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Mice deficient for galanin receptor 2 have decreased neurite outgrowth from adult sensory neurons and impaired pain-like behaviour

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Abstract

Expression of the neuropeptide galanin is markedly up-regulated within the adult dorsal root ganglia (DRG) following peripheral nerve injury. We have previously demonstrated that galanin knockout (Gal-KO) mice have a developmental loss of a subset of DRG neurons. Galanin also plays a trophic role in the adult animal, and the rate of peripheral nerve regeneration and neurite outgrowth is reduced in adult Gal-KO mice. Here we describe the characterization of mice with an absence of *GalR2* gene transcription (GalR2-MUT) and demonstrate that they have a 15% decrease in the number of calcitonin generelated peptide (CGRP) expressing neuronal profiles in the adult DRG, associated with marked deficits in neuropathic and inflammatory pain behaviours. Adult GalR2-MUT animals also have a one third reduction in neurite outgrowth from cultured DRG neurons that cannot be rescued by either galanin or a high-affinity GalR2/3 agonist. Galanin activates extracellular signal-regulated kinase (ERK) and Akt in adult wild-type (WT) mouse DRG. Intact adult DRG from GalR2-MUT animals have lower levels of pERK and higher levels of pAkt than are found in WT controls. These data suggest that a lack of GalR2 activation in Gal-KO and GalR2-MUT animals is responsible for the observed developmental deficits in the DRG, and the decrease in neurite outgrowth in the adult.

Keywords

dorsal root ganglia; galanin; GalR2; neurite outgrowth; pain; signalling

One of the largest changes observed in sensory neurons following peripheral nerve injury is the increase in the expression of the neuropeptide galanin (Hokfelt *et al.* 1987). In the intact adult dorsal root ganglia (DRG) galanin is expressed at low levels in only 2-3% of neurons (Hokfelt *et al.* 1987). Following nerve transection (axotomy) there is a marked up-regulation in the levels of the peptide, and 40-50% of DRG neurons express galanin while the nerve is regenerating (Hokfelt *et al.* 1994).

We have previously shown that galanin plays a critical role in the postnatal survival of a subset of small-diameter, unmyelinated peptidergic sensory neurons that are likely to be nociceptors (Holmes *et al.* 2000). These deficits may explain the marked reduction in both neuropathic and inflammatory pain behaviours observed in the galanin knockout (Gal-KO)

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animals (Kerr *et al.* 2001; Holmes *et al.* 2003). In addition to its role during development, galanin also appears to play a trophic role in the adult animal; Gal-KO animals have a 35% reduction in the rate of peripheral nerve regeneration following a crush injury to the sciatic nerve. Similarly, there is a 40% reduction in neuritogenesis from cultured adult DRG neurons (Holmes *et al.* 2000), which can be rescued by the addition of galanin peptide (Mahoney *et al.* 2003).

The above physiological effects of galanin are likely to be mediated by the activation of one or more of the three G-protein-coupled galanin receptor subtypes, designated GalR1, GalR2 and GalR3. All three receptors couple to $G_{i/o}$ and inhibit adenylyl cyclase (Habert-Ortoli *et al.* 1994; Smith *et al.* 1998), although GalR2 also signals via $G_{q/11}$ to activate phospholipase C (PLC) and protein kinase C (PKC) (Wittau *et al.* 2000). Rat GalR1 and GalR2 are both expressed in the DRG by differing, but overlapping, subpopulations of mainly medium-or small-diameter neurons, respectively, and the levels of both subtypes decrease after axotomy (Xu *et al.* 1996b; Sten Shi *et al.* 1997). Expression of GalR3 in the rat DRG is either very low, as determined by RT-PCR (Waters and Krause 2000), or undetectable using *in situ* hybridisation (Mennicken *et al.* 2002). An analysis of the function of each galanin receptor subtype has been hampered both by the lack of receptor-specific ligands to modulate their activity, and by specific antisera that may be used for immunohistochemistry to describe their cellular localization. The one tool that has been instrumental in describing the function of GalR1 and GalR2 is the galanin fragment galanin 2-11 (Gal2-11, also known as AR-M1896), which acts as an agonist with 500-fold selectivity for GalR2 compared with GalR1 (Liu *et al.* 2001). Use of Gal2-11 suggests that GalR2 appears to play either little or no role in the antiallodynic effects of galanin in neuropathic pain states (Liu *et al.* 2001). Studies in our laboratory using Gal2-11 have demonstrated that GalR2 appears to be the predominant receptor subtype that mediates neurite outgrowth from adult sensory neurons, in a PKC-dependent manner (Mahoney *et al.* 2003).

The intracellular signalling pathways that mediate the survival, trophic and nociceptive roles played by GalR2 activation are unknown, but there is an increasing body of literature to show that DRG activation of extracellular signal-regulated kinase (ERK) and the serine/threonine kinase Akt (also known as protein kinase B, PKB) occurs in nociceptors after nerve damage (Pezet *et al.* 2005). Furthermore, both kinases have been shown to play survival roles in adult sensory neurons (Liu and Snider 2001; Edstrom and Ekstrom 2003). Previous work using Chinese hamster ovary (CHO) cell lines that stably express either GALR1 or GALR2 showed that both receptors can elevate the activation of mitogen-activated protein kinase (MAPK, and hence ERK) by 2-3-fold (Wang *et al.* 1998). Furthermore, studies on small-cell lung cancer clonal cell lines that express GalR2 and galanin demonstrated that GalR2 activation increases ERK phosphorylation by 1.6-fold, in a PKC-dependent manner (Seufferlein and Rozengurt 1996; Wang *et al.* 1998). Most recently we have shown that hippocampal organotypic cultures from Gal-KO mice and mice with an absence of GalR2 gene transcription (GalR2-MUT) have deficits in the activation of Akt and ERK (manuscript under submission).

Here we describe the phenotypic analysis of mutant mice deficient for GalR2, and demonstrate that the receptor appears to transduce the neuritogenic effects of galanin in the adult DRG. We hypothesize that a GalR2-dependent alteration in the phosphorylation of the ERK and/or Akt signalling pathways in the adult may be responsible for the observed deficits in the GalR2-MUT and Gal-KO animals.

