Altered primary afferent anatomy and reduced thermal sensitivity in mice lacking galectin-1

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Abstract

The transmission of nociceptive information occurs along non-myelinated, or thinly myelinated, primary afferent axons. These axons are generally classified as peptidergic (CGRP-expressing) or non-peptidergic (IB4-binding), although there is a sub-population that is both CGRP-positive and IB4-binding. During neuronal development and following injury, trophic factors and their respective receptors regulate their survival and repair. Recent reports also show that the carbohydrate-binding protein galectin-1 (Gal1), which is expressed by nociceptive primary afferent neurons during development and into adulthood, is involved in axonal pathfinding and regeneration. Here we characterize anatomical differences in dorsal root ganglia (DRG) of Gal1 homozygous null mutant mice (Gal1+/−), as well as behavioural differences in tests of nociception. Gal1+/− mice have a significantly reduced proportion of IB4-binding DRG neurons, an increased proportion of NF200-immunoreactive DRG neurons, increased depth of central terminals of IB4-binding and CGRP-immunoreactive axons in the dorsal horn, and a reduced number of Fos-positive second order neurons following thermal (cold or hot) stimulation. While there is no difference in the total number of axons in the dorsal root of Gal1+/− mice, there are an increased number of myelinated axons, suggesting that in the absence of Gal1, neurons that are normally destined to become IB4-binding instead become NF200-expressing. In addition, mice lacking Gal1 have a decreased sensitivity to noxious thermal stimuli. We conclude that Gal1 is involved in nociceptive neuronal development and that the lack of this protein results in anatomical and functional deficits in adulthood.

Keywords: L-14; IB4; CGRP; DRG; Fos; c-fiber

1. Introduction

Small diameter dorsal root ganglion (DRG) neurons transmit nociceptive information from the periphery to the central nervous system (CNS). They have thinly-myelinated and unmyelinated small diameter axons and comprise approximately 70% of all neurons within the DRG (Snider and McMahon, 1998). Nociceptive neurons can be further subdivided based on neurochemistry and termination pattern within the spinal cord: those expressing the neuropeptide calcitonin gene-related peptide (CGRP) terminate in laminae I and II outer (IIo) (Averill et al., 1995), while non-peptidergic C afferents that express the ATP purinoceptor receptor P2X3 (non-peptidergic neurons) and bind the lectin Bandeiraea simplicifolia (IB4) terminate in lamina II inner (IIi) of the spinal cord (Vulchanova et al. 1998; Bradbury et al., 1998; Chen et al., 1995; Molliver et al., 1997). Large-caliber axons carrying proprio- and mechanoeceptive information terminate in deeper laminae (III-X) and are identifiable within the DRG by their expression of the large molecular weight neurofilament NF200 (Lawson et al., 1984).

During development, different neurotrophic factors regulate distinct functional classes of sensory neurons.
For example, the nerve growth factor (NGF)-specific receptor TrkA localizes to 70–80% of all DRG neurons early in development (Molliver and Snider, 1997; Molliver et al., 1995) and is required for their survival (Crowley et al., 1994; Silos-Santiago et al., 1995; Smeyne et al., 1994).

However, as development proceeds, half of the NGF-dependent neurons lose their TrkA expression and begin to express Ret, the signaling receptor for the glial cell line-derived neurotrophic factor (GDNF) family of neurotrophic factors (Molliver and Snider, 1997; Molliver et al., 1997), leading to an adult DRG population comprised of 40% TrkA- and CGRP-immunoreactive (IR) neurons and 30% Ret-expressing and IB4-binding neurons. During development and into adulthood in the rat, these small-diameter sensory somata as well as expression in spinal motor neurons (then termed RL-14.5) protein expression in 63% of primary sensory neurons began at E13–14 as they finished their final mitotic division and began their growth towards their targets within the dorsal horn of the spinal cord. When Gal1-expressing neurons reached their targets, Gal1 expression remained elevated, albeit at lower levels (Hynes et al., 1990; Regan et al., 1986; Sango et al., 2004; St John and Key, 1999). Furthermore, exogenous Gal1 protein promotes DRG axonal growth in vitro and in vivo (Horie and Kadoya, 2000; Horie et al., 1999, 2004). These data strongly suggest that Gal1 plays a role in nociceptive sensory neuronal outgrowth and maintenance. However, to date the role of Gal1 in sensory neuron development remains unknown.

