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L1 Cell Adhesion Molecule is Essential for the Maintenance of Hyperalgesia After Spinal Cord Injury

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Abstract

Spinal cord injury (SCI) results in a loss of normal motor and sensory function, leading to severe disability and reduced quality of life. A large proportion of individuals with SCI also suffer from neuropathic pain symptoms. The causes of abnormal pain sensations are not well understood, but can include aberrant sprouting and reorganization of injured or spared sensory afferent fibers. L1 is a cell adhesion molecule that contributes to axonal outgrowth, guidance and fasciculation in development as well as synapse formation and plasticity throughout life. In the present study, we used L1 knockout (KO) mice to determine whether this adhesion molecule contributes to sensory dysfunction after SCI. Both wild-type (WT) and KO mice developed heat hyperalgesia following contusion injury, but the KO mice recovered normal response latencies beginning at 4 weeks post-injury. Histological analyses confirmed increased sprouting of sensory fibers containing calcitonin gene related peptide (CGRP) in the deep dorsal horn of the lumbar spinal cord and increased numbers of interneurons expressing protein kinase C gamma (PKC γ) in WT mice 6 weeks after injury. In contrast, L1 KO mice had less CGRP⁺ fiber sprouting, but even greater numbers of PKC γ ⁺ interneurons at the six week time point. These data demonstrate that L1 plays a role in maintenance of thermal hyperalgesia after SCI in mice, and implicate CGRP⁺ fiber sprouting and the upregulation of PKC γ expression as potential contributors to this response.

Keywords

neuropathic pain; allodynia; protein kinase C; calcitonin gene-related peptide; sprouting; plasticity; primary afferent; sensory

Introduction

Spinal cord injury (SCI) results in a loss of normal motor and sensory function, leading to severe disability and reduced quality of life (Tate and Forchheimer, 2002; Anderson, 2004). While motor loss and recovery after SCI have been extensively studied in animal models, less emphasis has been placed on changes in sensation (Thuret et al., 2006). Recently, however, the phenomenon of neuropathic pain after SCI has received increased attention (Dobkin and Hayton, 2004; Rosenzweig and McDonald, 2004). A large proportion of individuals with SCI suffer from neuropathic pain (Stormer et al., 1997; Siddall et al., 2003;

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Widerstrom-Noga et al., 2006; Sawatzky et al., 2008) including hyperalgesia (an heightened response to a painful stimulus) and allodynia (a painful response to an innocuous stimulus). These types of pain are consuming, chronic, and largely refractory to treatment (Widerstrom-Noga et al., 2007; Sawatzky et al., 2008).

The study of sensation after SCI is particularly relevant as the plasticity, sprouting and reorganization that are crucial in many promising interventions may cause or increase sensory dysfunction (Hofstetter et al., 2005; Cameron et al., 2006; Deumens et al., 2008). While improved axonal growth in experimental conditions has long been a goal in SCI research (Bradbury and McMahon, 2006), there is evidence that excess sprouting, especially of sensory afferent fibers, can contribute to undesirable effects including hyperexcitability, spasticity, autonomic dysreflexia (Wong et al., 2000; Cameron et al., 2006) or behavior consistent with allodynia or hyperalgesia (Krenz and Weaver, 1998; Romero et al., 2000; Hofstetter et al., 2005). The causes of these conditions are poorly understood, but it is believed that developmental cues that are re-expressed following injury can contribute to inappropriate or ineffective guidance for sprouting or regenerating axons. Therefore, understanding the interactions between guidance molecules and the local environment is critical to improving the directed regrowth capacity of injured sensory fibers.