Materials and methods

Animals and genotyping

Mice deficient for the *GalR2* gene were generated and licensed from Lexicon Genetics (Lexicon Genetics, The Woodlands, TX, USA). The 5.17-kb gene-trap vector VICTR48 (viral construct for trapping) was inserted within the single intron of the murine *GalR2* gene in a 129Sv/EvBrd embrionic stem (ES) cell line clone (Zambrowicz *et al.* 2003). This Omnibank clone (OST105469; http://www.lexicon-genetics.com/discovery/omnibank.htm) was used to obtain germ-line transmission of the disrupted *GalR2* allele. Heterozygote pairs on the C57BL/6J × 129/SvEvBrd background were transferred to the University of Bristol and then bred to homozygosity, and have been maintained on that background.

Animals were genotyped by PCR from tail DNA using an intronic GalR2 forward primer (5′-CCCTTCCCACAGTAGAGCGGTGAAG-3′, IR2for2; AF042784 nt 1052-1076) located upstream of the gene-trap insertion site, a GalR2 exon 2 reverse primer (5′-ACTGACTGTAGTAGCTCAGGTAGG-3′, IR2rev; AF042784 nt 1579-1556) and a primer in the gene-trap long-terminal repeat (5′-AAATGGCGTTACTTAAGCTAGCTTGC-3′, IR2LTR, e.g. V01541 nt 129-104). Cycling conditions were 94 °C for 40 s, 65 °C for 60 s and 72 °C for 40 s (for 37 cycles), and amplified products were separated on 1.3% agarose gels.

RNA extraction, RT and RT-PCR of fusion transcripts

Total RNA isolation, DNase treatment and re-extraction and RT reactions with random hexamers were carried out as previously described (Kerr *et al.* 2004). The GalR2/Neo fusion transcript of GalR2-MUT was amplified using either1 μL (20 ng total RNA equivalent) or 5 μL of RT reaction in 50 μL RT-PCRs with FastStart High Fidelity Taq (Roche, East Sussex, UK), the GalR2 TaqMan forward primer GalR2-20F (see below) and the *Escherichia coli* transposon Tn5 neomycin phosphotransferase (Neo) antisense primer 5′-CCAGTCCCTTCCCGCTTCAGT-3′ (V00618, nt 423-403) (Beck *et al.* 1982). The vector/GalR2 fusion transcript of GalR2-MUT was amplified using primers IR2LTR and IR2rev (see above). Cycling conditions were 94 °C for 30 s, 64 °C for 30 s and 72 °C for 40 s (for 40 cycles). For each RT-PCR, the single product of interest was cut out from a 2% agarose gel, purified (GenElute; Sigma, St Louis, MO, USA) and cloned (Kerr *et al.* 2004).

Real-time quantitative (TaqMan) RT-PCR

Quantification of relative mRNA levels utilized real-time quantitative (TaqMan) RT-PCR assays (Giulietti *et al.* 2001). Primer and probe sets were designed using default parameters of Primer Express software (Applied Biosystems, Warrington, UK), unless specified, and were synthesized by Applied Biosystems. Assay probes contained fluorescent reporter dye FAM (6-carboxyfluorescein) at the 5′ end and quencher dye TAMRA (6-carboxy-tetramethyl-rhodamine) at the 3′ end, except for the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) control that had a VIC reporter dye.

The GalR1 primer set spanned intron 2 (~ 8 kb) (Jacoby *et al.* 1997; Wang *et al.* 1997), with the forward primer not encoding a transmembrane (TM) domain: 5′-GTCAAAAAAGTCTGAAGCATCCAA-3′ (mGalR1-105F); 5′-GCCAGGATATGCCAAATACTACAA-3′ (mGalR1-184R); and antisense probe 5′-ACCACCAGGACGGTCTGTGCAGTCTT-3′ (mGalR1-133TAQ). These correspond, respectively, to nt 1020-1043, 1099-1076 and 1073-1048 of Y15004 (Wang *et al.* 1997).

The GalR2 primer set spanned the single intron (~ 1.1 kb) (Pang *et al.* 1998), with the reverse primer (sense strand) not encoding a TM domain and the Primer Express default

settings for amplicon minimum length being adjusted to 100 bp: 5′-TCTGCAAGGCCGTTCATTTC-3′ (mGalR2-20F); 5′-TAGCGGATGGCCAGATACCT-3′ (mGalR2-119R); and probe 5′-CGCTGGCCGCTGTCTCCCTG-3′ (mGalR2-77TAQ). These correspond, respectively, to nt 287-306, 383-364 and 341-360 of AF077375 (Pang *et al.* 1998).

The GalR3 primer set is within exon 2 (AF042783), with default settings for amplicon maximum temperature (T_m) increased to 90 °C: 5′-GCTGGCGGCGCTCTTT-3′ (mGalR3-156F); 5′-TAGCCTGCGGCGAAGGT-3′ (mGalR3-297R); and probe 5′-ACGGCACGGTGCGCTACGG-3′ (mGalR3-201TAQ). These correspond, respectively, to nt 1494-1509, 1628-1612 and 1532-1550 of AF042783.

The galanin primer set was designed to span intron 2 (682 bp) (Kofler *et al.* 1996), with a low primer sequence identity (50%) to available galanin-like peptide (GALP) cDNA sequence (Jureus *et al.* 2001): 5′-CTGGCTCCTGTTGGTTGTGA-3′ (mGAL-180F); antisense probe 5′-CATCCCAAGTCCCAGAGTGGCTGACA-3′ (mGAL-203TAQ); and 5′-CCAACCTCTCTTCTCCTTTGCA-3′ (mGAL-252R). These correspond, respectively, to nt 30-49 and 78-53 of L38576, and to nt 89-69 of L38577 joined to nt 81 of L38576 (Kofler *et al.* 1996).