Gal1 homozygous null mutant (Gal1 <sup>−/−</sup>) mice are viable without obvious phenotypic abnormalities (Poirier and Robertson, 1993). Interestingly, in these mice, a neuronal subpopulation within the olfactory bulb that normally expresses Gal1 does not reach appropriate targets in olfactory glomeruli (Puche et al., 1996). These data suggest that Gal1 may be involved in axonal growth or pathfinding. Here we attempt to further elucidate the role of Gal1 by examining Gal1 expression in the DRG and spinal cord of wild-type (Gal1 <sup>+/+</sup>) mice, examine changes in neuronal populations and primary afferent terminations in Gal1 <sup>−/−</sup> mice, and correlate these changes with nociceptive behavioural responses.

2. Materials and methods

2.1. Animals

A total of 26 adult age-matched 129P3/J (Gal1 <sup>+/+</sup>, Jackson Labs, Maine) and 26 adult 129P3/J Gal1 <sup>−/−</sup> (Poirier and Robertson, 1993) were used for these experiments. The generation of Gal1 <sup>−/−</sup> mice has been described (Poirier and Robertson, 1993). All experiments were performed in accordance with the Canadian Council for Animal Care and approved by the University of British Columbia Animal Care Committee.

2.2. Behavioral testing

A total of 7 Gal1 <sup>+/+</sup> and Gal1 <sup>−/−</sup> mice were used for behavioral testing. Two tests (punctate pressure and either radiant heat or cold plate, see below) were carried out three times per day with at least 2 h between each trial. To avoid sensitization, the tests were repeated on four different days with three days between each testing day. Student’s t-test was used to determine differences between groups with statistical significance attained when P < 0.05.

Progressive plantar punctate force test: A mouse was placed on a metal grate and allowed to adjust to the surroundings for 10 min. A dynamic plantar aesthesiometer (model #37400 UGO Basile Biological Research, Comerio VA) with a dull metal wire was maneuvered under a paw. The ramp was set to 7 s, increasing to a maximum force of 20 g. Upon nocifensive withdrawal (including some or all of: sustained elevation, biting, licking, or shaking of the paw), the instantaneous force applied to the plantar surface eliciting the withdrawal was recorded automatically. Random (non-nocifensive) paw movements were not recorded.

Radiant heat. A mouse was placed on a Plexiglas surface and allowed to habituate to the surroundings for 20–30 min. When the mouse was still, an infrared beam connected to a timer was shone on the palmar or plantar surface of mouse paw (model #7371 UGO Basile Biological Research, Comerio VA). Latency to withdrawal was recorded. Only nocifensive movements were counted.

Cold plate test. A mouse was placed on a 1 °C cold plate (model #0134-002J Columbus Instruments, OH) and a timer was immediately started. The latency to the initial nocifensive response was recorded.

2.3. Fos activation

Fos protein expression is used as a marker for neuronal activation in the spinal cord (Hunt et al., 1987). We used a total of 8 Gal1 <sup>+/+</sup> and Gal1 <sup>−/−</sup> mice to determine if there were differences between Gal1 <sup>−/−</sup> and Gal1 <sup>+/+</sup> mice in second order neuron activation after noxious temperature stimulation. Under light anesthesia, the mouse’s left front paw was carefully submerged in a water bath (1 or 52 °C) three times for 10 s with a 30 s delay between each submersion. Animals were killed 2 h after thermal stimulation, when Fos activation in the dorsal horn is known to have peaked (Dai et al., 2001; Hunt et al., 1987).

2.4. Perfusion/cryosectioning

At the appropriate time, animals were injected with a lethal dose of chloral hydrate and monitored. Upon the loss of nociceptive reflexes, animals were perfused transcardially with 0.1 M phosphate buffered saline (PBS), followed by cold 4% paraformaldehyde in 0.1 M PBS (pH 7.4). The spinal cord and attached DRGs were removed and the tissue was post-fixed for 24 h in 4% paraformaldehyde at 4 °C, and then cryo-protected overnight in a 24% sucrose solution in PBS. Cryoprotected tissue was rapidly frozen in supercooled 2-methylbutane and, later, 14 µm cryosections were cut, thaw-mounted onto glass slides (Superfrost plus) and stored at −80 °C.
2.5. Immunohistochemistry