One such developmental cue is L1, a cell adhesion molecule (CAM) that is the mouse and human homolog to avian Ng-CAM, rat NILE/L1-CAM, and L1.1 and L1.2 found in zebrafish (Becker et al., 2004; Becker et al., 2005). L1 is a widely expressed neural cell adhesion molecule of the immunoglobulin superfamily important in many aspects of nervous system development including axonal outgrowth, guidance and fasciculation (Burden-Gulley et al., 1997; Castellani et al., 2000; Kamiguchi, 2003; Whittard et al., 2006; Chen et al., 2007), as well as synapse formation (Godenschwege et al., 2006), signaling (Panicker et al., 2003; Maness and Schachner, 2007), and establishment of long term potentiation (LTP) (Luthi et al., 1994; Luthi et al., 1996). Expression of L1 is required for normal development of the corticospinal tract (CST) (Wong et al., 1995; Dahme et al., 1997; Cohen et al., 1998; Jakeman et al., 2006), as well as hippocampal and cerebellar projections (Fransen et al., 1998; Demyanenko et al., 1999). L1 acts through a variety of binding partners, using homophilic and heterophilic interactions to mediate cell-cell adhesion as well as activation of a number of intracellular signaling cascades (Burden-Gulley et al., 1997; Kamiguchi, 2003). In the mature CNS, the presence of L1 also influences learning and memory (Arami et al., 1996; Wolfer et al., 1998; Law et al., 2003; Venero et al., 2004). Changes in L1 expression patterns may be associated with neuropathic pain after sciatic nerve transection (Yamanaka et al., 2007). Given the indications of the multifaceted role of L1 in nervous system development and function, it is likely that L1 also plays a number of important roles in axon guidance and plasticity after SCI.

We have shown previously that the absence of L1 in mice results in impaired locomotion and leads to enhanced sprouting of injured corticospinal fibers rostral to the site of an SCI (Jakeman et al., 2006). Given the robust expression of L1 in primary sensory afferents throughout adulthood (Akopians et al., 2003; Runyan et al., 2005), and the reported sensory deficiencies in L1 knockout (KO) mice (Haney et al., 1999; Thelin et al., 2003), we hypothesized that L1 deficient mice would also have decreased sensory responses and reduced sensory fiber sprouting after injury. To test this, and to extend the previous findings, we compared sensory responses, locomotor ability, and histology in L1 KO and wild-type (WT) littermates before and for six weeks after a moderate contusion injury.

We found that while WT mice demonstrated hyperalgesia after SCI for the duration of the study, L1 KO mice recovered normal sensory responses to heat stimuli beginning at 4 weeks post-injury. Hyperalgesia was associated with increased CGRP sprouting in the deep dorsal

horn of the lumbar spinal cord in WT mice 6 weeks after injury, while KO mice had reduced CGRP sprouting. We also found increases in the number of cells expressing protein kinase C gamma (PKC γ) in lamina II of the lumbar spinal cord in injured mice, with significantly more PKC γ ⁺ cells in KO mice compared to WT littermates. These data demonstrate that L1 plays a role in maintenance of thermal hyperalgesia after SCI in mice, and implicate CGRP fiber sprouting and the regulation of PKC γ expression as contributors to this response.

Methods

Mice

All procedures were performed in accordance with the Ohio State University Animal Care and Use Committee and the NIH Guide to Care and Use of Laboratory animals. Adult, male KO and WT littermate mice (8-14 weeks of age) on a B6;129S7 background were generated from non-sibling crosses from breeders obtained from Jackson Laboratories [B6;129S7^{11camtm1sor1}] as previously described (Jakeman et al., 2006). At weaning, tail samples from all male progeny were screened to identify genotype using PCR primers as indicated by the supplier. Genotypes were also confirmed with immunohistochemistry. A total of 17 WT and KO mice from the colony were subjected to SCI and evaluated behaviorally and histologically. One mouse was excluded because of an unacceptable injury, and two mice died before the completion of the study, leaving groups of five KO mice and nine WT littermates. An additional seven WT mice of the same genetic background as the transgenic mice were obtained commercially (C57BL/6 $\times$ 129S7 hybrid F1 generation, Taconic) and used to compare anatomical responses in injured and uninjured WT mice. All mice were housed two to three per cage and were maintained on a 12-hour light-dark cycle with food and water *ad libitum* for the duration of the study.

Spinal Cord Injury

Mice received a moderate contusion injury to the mid-thoracic spinal cord as described previously (Jakeman et al., 2000; Ma et al., 2001). Briefly, all animals were anesthetized intraperitoneally with ketamine (80mg/kg) and xylazine (10 mg/kg) and subjected to a T9 vertebral level laminectomy. Two wild-type animals received only the laminectomy to provide uninjured tissue for immunohistochemical comparison, and all others were subjected to a moderate (0.5 mm rapid displacement) contusion injury with the OSU electromagnetic spinal cord injury device (ESCID). (Jakeman et al., 2000). Incisions were closed, and mice were allowed to recover in a warm cage overnight. Post-operative care included saline injections and antibiotics (gentocin; 5 mg/kg) for five days following surgery, and bladder expression twice a day for the duration of the study.