Endogenous control GAPDH primers were: 5′-GCAGTGGCAAAGTGGAGATTG-3′ (GAP65F); 5′-CTGGAACATGTAGACCATGTAGTTGA-3′ (GAP138R); and probe 5′-CCATCAACGACCCCTTCATTGAC-3′ (GAP89Taq). These correspond, respectively, to nt 111-131, 184-159 and 135-157 of M32599.

Replicate 25 μL PCRs included 12.5 μL TaqMan Universal PCR Master Mix (Applied Biosystems), water (Sigma), primers, probe and template in a 96-well microtitre plate (Applied Biosystems). Standard cycling conditions (95 °C for 10 min, followed by 95 °C for 15 s and 60 °C for 1 min, for 50 cycles) were used in an ABI PRISM 7900 Sequence Detection System (Applied Biosystems). Primer and probe concentrations were optimized against either corresponding cDNA plasmids or RT reactions (GalR3) (data not shown).

Each total RNA sample was used for two duplicate reactions containing reverse transcriptase (RT+), and one without enzyme (RT−) as a control for the RT-dependence of the product. No product was detected at 50 cycles in RT-samples of either GalR1-3 or galanin assays (data not shown), and a single product of the expected size was made in RT+ samples from DRG, as determined on a 4% agarose gel (data not shown). Data analysis was performed using SDS2.0 software (Applied Biosystems), with default fluorescence threshold settings.

DRG neurite outgrowth

Dispersed DRG cultures were prepared from age-matched female mice (8 weeks) and enriched for neuronal cells by pre-plating for 16 h as previously described (Holmes *et al.* 2000). Cells were replated on to 24-well plates treated with 0.5 mg/mL polyornithine and 5 μg/mL laminin, 100 000 cells per well in Dulbecco's modified Eagle's medium (DMEM)/F12 medium supplemented with 5% horse serum, 1 mM glutamine and 10 ng/mL gentamycin, and cultured for a further period of 4 h at 37°C in a humidified incubator with 95% air, 5% CO_2. At the end of the 20-h experiment, cultures were washed with phosphate-buffered saline (PBS) and fixed with 4% parafomaldehyde/PBS for 20 min at room temperature (22°C). Cells were visualized using differential interference contrast using a Leica DMIRB microscope (Leica, Milton Keynes, UK) and images were captured using a Leica DC500 camera. The percentage of cells bearing neurites and the neurite length were

both measured using NIH IMAGE (Scion, Frederick, MD, USA). Data are presented as mean ± SEM and the Students *t*-test was used to analyse significance.

Histological analysis

For immunohistochemistry, age-matched (10-12 weeks), male GalR2-MUT and WT mice were deeply anaesthetized and intracardially perfused with 4% paraformaldehyde/PBS, and post-fixed for 4 h at room temperature (22°C). L4 and L5 DRGs were dissected and equilibrated in 20% sucrose overnight at 4°C, embedded in OCT mounting medium, frozen on dry ice and then cryostat sectioned at 8-μm thickness. Sections were blocked and permeabilized in 10% normal donkey serum/PBS 0.2% Triton X-100 (PBST) for 1 h at room temperature (22°C). Sections were then incubated in PBST overnight at room temperature, with IB4 (Sigma), primary antisera against calcitonin gene-related peptide (CGRP; Biomol, Exeter, UK), galanin (Biomol) or neurofilament 200 (Sigma), as previously described (Holmes *et al.* 2000, 2003). Sections were then washed 3 × 10 min in PBS and incubated in the appropriate fluorescein isothiocyanate (FITC)-conjugated secondary antibody (Sigma) at a ratio of 1 : 200 for 3 h at room temperature (22°C). After washing, sections were mounted in Vectashield (Vector Laboratories, Burlingame, CA, USA). Images were taken using a Leica fluorescent microscope with a Leica DC500 camera. Cells with a visible nucleolus were counted and six sections were counted per DRG ($n = 4$).

Galanin expression in cultured DRG neurons was detected using immunocytochemistry. Cultures were prepared and fixed with 4% paraformaldehyde/PBS as described above, and were pre-plated for 16 h followed by 4 h of re-plating on glass coverslips coated with polyornithine and laminin. Cells were blocked and permeabilized in 10% normal donkey serum/PBST for 1 h at room temperature (22°C). Cells were then incubated overnight at room temperature (22°C) in PBST containing galanin primary antibody at a ratio of 1 : 1000 (Peninsula Laboratories Inc., Bachem, Weil am Rhein, Germany). Cells were washed 3 × 10 min in PBS and incubated with anti-rabbit FITC (Sigma) at 1 : 200 for 3 h at room temperature (22°C). After washing coverslips were mounted with Vectashield (Vector Laboratories). Cells were visualized using both differential interference contrast and fluorescence using a Leica DMIRB.

Surgery

Age-matched male adult mice (10-12-weeks-old, 25-30 g) were used, with a group size of at least eight animals per genotype. Animals were fed standard chow and water *ad libitum*. All experiments were carried out in accordance with the United Kingdom Animals (Scientific Procedures) Act 1986.

A modification of the spared nerve injury model (SNI) of neuropathic pain, originally described in the rat, was used (Decosterd and Woolf 2000). In this model the tibial branch of the mouse sciatic nerve is spared (cutting the common peroneal and sural branches) producing a robust and consistent model of allodynia (Shields *et al.* 2003). Surgery was performed as previously described (Holmes *et al.* 2003).

Behavioural Testing

In all tests the examiner was blind to the mouse genotype. Thermal thresholds were measured according to the method of Hargreaves *et al.* (1988) as previously described (Holmes *et al.* 2003). Mechanical thresholds were measured by stimulating the medial plantar surface (tibial nerve territory) of the hindpaw with a series of calibrated von Frey filaments (Stoelting) as previously described (Holmes *et al.* 2003).

The formalin test was performed based on the method of Dubuisson and Dennis (1977) as previously described (Kerr *et al.* 2001). Formalin (30 μL of 5% formalin) injection elicits a characteristic biphasic response (Tjolsen *et al.* 1992) and the results were scored as time spent in pain-like behaviour in the first (0-10 min) and the second phase (10-60 min).