Standard immunohistochemical techniques and controls for indirect immunofluorescence were used in order to visualize specific antigens on cryosectioned tissue. Slides were washed in 0.1 M PBS for 20 min, blocked for 20 min in 10% normal goat serum and then goat anti-Gal1 (1:500 R&D systems, MN), rabbit anti-NeuN (1:100, Chemicon, Temecula CA) (McPhail et al., 2004), rabbit anti-CGRP (1:2000, Sigma, Oakville, ON), biotin-conjugated IB4 (1:50, Vector Labs), mouse anti-bIII tubulin (1:500, Sigma), mouse anti-NF200 (clone N52, 1:500, Sigma) or rabbit anti-Fos (1:5000, Oncogene, Cambridge, MA), in 0.1 M PBS (in 0.2% Triton X-100, and 0.1% sodium azide) was applied to the slides overnight. After washing, secondary antibodies raised in donkey and conjugated to Cy3, Alexa 488, AMCA (1:300, Jackson Immunological Research, West Grove, PA) or extravidin conjugated Cy3 or FITC (1:500, Sigma) was applied for 1 h at room temperature. After a final wash, slides were coverslipped with a 3:1 solution of glycerol:PBS. A fluorescent microscope (Carl Zeiss, Axioskop, Toronto, ON) was used to visualize chromophore-labeled tissue and then greyscale images were captured using a digital camera (Carl Zeiss, Axioskop, Toronto, ON) in combination with Northern Eclipse software (Empix Inc, Mississauga ON). All images for an individual antigen were taken at the same time and under the same light intensities.

2.6. DRG quantification

DRG quantification was carried out as described by Ramer et al. (2001b). Briefly, triple-labeled images of Gal1, CGRP and IB4 were imported into SigmaScan Pro 5.0 (SPSS Inc., Chicago, IL). The DRG cell bodies were outlined creating an image overlay. The average intensity and feret diameter of each object identified by the overlay was automatically measured. The threshold for immunopositivity for Gal1, IB4 and CGRP was determined by averaging three cell bodies in each section that were judged to be minimally positive. Using recursive translation, a stereological counting method which reconstructs cell populations based on size-distribution of profiles (Rose and Rohrlich, 1988), the proportion of cells expressing an antigen and the exact somata size was determined. Proportions of labeled neurons were compared using Student’s t-test.

2.7. Thin sectioning and electron microscopy

To compare total numbers of primary afferent neurons, dorsal roots from 4 Gal1+/+ and Gal1−/− adult mice were examined at the electron microscopy level. A 2–3 mm length of dorsal root just central to the DRG was harvested from each mouse. Deeply anesthetized mice were transcardially perfused with a mixture of 4% paraformaldehyde and 1% glutaraldehyde. Tissue processing was performed using the Pelco 3450 laboratory microwave with a Pelco coldspot (Ted Pella, Inc., Redding, CA) in place. Fixation of the tissue, in 2.5% glutaraldehyde, was completed with a cycle of 2 min on, 2 min off, 2 min on, under vacuum, at 100 W. The fixative was washed out with two cycles of 40 s at 100 W, using fresh buffer with each wash. Tissue was then placed in 1% osmium tetroxide in 0.1% potassium ferrocyanide for membrane enhancement and microwaved for 2 min on, 2 min off, 2 min on at 100 W (repeated once without changing solution). After two distilled water washes (40 s, 100 W), the tissue was dehydrated through an ethanol series (50, 70, 90, 100, 100%, each at 40 s, 250 W). Microwave infiltration of the 1:1 Spurr’s/Epon resin (1:1 aceton:acetyl, followed with three 100% resin changes) was completed with 3 min, 300 W, under vacuum cycles. Resin polymerization was completed at 65 °C for 16 h and thin sections were cut using a Leica Ultracut T (Leica Microsystems AG, Wetzlar, Germany) on a 45° Diatome diamond knife. Sections were picked up on copper grids and stained in 2% UA (12 min) and Reynold’s lead citrate (6 min). Sections were viewed in a Hitachi H-7600 transmission electron microscope (Hitachi, Ltd, Tokyo, Japan) and photos were taken using a built-in AMT digital camera (American Technologies, Corp., San Francisco, CA). Counting of myelinated axons was performed on thin plastic sections using micrographs taken using bright field microscopy. In electron micrographs the number of unmyelinated axons in a randomly sampled area was counted, from which the total number per root was extrapolated.

2.8. Dorsal horn quantification

Dorsal horn quantification was carried out as described previously (Gaudet et al., 2004; Ramer et al., 2001a, 2003). Briefly, for each mouse cervical spinal cord level, three images of CGRP- and IB4-stained tissue were imported into SigmaScan Pro 5.0 (SPSS Inc., Chicago, IL) where a threshold was applied. Staining was measured along three non-overlapping 50 μm wide strips starting from uppermost border of grey matter and extending 450 μm ventrally. Measurements at each depth were averaged across all sections per mouse and mean ± SEM axonal density was plotted as a function of depth. For every 10 μm in depth, the average axon density was determined. Using Student’s t-test, differences between Gal1+/+ and Gal1−/− mice were determined at 10 μm intervals.

