phosphorylated ERK and Akt in WT mouse organotypic hippocampal cultures [43]. Furthermore, the use of specific inhibitors of ERK and Akt confirms a GalR2-dependent modulation of both the ERK and Akt signalling pathways in hippocampal neuroprotection [43]. These data therefore indicate a role for both the ERK and Akt signalling pathways in a number of the trophic roles played by galanin; however, additional studies are needed to further delineate these pathways.

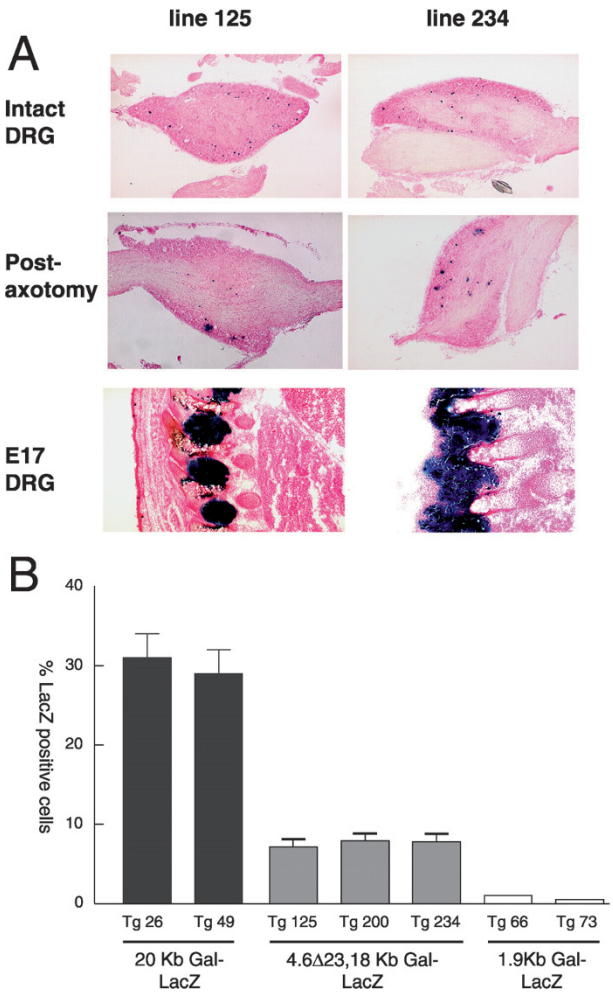

Figure 3. (*A*) Representative photomicrographs demonstrating expression in two expressing 4.6Δ23,18-kb lines. For each line the expression pattern was determined for a minimum of 5 animals. Expression similar in distribution and intensity to the 20-kb line was noted in the intact DRG (top panel) and in the embryonic DRG harvested at day 17 of gestation (bottom panel). In contrast, expression in the adult DRG after axotomy was completely abolished. (*B*) Quantitation of the number of β-galactosidase-positive cells in adult DRG neurons after 3 days in culture. 30±3 % of adult DRG neurons from the 20-kb transgenic animals were strongly positive for β-galactosidase staining. No β-galactosidase staining was noted in neurons harvested from any of the 1.9-kb lines. Only 8±1 % of DRG neurons exhibited β-galactosidase staining in the three 4.6Δ23,18 lines. Figure reproduced from [59], with permission (copyright 2007 by Society for Neuroscience).

Conclusion

There is an increasing body of evidence to suggest that galanin plays developmental survival roles within neuronal subpopulations of the PNS and CNS. Fol-

lowing injury, galanin also appears to stimulate neurite outgrowth in the adult DRG and play a neuroprotective role in the hippocampus. In addition, studies suggest that galanin may play a similar neuroprotective role in AD protecting neurons from β-amyloid-induced cell death. Many of these survival and trophic effects appear to be mediated via activation of GalR2, which can in turn activate both the ERK and Akt signalling pathways. These studies imply that GalR2 agonists might have clinical utility in a range of diseases that might include neuropathy, neuropathic pain, stroke and AD.

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