Data are presented as the mean ± SEM. The Student's *t*-test was used to analyse the difference in baseline thermal withdrawal thresholds and responses to formalin. Non-parametric Mann-Whitney *U post hoc* tests were used to analyse differences between genotypes and at different time points following SNI. A p-value of < 0.05 was considered to be significant.

Western blotting

DRG explants were lysed in 45 μL of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE) sample buffer containing 62.5 mM Tris-HCl (pH 6.8), 2% (w/v) SDS, 10% glycerol and 5% β-mercaptoethanol. Extracts were heated to 95-100°C for 5 min, sonicated for 5 min in an ice-cold water bath and centrifuged at 16 000 *g* for 5 min to remove insoluble material. Samples were then subjected to gel electrophoresis using discontinuous SDS–PAGE slab gels (12% running; 5% stacking). Molecular weight markers were also electrophoresed, and the resolved proteins were then transferred to a nitrocellulose membrane at 100 mA for 75 min. The membrane was then blocked with Trisbuffered saline (TBS) containing 5% non-fat dry milk and 0.1% Tween-20 at room temperature for 1 h, and subsequently incubated overnight at 4°C in 1 : 1000 primary antibody solutions of phospho-ERK, phospho-Akt, total ERK or total Akt (Cell Signalling Technology Inc., Beverly, MA, USA) in TBS containing 5% bovine serum albumin and 0.1% Tween-20. Nitrocellulose membranes were washed three times with TBS-Tween-20 (0.1%), and incubated with goat anti-rabbit antibody conjugated with horseradish peroxidase (1 : 2000 in blocking solution) for 1 h at room temperature. Following washing three times with TBS-Tween-20 (0.1%), immunoreactivity was detected by enhanced chemiluminescence (Pierce, Rockford, IL, USA) onto Hyperfilm ECL (Amersham Pharmacia Biotech, Piscataway, NJ, USA). Band density was analysed using NIH IMAGE and bands were normalized to a percentage of control value ($n = 4$).

Results

The *GalR2* gene-trap mutation results in an absence of *GalR2* gene transcription

Genotyping by PCR from genomic DNA demonstrated a 383 bp product from GalR2-MUT, whereas the endogenous GalR2 product of 528 bp was detected in WT mice; both products were apparent in heterozygote animals (Fig. 1). The DNA sequence of the endogenous GalR2 product was identical to that previously published (Pang *et al.* 1998), and the gene-trap vector was inserted between nt 858/859 of the 1.06-kb intron (AF042784, nt 1246/1247).

GalR2 mRNA containing exons 1 and 2 was detected in whole brain, DRG, trigeminal ganglia and kidney of WT mice using real-time quantitative RT-PCR, as expected from previous studies in the rat (Smith *et al.* 1997; Sten Shi *et al.* 1997; Ahmad *et al.* 1998; O'Donnell *et al.* 1999; Burazin *et al.* 2000). In contrast, no product was detected in mice homozygous for the *GalR2* mutation (Table 1), and expression levels were reduced by approximately 50% in heterozygous animals (data not shown).

Insertion of the targeting vector would be expected to replace the endogenous GalR2 transcript with two fusion transcripts: (a) from the endogenous GalR2 promoter, a transcript of GalR2 exon 1 spliced to *Neo*, and (b) from the vector phosphoglycerate kinase-1 (*Pgk1*) promoter, the first exon of the mouse Bruton's tyrosine kinase gene (*Btk*) spliced to exon 2

of GalR2. The GalR2/Neo transcript was readily detected as a single product of 392 bp by RT-PCR in GalR2-MUT, but not in WT DRG. DNA sequencing of multiple cDNA clones confirmed the presence of two in-frame stop codons downstream of GalR2 exon 1 (data not shown), thereby allowing the translation of a non-functional GalR2 protein containing only the first three transmembrane α-helices. The second fusion transcript, Btk/GalR2, was not detected by RT-PCR using a number of different forward and reverse primer pairs (data not shown). However, an abundant product extending from the vector, through the downstream intron of GalR2 and into exon 2, was readily detected. DNA sequencing confirmed the GalR2 intron included multiple stop codons in all reading frames (data not shown and AF042784), and the exon 2 sequence has open reading frames encoding either only 8 or 14 amino acids each out-of-frame compared with GalR2.

Lack of apparent compensatory changes in either GalR1 or galanin expression in the DRG of GalR2-MUT mice

Real-time quantification RT-PCR assays were used to address potential compensatory changes in the expression of galanin and the other galanin receptors, analogous to the up-regulation of GalR1 detected in Gal-KO animals (Hawes *et al.* 2005) and galanin over-expressing animals (Hohmann *et al.* 2003). Similar levels of both GalR1 and galanin mRNAs were observed in intact lumbar DRG, whole brain, trigeminal ganglion and kidney of adult WT and GalR2-MUT mice (Table 1). Expression of GalR3 mRNA was detectable and RT-dependent, but levels were too low for reliable comparisons between genotypes (data not shown), consistent with the previously reported low levels of expression in the rat (Waters and Krause 2000).

One week after axotomy the expression levels of galanin mRNA in the DRG rose to a similar extent in WT and GalR2-MUT mice (35.4- and 41.2-fold, respectively), whereas GalR1 mRNA expression fell by 37% in both genotypes. GalR2 expression decreased by 28.2% in WT animals and was undetectable in the GalR2-MUT animals.

DRG neurite outgrowth is reduced in GalR2-MUT cultures

Before using an *in vitro* model to study the role played by galanin in neuritogenesis, it was important to confirm that the up-regulation of galanin observed in the DRG after axotomy also occurs in our culture paradigm. Immunocytochemistry for galanin was therefore performed on dispersed adult WT sensory neurons 20 h after removal from the animal, demonstrating that 40 +/− 3.5% of DRG neurons express the protein at that time point (Fig. 2). This finding is in keeping with a previous study which demonstrated that cultured adult rat sensory neurons express measurable quantities of galanin 12 h after removal from the animal (Kerekes *et al.* 1997).