2.9. Fos quantification

Three images were captured for each cervical section of the left dorsal horn for each animal. These images were imported into SigmaScan Pro 5.0 (SPSS Inc., Chicago, IL), a threshold was applied to remove any background staining and then Fos-positive cells were automatically counted.

2.10. NeuN quantification

For each animal, three randomly-selected images were captured for each cervical section (C7–C8) of the left dorsal horn. These images were then imported into Photoshop (Adobe Systems, San Jose, CA) and only laminae I–II were selected. Using the Image Processing toolkit 3.0 (Reindeer Graphics, Asheville, NC) a threshold was used to separate the individual NeuN positive cells. The cells were then automatically counted.

2.11. In situ hybridization

The mouse Gal1 probe was a 51-mer oligonucleotide complementary to the 3'-untranslated sequence of Gal1 and 5'-TCA TCT AAA GGC CAC GCA CTT AAT CTT GAA GTC TCC ATC CGC CGC CAT GTA -3' (GenBank accession number BC002063). The Gal1 probe was complementary to bases 424–474. The mouse probes were end-labeled with 32P-dATP.
(Perkin–Elmer, Woodbridge, ON) by using deoxynucleotide terminal transferase according to a standard protocol (Kobayashi et al., 1996). Perfusion-fixed sections were hybridized to 1.2 × 10^6 cpm of probe for 16–18 h at 44 °C. The slides were dipped in Kodak NTB-2 emulsion and exposed for 3 days. Slides were then dehydrated in a series of alcohols and stored at room temperature. Spinal cord sections were later re-hydrated in dH2O for 1 h and then the fluorescent Nissl stain; Neurotrace (1:200, Molecular Probes Inc. Eugene, OR) was added to the slides. Slides were then dehydrated in a series of alcohols and coverslipped with Entallen (Fisher Scientific, Nepean, ON).

2.12. Images and statistics

All images were imported into Photoshop (7.0, Adobe, Ottawa, ON) and adjustments were made to brightness and contrast to the whole image. Some images were false-coloured in Photoshop to provide clarity. Quantification was performed blind with respect to the treatment groups. All results were analyzed using SigmaStat 3.0 (SPSS Inc., Chicago, IL) and the criterion for significance was P<0.05. Unless otherwise stated, Student’s t-test was used to determine significance. All results are stated as mean±standard error of the mean (SEM).

3. Results

3.1. Gal1−/− mice have increased thermal nociceptive thresholds

The responses to both noxious and non-noxious stimuli of transgenic mice carrying the homozygous null mutation of the Gal1 gene (Gal1−/−) were compared to those of the inbred Gal1+/+ mice. When placed on a 1 °C cold plate, null mutant mice displayed significantly longer latencies before exhibiting a nociceptive withdrawal response (involving some or all of: sustained elevation, biting, licking or shaking of the paw) when compared to Gal1+/+ (75.4±6.8 for Gal1−/−, mean±SEM, compared to 18.4±1.3 s for Gal1+/+, ANOVA, P<0.001, Fig. 1). In addition, Gal1−/− mice had increased withdrawal latency from radiant heat (8.3±0.3 s, front paw; 8.7±2.6 s, hind paw) compared to Gal1+/+ mice (5.3±0.3 s, front paw; 5.4±2.6 s, hind paw, ANOVA, P<0.012).

A dynamic plantar punctate pressure test was used to assess the amount of force (grams) at which a mouse would withdraw. Here, no significant difference was observed between the Gal1−/− (front: 6.7±0.6 hind paw: 7.5±2.6 gm of force) compared to Gal1+/+ groups (front: 5.7±1.3 hind paw: 6.7±0.6 gm of force, Fig. 1).

3.2. Gal1 expression in Gal1+/+ mice

A sub-population of small-diameter nociceptive afferents express the ATP receptor P2X3 and bind IB4, terminate in lamina II inner of the dorsal horn in the spinal cord, and...
require the neurotrophin GDNF for development (Chen et al., 1995; McMahon and Moore, 1988; Molliver et al., 1997). Gal1 protein was expressed in 68 ± 8.3% of somata (of all sizes) within the C7 and C8 DRGs of Gal1+/+ mice (Fig. 2, histogram). 32 ± 4% of Gal1-IR neurons expressed CGRP, while 37 ± 2% of Gal1-positive neurons were IB4-binding (Fig. 2, histogram inset). 31 ± 3% of Gal1+/+ neurons were immunoreactive for neither CGRP nor IB4-binding. While Gal1 expression was evident in neurons of all sizes, Gal1 immunoreactivity was most intense in small-diameter neurons.