Behavioral Analyses

Prior to injury, mice were acclimated by daily handling and exposure to all behavioral testing apparatus for one week. Baseline testing was performed from one to three days prior to injury. Post-operative testing began when recovery was sufficient to perform the task (e.g. hindlimb plantar placement with weight support for sensory tests), and continued until six weeks post-injury. All behavioral testing was done by examiners who were not knowledgeable of the mouse genotype.

Motor Testing

Basso Mouse Scale (BMS) testing: The Basso Mouse Locomotor Scale is a 0 to 9 point scale (0= hind limb paralysis, 9= normal locomotion), which was developed specifically to describe recovery of function after thoracic spinal cord contusion injury in mice (Engesser-Cesar et al., 2005; Basso et al., 2006). Testing sessions were conducted by a team of two

trained investigators prior to injury, at one, three, seven, and ten days post injury (dpi), then weekly until six weeks post injury. BMS scores were calculated for left and right hindlimbs and averaged to obtain a single value per mouse.

Beam Cross: To assess locomotion in a more challenging setting, mice were observed and scored while crossing a narrow beam (Feeney et al., 1982; Metz et al., 2000; Karl et al., 2003). During this test, mice traversed a 1.3 cm wide by 80 cm long rectangular wooden beam. Each mouse was given 3 trials. Two raters, one on each side of the beam, evaluated their performance on a 10 point scale. To assess their maximum capabilities, the highest scoring trial was counted. See Supplementary Data for a more detailed description of the testing criteria.

Sensory Testing

Plantar Heat Test: Thermal sensitivity of the plantar hindpaws was tested based on the method developed by Hargreaves (Hargreaves et al., 1988; Mogil et al., 1999). Mice were placed unrestrained in individual clear plastic compartments (11 cm x 17 cm x 14 cm). An infrared radiant heat source (25 watts, Ugo Basile, Italy) was applied through a glass floor to the middle of the plantar surface of the hindpaw, between the foot pads. A photocell automatically stopped the heat source and the timer when the mouse lifted its paw. Each mouse was tested for five trials on each hindpaw, with at least one minute between trials. A 20 second maximum cutoff was established to prevent tissue damage. Latency (in seconds) to withdrawal from the heat source was measured. High and low latencies were dropped for each paw, and the remaining latencies were averaged for each mouse.

Von Frey Hair Test: The withdrawal threshold to tactile stimulation was measured using calibrated von Frey hairs (Mogil et al., 1999; Kloos et al., 2005). Mice were placed in a small, clear compartment (8 cm x 12 cm x 5.5 cm) on an elevated wire mesh screen to allow the investigator free access to the plantar surface of the paws. Hindpaws were tested in a random order, and the investigator determined the 50% withdrawal threshold using the up-down method (Dixon, 1980; Chaplan et al., 1994). Each hindpaw was tested for 10 trials. Since this test stimulated the same area of skin as the plantar heat test, these tests were conducted on different days.

Anatomical Analyses

Sensory Fiber Labeling—To label large diameter sensory fibers, the sciatic nerves in a subgroup of mice (n=9, 4 KO and 5 WT mice) were labeled unilaterally with a high salt formulation of the β subunit of Cholera Toxin (CTB) (List Biological Lab, CA) (Krenz and Weaver, 1998; Bradbury et al., 1999; Romero et al., 2000; Lu et al., 2003). Mice were anesthetized and the common sciatic nerve exposed. A Hamilton syringe with a blunted needle was used to inject 2 μ l of 1% CTB solution over 1 minute. The needle was held stationary for 2 additional minutes, and then removed. The nerve was cut distal to the injection site. The muscles were then sutured, and the wound was closed with clips. Mice were allowed to survive for four days, the perfused as described below.

Tissue Preparation—Six to seven weeks after SCI, mice were deeply anesthetized with a lethal dose of ketamine (120 mg/kg) and xylazine (15 mg/kg), and were transcardially perfused with phosphate buffered saline (PBS) followed by 4% paraformaldehyde. The spinal cords were removed and post-fixed for two hours. After an overnight rinse in phosphate buffer and cryoprotection in 30% sucrose, they were frozen in optimal cutting temperature (OCT) compound in 7 mm blocks centered on the injury epicenter and the lumbar enlargement. Each block was cut on a cryostat in 10 μ m transverse sections, mounted on slides in 10 alternating sets so that each section on a slide was 100 μ m apart,

and stored at -20°C until they were stained (Ma et al., 2001; Ma et al., 2004). Specimens from both genotypes were blocked together and mounted and stained on the same slides. All histological analyses were done with coded sections and the investigator was blind to genotype.