Our previous data indicated that galanin-mediated neuritogenesis in the adult DRG (Holmes *et al.* 2000) is predominantly a GalR2-dependent process (Mahoney *et al.* 2003), although some involvement of GalR1 and/or GalR3 could not be excluded. The addition of either 100 nM galanin or 100 nM Gal2-11 significantly increased neurite length in WT cultures (Fig. 3a), as previously demonstrated (Mahoney *et al.* 2003). GalR2-MUT cultures demonstrated significant deficits in neurite length (68 μm vs. 90 μm, Fig. 3a) and the percentage of neurons able to extend neurites (20% vs. 35%, Fig. 3b), compared with WT controls. These deficits were not rescued by the addition of either 100 nM galanin or 100 nM Gal2-11 (Figs 3a and b).

Markers for subpopulations of DRG neurons in GalR2-MUT mice

In light of the possibility that GalR2 mediates the previously described developmental survival role played by galanin, we measured the number of neuronal profiles in the adult

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DRG of WT and GalR2-MUT that expressed a number of standard markers. No differences were noted in the percentage of neuronal profiles expressing neurofilament, IB4 or galanin between adult GalR2-MUT and WT mice (data not shown). In contrast, there was a significant ~15% decrease in the number of neuronal profiles that express CGRP (Fig. 4).

GalR2-MUT animals have decreased neuropathic and inflammatory pain-like behaviours

Baseline thresholds were measured in intact adult GalR2-MUT and age-matched WT controls. The results demonstrate that both genotypes had similar thresholds to both mechanical (1.299 ± 0.109 g vs. 1.382 ± 0.163 g in WT mice, Fig. 5a) and thermal stimuli (12.9 ± 0.3 s vs. 13.95 ± 0.05 s in WT mice). These data are consistent with two recent studies that also demonstrate no differences in thermal and/or mechanical thresholds, respectively, in two other lines of mice deficient in *GalR2* gene transcription (Gottsch *et al.* 2005; Shi *et al.* 2006).

GalR2-MUT mice were then tested for mechano-sensory allodynia for 14 days following an SNI model (Holmes *et al.* 2003) of neuropathic pain (Fig. 5a). WT mice developed mechanical allodynia of the plantar hindpaw ipsilateral to the injured nerve within 2 days of SNI surgery, which was maintained for the duration of the experiment. Following SNI, GalR2-MUT mice failed to develop allodynia throughout the experiment. Un-operated and sham-operated animals showed no significant change in withdrawal thresholds over the testing period (data not shown).

We also tested the GalR2-MUT mice and WT controls in the formalin model of inflammatory pain (Fig. 5b). WT mice demonstrated a typical biphasic response to the acute inflammation elicited by the injection of formalin into the hindpaw. No difference was seen in the time spent in pain-like behaviour between GalR2-MUT and WT mice during the first phase. However, during the second phase GalR2-MUT mice spent a significantly shorter length of time in pain-like behaviour than that observed for WT controls. No differences in hind-paw swelling or inflammation were noted between the genotypes (data not shown).

Downstream signalling pathways utilized by GalR2

We used western blotting to study whether exogenous galanin could increase the phosphorylation of ERK and/or Akt in acutely extirpated DRG from adult WT and GalR2-MUT animals. Results demonstrate that ERK1 is rapidly activated within 5 min of the addition of galanin in the WT DRG (Fig. 6a), but no significant activation was observed in GalR2-MUT animals (data not shown). In contrast, galanin significantly stimulated pAkt in both WT and GalR2-MUT animals within 5 min (Figs 6b and c). We then looked for differences in the expression of phosphorylated ERK1 and Akt in the DRG from intact adult WT and GalR2-MUT animals. Results demonstrate that phosphorylated ERK1 is decreased by 24% in GalR2-MUT animals compared with WT controls (Fig. 6d), whereas phosphorylated Akt levels are increased by 35% (Fig. 6e).

Discussion

The aim of the present study was to use mice deficient for GalR2 to further define the role played by the receptor in sensory neuron survival, neuritogenesis in the adult and in the modulation of pain behaviour. GalR2-MUT mice were generated by the insertion of a gene-trap vector within the single intron of the murine *GalR2* gene. The data presented here demonstrate that a *GalR2*-deficient mouse has been generated in which the GalR2 mRNA has been replaced by two non-functional fusion transcripts.

Previous studies have shown that both Gal-KO and galanin over-expressing animals demonstrate an up-regulation in GalR1 levels (Hohmann *et al.* 2003; Hawes *et al.* 2005). We

therefore used RT-PCR to address any such potential compensatory changes in GalR2-MUT animals. No change in either galanin or GalR1 expression was detected in intact tissues of GalR2-MUT mice compared with WT controls. In addition, following axotomy the levels of galanin mRNA in the DRG rose to a similar extent in both GalR2-MUT and WT animals, whereas GalR1 mRNA expression fell in both genotypes. The fall in GalR1 and GalR2 expression after axotomy in the mouse is consistent with previous findings in the rat (Sten Shi *et al.* 1997; Xu *et al.* 1996b). We conclude therefore that both galanin and GalR1 mRNAs are independent of *GalR2* expression in the intact adult DRG and in the DRG following axotomy.

We have previously demonstrated, using Gal2-11, that GalR2 appears to be the predominant receptor mediating both neurite outgrowth from adult sensory neurones (Mahoney *et al.* 2003) and the neuroprotective effects of the peptide in the hippocampus (Elliott-Hunt *et al.* 2004). However, recently Gal2-11 has also been shown to bind and activate GalR3 in a transfected cell line with a similar affinity to GalR2 (Lu *et al.* 2005). Although with such low levels of GalR3 present in the DRG it is unlikely that the significant effect of Gal2-11 on neuritogenesis is mediated through this receptor, although some involvement of GalR1 and/or GalR3 in neuritogenesis could not be excluded. Here we report that GalR2-MUT cultures demonstrated significant deficits in both neurite length and the percentage of neurones able to extend neurites compared with WT controls. These findings are similar to those previously described in the Gal-KO (Mahoney *et al.* 2003) and confirms that GalR2 plays a key role in neurite outgrowth. As the addition of either galanin (which would activate GalR1 and GalR3) or Gal2-11 (which would activate GalR3) failed to rescue the deficits in the GalR2-MUT cultures, these findings imply that neither the GalR1 nor GalR3 receptor subtypes play a major role in galanin-mediated neuritogenesis of adult sensory neurons.