3.3. Gal1−/− mice have a smaller proportion of IB4-binding DRG neurons, a larger proportion of NF200-expressing DRG neurons, and more myelinated sensory axons

Using radioactive in situ hybridization for Gal1 mRNA followed by autoradiography, Gal1 mRNA was observed in the DRG somata (Fig. 3) and the silver grains predominantly co-localized to neurons within the DRG (Fig. 3). Silver grain density was at background levels in Gal1−/− tissue sections, even when exposed for 2 days longer than Gal1+/+ sections, confirming that Gal1 mRNA expression was undetectable in Gal1−/− mice (Fig. 3 middle panel).

Differences in the proportions of large-diameter mechanoreceptive (NF200-IR) and nociceptive (peptidergic, CGRP-expressing; and non-peptidergic, IB4-binding) neurons within Gal1−/− and Gal1+/+ mouse DRGs were examined. There was no significant difference observed in CRGP-IR between groups, with 56 ± 4% CRGP-positive somata in Gal1+/+ mice and 50 ± 4% CRGP positive somata in Gal1−/− mice (Fig. 4). Interestingly, there was a significant reduction in the proportion of cells binding IB4 from 59 ± 3% in Gal1+/+ to 38 ± 5% in Gal1−/− mice (P < 0.05, Fig. 4). In addition, the proportion of neurons that showed co-localization of both IB4-binding and CRGP-IR was also significantly decreased from 38 ± 3% in Gal1+/+ mice to 21 ± 3% in Gal1−/− mice (P < 0.05, data not shown). In contrast with the decreased proportion of IB4-binding neurons, there was a significant increase in the proportion of NF200-IR DRG neurons in Gal1−/− (29 ± 3%) compared to Gal1+/+ (20 ± 3%) mice (P < 0.05, Fig. 4).

The morphology and number of axons in the L5 dorsal root of Gal1−/− mice were quantified (Fig. 5). We found that there was no significant difference in the total number of axons projecting centrally from the DRG. However, there was a significant increase in the number of myelinated axons in Gal1−/− mice when compared with Gal1+/+ mice (Gal1+/+: 1490 ± 90, Gal1−/−: 1980 ± 170 axons). No significant difference between Gal1−/− and Gal1+/+ mice in the axonal-Remak cell (ensheathing, non-myelinating Schwann cell) relationship, axonal size distribution, nor in the g-ratio (ratio of axon diameter to axon plus myelin diameter) was observed (data not shown).

The above data (a decrease in the proportion of IB4-binding neurons, an increase in the proportion of NF200-expressing DRG neurons, but no change in the total number of dorsal root axons) indicate that some neurons which otherwise would be IB4-binding may take on the NF200-expressing phenotype. We therefore examined the size-distribution of NF200-expressing cells to determine if the mean diameter of these neurons differed between genotypes. The mean diameter of these neurons was 57 ± 2 mm in Gal1+/+ mice, and 54 ± 2 mm in Gal1−/− mice (P = 0.028, t-test). This small but statistically significant difference in neuronal diameter supports the acquisition of NF200-significance.
expression by otherwise small-diameter IB4-expressing cells.

3.4. Altered central distribution of nociceptive axons in the spinal cord of Gal1<sup>−/−</sup> mice

Afferents that bind IB4 normally terminate in lamina II. In Gal1<sup>−/−</sup> mice, these axons extended deeper into the dorsal horn than in Gal1<sup>+/+</sup> mice (middle panel Fig. 6). In Gal1<sup>−/−</sup> mice, the peak IB4 binding density occurred between 70–80 μm ventral to the top of lamina I, whereas in Gal1<sup>+/+</sup> mice, the maximum binding intensity occurred 30–40 μm deep in the dorsal horn (Fig. 6 bottom right graph). CGRP-positive axons also terminated deeper in the dorsal horn of Gal1<sup>−/−</sup> mice than Gal1<sup>+/+</sup> mice (Fig. 6, bottom left graph).