Galanin has been shown to play an important role in neuronal cell survival in late development, as Gal-KO mice display a 13% decrease in the total cell number of the DRG (Holmes *et al.* 2000) and a loss of a third of the cholinergic neurons of the basal forebrain (O'Meara *et al.* 2000). The observed increase in apoptosis in the DRG that occurs in the Gal-KO animals at P3/4 is consistent with the reported high levels of galanin in the DRG and dorsal horn of the spinal cord, shortly after birth (Xu *et al.* 1996a). Our findings that the GalR2-MUT animals demonstrate a 15% decrease in the number of CGRP-positive neuronal profiles in the DRG compared with WT controls, analogous to that previously described in the Gal-KO animals (Holmes *et al.* 2000), implies that this survival role is a GalR2-dependent process. During the preparation of this manuscript a study of another *GalR2*-deficient mouse has been published; the major finding being that the absence of GalR2 gene transcription causes a loss of 20% of neurons in the DRG, as measured by unbiased stereological counting (Shi *et al.* 2006). These findings are in agreement with our own data reported above, and further substantiate our hypothesis that the developmental survival role played by galanin is mediated by the activation of GalR2.

The loss of a subset of neurons in the DRG of the GalR2-MUT animals may explain our findings that the animals have substantial deficits in neuropathic and inflammatory pain behaviours, similar to that previously described in the Gal-KO animals. It is noteworthy that the recent description of another GalR2-MUT line demonstrated no significant differences in allodynia between GalR2-MUT and WT mice following the Chung spinal nerve injury model (Shi *et al.* 2006). The most obvious explanations for the apparent discordance in nociceptive behaviours between these two different GalR2-MUT lines are that each study has used a different model of neuropathic pain, and the animals have been generated on differing genetic backgrounds (strain differences have been shown to play a significant role in pain perception; Mogil *et al.* 1999). In order to define which of these explanations are

correct, it would be necessary to perform the spinal nerve injury and SNI models of neuropathic pain on both GalR2-MUT transgenic lines.

An alternative explanation for the significant deficits in the neuropathic and inflammatory pain behaviours observed in the GalR2-MUT mice is that GalR2 may play a pronociceptive role following nerve injury, as previously suggested (Hua *et al.* 2004). This hypothesis is compatible with the work of Carlton and colleagues who have demonstrated that low-dose intraplantar injection of either galanin or Gal2-11 both increase capsaicin-induced inflammatory pain behaviour (Jimenez-Andrade *et al.* 2004). However, this would not be in keeping with the findings of Liu *et al.* (2001), who demonstrated that high doses of Gal2-11 had no effect on mechanosensory thresholds in either allodynic or non-allodynic rats (using the chronic constriction injury model of neuropathic pain), and concluded that the antiallodynic effects of high-dose galanin in various neuropathic pain models may be mediated via GalR1. Thus, a further interpretation of our data is that the high levels of galanin in the DRG after nerve injury in the GalR2-MUT animals activates GalR1 in the absence of a functional GalR2, explaining the increase in withdrawal thresholds after SNI, especially at days 5 and 7 after injury.

As GalR2 activation appears to be a key function in both the developmental survival and the neuritogenic role played by galanin in the adult DRG following injury, we asked which downstream intracellular signalling pathways might transduce these effects and focussed on ERK and Akt. Here we show that exogenous galanin activates ERK in the adult WT DRG, and the levels of phosphorylated ERK are decreased in GalR2-MUT DRG compared with WT controls. These findings are consistent with previous studies demonstrating that GalR2 couples to PKC and ERK (Seufferlein and Rozengurt 1996; Wang *et al.* 1998), and with recent work showing that an inhibition of ERK significantly impaired neurite outgrowth in the adult DRG (Chierzi *et al.* 2005). This in turn may explain the decrease in neurite outgrowth observed in the Gal-KO and GalR2-MUT animals. The finding that galanin stimulates Akt activation in the WT DRG, and yet levels are increased in the GalR2-MUT animals, is unexpected and intriguing. An increasing body of work has demonstrated cross-talk between the ERK and Akt signalling pathways (Campbell *et al.* 2004; Merighi *et al.* 2005). Furthermore, Akt has been shown to inhibit the ERK pathway in a tissue-specific manner (Rommel *et al.* 1999; Zimmermann and Moelling 1999; Moelling *et al.* 2002). It is possible therefore that the increase in phosphorylated Akt is a consequence of the deficit in either ERK activation or another, as yet unidentified, signalling pathway that couples to GalR2. An alternative explanation, consistent with the observed stimulation in pAkt by exogenous galanin in the GalR2-MUT animals, is that GalR1 and/or GalR3 activates Akt, which would explain the rise in pAkt observed in the GalR2-MUT DRG. In support of this the inhibitory G-protein $G_{i/o}$ (utilized by GalR1 and GalR3) has been shown to couple to phosphatidylinositol-3-kinase and Akt (Wu and Wong 2005).

In summary, the absence of GalR2 expression at a critical point in DRG development in both GalR2-MUT and Gal-KO animals appears to induce the loss of a subset of sensory neurons that are likely to be nociceptors. This neuronal loss may underlie the observed deficits in neuropathic and inflammatory pain behaviours in both strains of animal, although the possibility that GalR2 plays a pro-nociceptive role after either nerve injury or tissue damage cannot at present be discounted. Similarly, the lack of GalR2 activation may well explain the observed deficits in neuritogenesis in the adult GalR2-MUT and Gal-KO animals. The above data would be compatible with the hypothesis that a GalR2-dependent alteration in the phosphorylation of either one or more of the downstream signalling pathways activated by GalR2 (such as ERK and/or Akt), may be responsible for the observed deficits in both strains of animals.