3.5. Fos expression is reduced following noxious stimulation in Gal1<sup>−/−</sup> mice

Using Fos expression as a marker for neuronal primary afferent-elicited activity in second-order neurons in the dorsal horn (Dai et al., 2001; Hunt et al., 1987), we tested whether Gal1<sup>−/−</sup> and Gal1<sup>+/+</sup> mice differed in their ability to transmit nociceptive information after thermal stimulation. Two hours after exposure of the front paw to noxious thermal stimuli, the dorsal horns of spinal segments C7 and C8 were examined. These two segments were examined since these two spinal levels receive the majority of sensory input from the forepaw. After exposure to 1 °C water (Fig. 7, left panel), Gal1<sup>−/−</sup> mice had 14.7 ± 0.9 Fos-positive nuclei per section C7 and 13.1 ± 0.6 Fos-positive nuclei at C8. Gal1<sup>+/+</sup> mice had 21.1 ± 1.9 Fos-positive nuclei per section at C7 and 24.1 ± 2.7 Fos-positive nuclei per section at C8 (P < 0.05 compared at the same level between Gal1<sup>−/−</sup> and Gal1<sup>+/+</sup> animals, Fig. 7).

After exposure to 52 °C water, Gal1<sup>−/−</sup> mice had 18.7 ± 2.1 Fos-positive nuclei per section at C7 and 28.0 ± 1.9 Fos-positive nuclei per section at C8, while Gal1<sup>+/+</sup> mice had 27.5 ± 2.9 Fos-positive nuclei at per section C7 and 34.5 ± 1.5 Fos positive nuclei per section at C8 (P < 0.05 compared at the same level between Gal1<sup>−/−</sup> and Gal1<sup>+/+</sup> animals, Fig. 7). The neuronal-specific antibody NeuN was used to determine if the differences in Fos expression could be attributed to differences in the number of second order neurons. There was no significant difference in the number of neuronal cell bodies at either the C7 or C8 spinal levels between Gal1<sup>−/−</sup> and Gal1<sup>+/+</sup> mice (Fig. 8).

4. Discussion

The principal findings of this study are (1) that Gal1<sup>−/−</sup> mice have reduced sensitivity to noxious thermal stimuli; (2) that there are proportionally fewer IB4-binding neurons and proportionally more NF200-expressing neurons in Gal1<sup>−/−</sup> DRGs; (3) that the increase in large-diameter DRG neurons is coincident with an increase in the number of myelinated dorsal root axons; (4) that the terminal fields of nociceptive DRG neurons is deeper in the dorsal horn of Gal1<sup>−/−</sup> mice than Gal1<sup>+/+</sup> mice; and (5) that transmission of thermal nociceptive information to second order
neurons in the dorsal horn is reduced in Gal1\textsuperscript{-/-} mice. These results suggest that Gal1 is required for the appropriate developmental specification of neuronal phenotypes in the DRG, axonal targeting in the spinal cord, and nociception in the adult.

Nociception serves to prevent actual or potential tissue damage through the detection of noxious thermal, chemical, and mechanical stimuli. These diverse sensory inputs are transmitted along two major nociceptive pathways that terminate primarily in laminae I and II (Snider and McMahon, 1998). The segregation of these small-diameter afferent fiber projections into a laminar-specific topology occurs during development and suggests different functional roles. Different classes of sensory neurons enter the spinal cord in sequence: large-diameter sensory fibers enter the

Fig. 4. Differences in distribution of CGRP-expressing (green), IB4-binding (red), and neurofilament (NF)200-immunoreactive neurons in the DRG between Gal1\textsuperscript{-/-} and Gal1\textsuperscript{+/+} mice (n=4 for both groups). 56±4% of C7 and C8 DRG neurons expressed CGRP in Gal1\textsuperscript{+/+} mice (top left panel), and 50±4% of neurons were CGRP-positive in Gal1\textsuperscript{-/-} mice (top right panel). 59±3% of neurons were IB4-binding in Gal1\textsuperscript{+/+} mice (middle left panel) which was significantly different from the 39±5% of IB4-binding neurons in Gal1\textsuperscript{-/-} mice. In Gal1\textsuperscript{+/+} mice, 20±3% of DRG neurons were NF200-IR, while 29±3% of DRG neurons were NF200-positive in Gal1\textsuperscript{-/-} mice. * P<0.05, scale bar: 100\textmu m.

Fig. 5. Thin plastic sections (top) and high power electron micrographs (middle) demonstrating axonal morphology dorsal roots of Gal1\textsuperscript{-/-} and Gal1\textsuperscript{+/+} mice. There was no significant difference in morphology of myelinated axons or unmyelinated axons in the dorsal root of Gal1\textsuperscript{-/-} mice compared to Gal1\textsuperscript{+/+} mice. There was a significant increase in the number of myelinated axons in Gal1\textsuperscript{-/-} compared with Gal1\textsuperscript{+/+} mice. There was no significant difference in the total number of axons (myelinated plus unmyelinated) between groups (9100±1500 axons in Gal1\textsuperscript{+/+} vs. 8500±1500 in Gal1\textsuperscript{-/-} mice). * P<0.05, scale bar: 25\textmu m (top panels), 500 nm (bottom panels).
spinal cord at E14.5, followed by small-diameter fibers at E15.5 (Ozaki and Snider, 1997). Interestingly, Gal1 expression was first demonstrated in the rat DRG at E14, just as small-diameter fibers were approaching the spinal cord (Regan et al., 1986). In the spinal cord, Gal1 is expressed in superficial laminae from P0, with most intense staining occurring between P0 and P7. This corresponds to the time at which appropriate connections are established in the dorsal horn of the spinal cord (Regan et al., 1986). These observations led to speculation that Gal1 was involved in either axonal outgrowth or synaptic stability of nociceptive fibers during development (Dodd and Jessell, 1986). Once connections are made, these fibers continue to express Gal1 at lower levels.

4.1. Gal1 distribution

Since this is the first report of Gal1 expression in mouse DRGs, these results can only be compared to previous findings in rat DRGs. Approximately 68% of all neurons within the cervical DRGs are Gal1-IR in Gal1+/+ mice, and small-diameter cells were particularly intensely staining. These results are in agreement with those for adult rats, in which 63% of DRG neurons were Gal1, and 46% of neurons were intensely stained (Regan et al., 1986). Others have reported that most DRG neurons from the 4th and 5th lumbar levels in the rat have some Gal1-IR, with 20–26% intensely Gal1-IR (Imbe et al., 2003; Sango et al., 2004).

There is a decrease in the proportion of IB4-binding DRG neurons, and an increase in the proportion of neurons that are NF200-IR in adult Gal1−/− mice. Since Gal1 is highly expressed in neurons that bind IB4 in the Gal1+/+ mouse, this indicates that Gal1 is required to specify the development of the full complement of IB4-binding neurons and/or the switch to the IB4-binding phenotype during development. Since there is no change in the total number of axons in the dorsal root, we infer that some DRG neurons, rather than taking on the IB4-binding phenotype, instead become NF200-expressing. This is supported by increased number of myelinated axons in dorsal roots of Gal1−/− mice. Therefore, Gal1 is required for proper determination of DRG neuron phenotype during development.

Gal1−/− mice have no gross morphological differences from Gal1+/+ mice, no known compensatory changes in the expression of other galectins, and no changes in immune cell numbers (Poirier and Robertson, 1993). During olfactory development, Gal1 is expressed by Dolichos biflorus agglutinin (DBA)-binding neurons as they are growing towards their targets and this expression continues, albeit at lower levels, once connections have
been made (Puche et al., 1996). Furthermore, Gal1 increases olfactory neurite outgrowth in vitro (Puche et al., 1996). During development in Gal1\(^{-/-}\) mice, these DBA-binding neurons do not reach their appropriate targets (Puche et al., 1996). In parallel with the altered olfactory system, small-diameter primary afferents (which normally express Gal1) have a significantly different terminal distribution in the dorsal horn of Gal1\(^{-/-}\) mice compared to Gal1\(^{+/+}\) mice. The altered depth profile of nociceptive axons in the dorsal horn and the decrease in Fos activation after noxious thermal stimulation in mice lacking Gal1, suggest that the significant increase in noxious pain threshold that is observed is in part due to the inappropriate connections of the small-diameter fibers in lamina II.

There are many putative mechanisms by which Gal1 could maintain sensory neuronal function. Gal1 stimulates DRG outgrowth in vitro and the rate of sensory and motor regeneration in vivo. This occurs by increasing Schwann cell migration and eliciting the release of an unidentified growth-stimulating factor from macrophages (Fukaya et al., 2003; Horie and Kadoya, 2000; Horie et al., 1999, 2004). Furthermore, we have shown recently that neuronal Gal1 expression is correlated with regenerative potential in spinal motoneurons and rubrospinal neurons (McGraw et al., 2004). A neuronal Gal1 receptor has not been identified, but under normal circumstances Gal1 is secreted by Schwann cells and dorsal root ganglion neurons via a non-classical pathway (Sango et al., 2004). Secreted Gal1 may then act in an autocrine/paracrine fashion on sensory neurons and/or glial cells within the DRG during development and following axotomy. Therefore, the lack of Gal1 in the null-mutant mouse may lead to reduced trophic support within the DRG, resulting in a decreased number of IB4-binding neurons.