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Abbreviations used

Btk	Bruton's tyrosine kinase gene
CGRP	calcitonin gene-related peptide
CHO	Chinese hamster ovary
DRG	dorsal root ganglia
ERK	extracellular signal-regulated kinase
ES	embrionic stem
Gal2-11	galanin 2-11
Gal-KO	galanin knockout
GalR2-MUT	mice with an absence of *GalR2* gene transcription
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
PBS	phosphate-buffered saline
PBST	PBS with 0.2% Triton X-100
PKC	protein kinase C
SDS–PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SNI	spared nerve injury model
TBS	Tris-buffered saline
TM	transmembrane
VIC	viral construct
WT	wild-type

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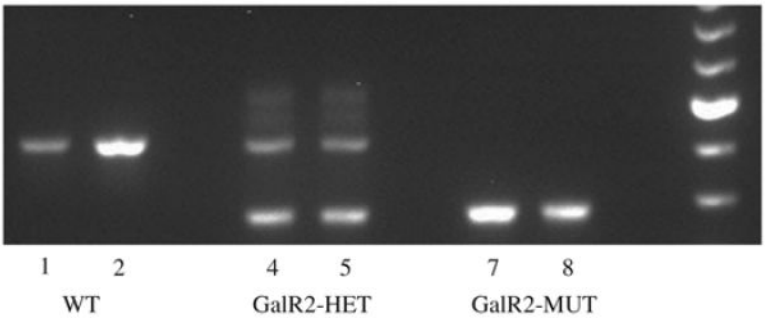

Fig. 1.

Detection of the gene-trap vector within the GalR2 intron. Genotyping of mouse tail DNA by PCR resulted in products of 528 bp from two WT mice (Lanes 1 and 2), products of 528 and 383 bp from two heterozygotes (GalR2-HET, lanes 4 and 5) and products of 383 bp from two mice homozygous for the GalR2 gene-trap mutation (lanes 7 and 8). The 383-bp product extends from the gene-trap vector LTR into the intron of GalR2. Lanes 3 and 6 are blank.

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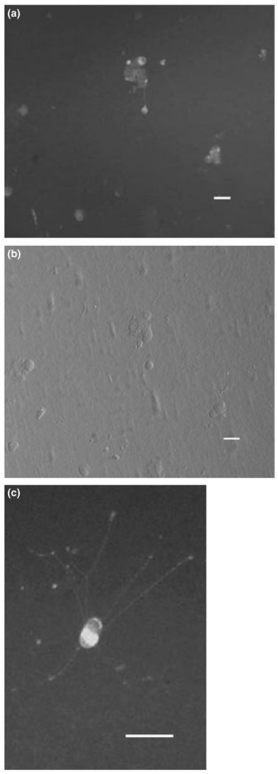

Fig. 2.
(a) Galanin staining in DRG cultures after 20 h in culture; (b) the same neurons viewed with differential interference contrast; and (c) a high-powered image of a galanin-positive neuron. Scale bars indicate 50 μm.

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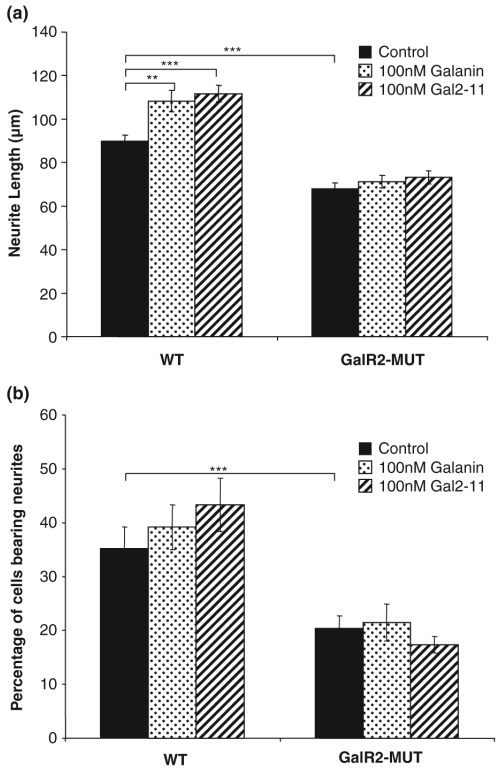

Fig. 3.

Neurite length (a) and the percentage of cells bearing neurites (b) from dissociated DRG cultures isolated from wild-type (WT) mice and mice with an absence of *GalR2* gene transcription (GalR2-MUT) in the presence and absence of either 100 nM galanin or 100 nM Gal2-11 4 h after replating. The addition of either galanin or Gal2-11 to WT cultures significantly increased neurite length, whereas no significant differences were observed in the percentage of cells producing neurites. GalR2-MUT animals demonstrated significant deficits in the number of cells producing neurites and neurite length compared with WT controls. These deficits could not be rescued by the addition of either galanin or Gal2-11 (Student's *t*-test: $^{**}p < 0.01$; $^{***}p < 0.001$; $n = 4$).

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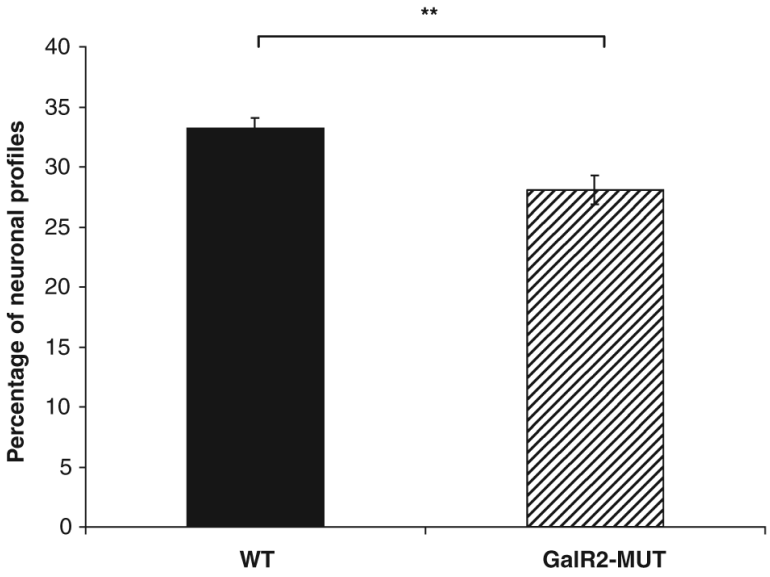

Fig. 4.