Gal1 also promotes axonal growth by altering adhesion properties of the extracellular matrix. For example, Gal1 mediates self-aggregation of primary olfactory neurons through the cross-linking of carbohydrate ligands, and facilitates fasciculation of DRG neurites in vitro (Mahanthappa et al., 1994; Outenreath and Jones, 1992). The absence of Gal1 in the null mutant mouse may thus lead to inappropriate targeting by either hindering axonal fasciculation or altering cellular adhesion during innervation of the spinal cord by nociceptive axons.

The fact that IB4-positive neurons overshoot their normal targets in the Gal1\(^{-/-}\) superficial dorsal horn is interesting, given Gal1 is also implicated in axonal elongation: simplistically, one might expect the Gal1-negative axons to fall short of their targets. The developmental expression of
Gal1 (high until target connection), and the mis-direction of axons in the Gal1 K/K mouse olfactory system indicates that Gal1 is important for target specification early in development, but can also promote neurite outgrowth following injury in the adult.

4.2. IB4 neurons in thermal nociception

Both peptidergic (CGRP-IR) and non-peptidergic (IB4-binding) small-diameter sensory neurons are implicated in thermal nociception, although their precise roles remain elusive (Snider and McMahon, 1998). These two neuronal populations are functionally distinct in their response to heat (Stucky and Lewin, 1999), and heterogeneity in thermal responses exists within the non-peptidergic neurons: only half of the IB4-binding nociceptors are sensitive to noxious heat (Stucky and Lewin, 1999). This is most likely due to differences in their expression of thermally sensitive receptors. Although 78% of rat IB4-binding neurons express the capsaicin—and heat—sensitive vanilloid receptor TRPV1 (VR1), which has been implicated in thermal hyperalgesia (Garcia-Martinez et al., 2002; Jarvis et al., 2002), the function of this protein in the mouse is less clear. TRPV1 is expressed in only 2–3% of IB4-binding neurons in mice (Zwick et al., 2002). In addition, heat-sensitive nociceptors from mice lacking both TRPV1 and TRPV2 have normal thresholds (Woodbury et al., 2004). Thus, although IB4-binding neurons are implicated in the sensation of noxious heat, the full complement of proteins involved in heat transduction in these afferents has not been elucidated. Regardless, our data indicate that IB4-binding neurons do play a role in thermal nociception. The reduction in the proportions of IB4-binding cells observed in this report as well as the alterations in laminar termination of these neurons suggest that this contributes to the observed attenuated thermal nocifensive responses in Gal1 K/K mice.

There have been two ion channels that have been implicated in sensing noxious cold, TRPM8 (CMR1, McKemy et al., 2002) and TRPA1 (ANKTM1, Story et al., 2003). TRPA1 is activated by noxious cold (<17 °C), and is expressed by a subset of TRPV1-positive, TRPM8-negative, CGRP-IR somatic neurons (Story et al., 2003). TRPM8 is activated by a range of temperatures that include noxious and innocuous stimuli (8–29 °C), and is present in about 15% of all DRG neurons (McKemy et al., 2002). This non-selective cation channel is thought to be present in a subpopulation of nociceptors (McKemy et al., 2002), although the distribution of TRPM8 in IB4-binding and CGRP-IR neurons in mice is currently not known. We suggest that the decrease in the number of IB4-binding (and potentially TRPM8-containing) neurons is one possible mechanism by which the threshold for noxious cold may be increased in Gal1 K/K mice.

In summary, the results of the present work show that the absence of Gal1 expression leads to subtle but significant shifts in the phenotypic distribution of primary afferent neurons, their terminal fields in the spinal cord, and substantial changes in the responses to noxious thermal stimuli. Understanding what molecules are important for the specification of nociceptive signaling systems during development have the potential to indicate therapeutic targets for the treatment of acute or chronic pain. Given its roles in neurite outgrowth and the development of nociceptive neurons, Gal1 may be one such molecule.

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References


Makrenchappa NK, Cooper DN, Barondes SH, Schwarting GA. Rat olfactory neurons can utilize the endogenous lectin, L-14, in a novel adhesion mechanism. Development 1994;120:1373–84.