The percentage of neuronal profiles expressing calcitonin gene-related peptide (CGRP) in the adult DRG of wild-type (WT) mice and mice with an absence of *GalR2* gene transcription (GalR2-MUT). GalR2-MUT animals demonstrated a significant decrease in the percentage of neuronal profiles expressing CGRP. (Student's *t*-test: $^{**}p < 0.01$; $n = 4$).

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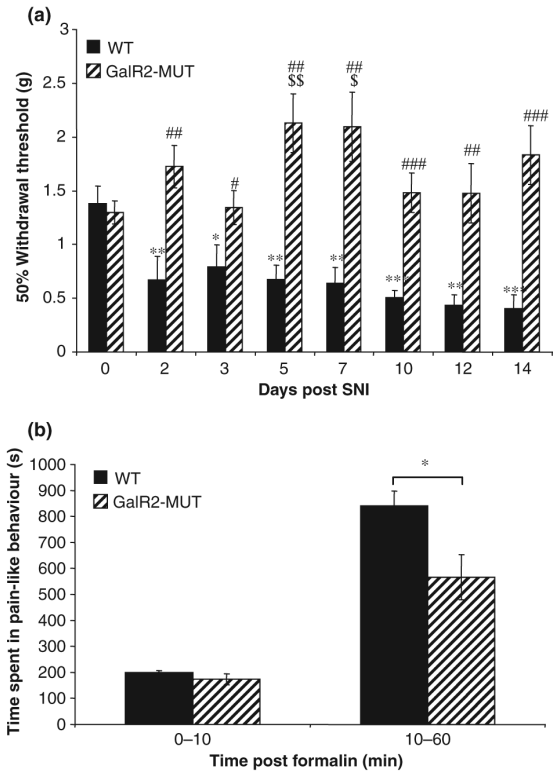

Fig. 5.

(a)Response of wild-type (WT) mice and mice with an absence of *GalR2* gene transcription (GalR2-MUT) ($n = 12$) to mechanical stimuli. Pre-surgery 50% withdrawal thresholds are denoted as day 0 and were not significantly different between genotypes. WT animals developed robust allodynia within 2 days of spared nerve injury (SNI) (Mann–Whitney *U* test: $p < 0.01$), which was maintained for the duration of the experiment. GalR2-MUT animals failed to develop allodynia throughout the duration of the experiment. *WT day 0 group compared with WT groups at each time point; $^{\$}$GalR2-MUT day 0 group compared with GalR2-MUT groups at each time point; $^{\#}$WT compared with GalR2-MUT groups at each time point (*$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$; $^{\$}p < 0.01$ and $p < 0.001$; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.01$). (b) Responses of WT and GalR2-MUT mice (n = 12) in the formalin test. Analysis of behaviour in the distinct phases of formalin responding reveals that the GalR2-MUT animals show similar pain behaviour in the acute first phase, but a significant reduction in pain behaviour in the prolonged second phase post-formalin injection (Student's *t*-test: *$p < 0.05$).

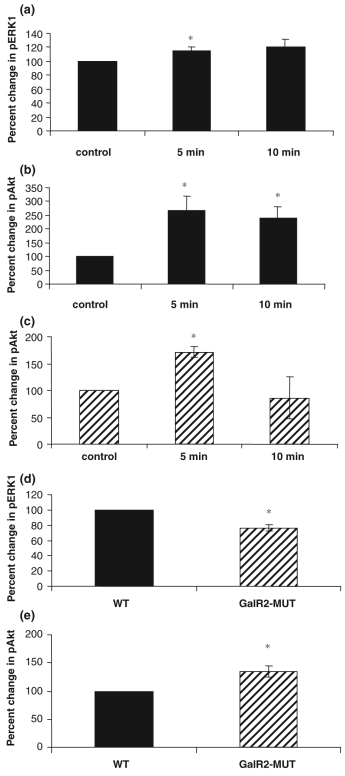

Fig. 6.

Graphical representation of protein expression levels of phosphorylated extracellular signal-regulated kinase 1 (pERK1) (a) and pAkt (b and c) from acutely extirpated adult DRG from wild-type (WT) mice, and pAkt from acutely extirpated adult DRG from mice with an absence of *GalR2* gene transcription (GalR2-MUT) following exposure to 100 nM galanin. The results demonstrate that ERK1 and Akt are phosphorylated within 5 min of the addition of galanin in WT DRG and in pAkt in GalR2-MUT animals. Panels (d) and (e) show graphical representations of protein expression levels for pERK1 (d) and pAkt (e) from intact adult WT and GalR2-MUT animals, demonstrating a decrease in pERK1 and an increase in pAkt. (Student's *t*-test: $*p < 0.05$, $n = 4$).

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Table 1

The relative expression levels of GalR1, GalR2 and galanin mRNAs in tissues from mice with an absence of *GalR2* gene transcription (GalR2-MUT) compared with wild-type (WT) animals

Transcript	Genotype	DRG	Brain	TG	Kidney
GalR2 mRNA	WT	100.0%	100.0%	100.0%	100.0%
	GalR2-MUT	ND	ND	ND	ND
GalR1 mRNA	WT	100.0%	100.0%	100.0%	100.0%
	GalR2-MUT	87.9%	104.7%	92.6%	78.6%
Galanin mRNA	WT	100.0%	100.0%	100.0%	100.0%
GalR2-MUT	124.9%	91.9%	111.8%	93.7%	

Quantitative RT-PCR assays were performed on intact (unaxotomised) DRG from five pairs of either wild-type (WT) mice or mice with an absence of *GalR2* gene transcription (GalR2-MUT). Brain, trigeminal ganglion (TG) and kidney were screened using a pool of either WT or GalR2-MUT RNA. No major differences were detected in either galanin or GalR1 expression between the genotypes in any of the tissues. No GalR2 mRNA was detected (ND) in GalR2-MUT animals.