Histology—One set of equally spaced sections from the lesion site was stained with Eriochrome Cyanine (EC) to define the extent of damage based on the distribution of myelin (Jakeman et al., 2006). The epicenter was identified as the section of tissue with the smallest area of darkly stained white matter in the rim. Computer assisted imaging with the MCID Analysis System (Imaging Research; St Catherine's, Ontario, Canada) was used to measure the cross sectional area of the spared white matter (SWM) and the total cross sectional area of the tissue section (TCA), and then the percent cross-sectional area at the lesion epicenter was calculated (SWM/TCA).

Immunohistochemistry—Adjacent sets of sections from thoracic and lumbar spinal cord were stained following overnight incubation with antibodies raised against markers identifying axons, neurons or glial cells (Details in Table 1). Antibody detection was performed following incubation in biotinylated secondary antibodies (Vector Laboratories, Burlingame, CA) using the Avidin-Biotin peroxidase Complex (ABC) with diaminobenzidine (DAB) as the chromagen (Vector Laboratories) or with fluorescent tagged secondary antibodies (Alexa Fluor, Invitrogen, Carlsbad, CA). For ABC detection, endogenous peroxidases were first quenched with 6% hydrogen peroxide in methanol. In all cases, non-specific binding was blocked with solution containing 5-10% normal serum from the same species as the secondary antibody. Specificity of the immunohistochemical markers was confirmed by examining the anatomical distribution in regions of known positive and negative staining and by control stains that included omission of the primary and/or secondary antibody.

Immunohistochemical Analyses—Because primary afferents branch and enter the dorsal horn in bundles, it is important to sample afferents in the dorsal horn across more than one section. To quantify the distribution of CGRP-containing afferents at the lesion site, images of the epicenter section and sections 200 μm rostral and caudal to the epicenter were captured using a 20x objective and Sony CCD camera. The intensity threshold for positive staining was determined for each section and the area of staining for the dorsolateral quadrant was measured using the MCID Analysis System analysis program on left and right sides using a sampling box ($100,000\text{ }\mu\text{m}^2$) that was placed in alignment with the midline and the dorsal section border. Left and right sides were summed and then a single value was obtained for each animal by averaging areas from the three sections.

The distribution of CGRP⁺ hindlimb afferent fibers was measured in the lumbar spinal cord. In order to provide unbiased sampling throughout the entire lumbar enlargement, CGRP was measured from 10 sections equally spaced 600 μm apart (6000 μm total distance) beginning at the rostral end of lumbar enlargement, at approximately L1 spinal level, and continuing through to the caudal end, approximately L6. For each measured section, images of the left and right dorsal horns were captured at 20x magnification. The threshold for positive staining was established for each section and the stain area was measured in outlines of the superficial and deep dorsal horn regions (Krenz and Weaver, 1998). Values for left and right sides were averaged to obtain a single value at each section level for each specimen.

For assessment of PKC γ ⁺ interneurons in the lumbar spinal cord (Polgar et al., 1999), a similar strategy was used except that four equally spaced sections, starting at the L3 spinal level and spanning the middle of the lumbar enlargement (800 μm apart for a total of 3200 μm length) were viewed using a 40x objective. Laminae I, II, III, IV-V and X were

estimated based on neuropil background staining, and all cell bodies with clearly distinguishable cytoplasmic staining and a pale nuclear center in each region were counted manually. The section thickness (10 μm) precluded stereological cell counts in this study. However, there were no differences in the diameters of labeled cell bodies chosen at random from both groups, suggesting that all cells had an equivalent probability of being counted, and the numerical estimates from direct counts in each region were a reasonable approximation of relative cell numbers.

Growth of CTB-labeled large-diameter afferents was examined in transverse sections at the lesion site and throughout the lumbar enlargement of the spinal cord of animals that received CTB injections to the sciatic nerve. For qualitative analysis of the fiber distribution pattern of CTB stained sections, images of afferent fiber-labeling were printed, and the distribution of labeled profiles was outlined and reviewed by an observer who was blind to the genotype. For quantitative analysis at the epicenter level, the proximity of CTB-labeled axons to the lesion epicenter was measured. In the lumbar spinal cord, images of 9 equally spaced sections spaced 600 μm apart and spanning the lumbar enlargement of the spinal cord were captured for quantitative analysis. The total area of CTB labeled fiber staining was measured in the dorsal horn using MCID and compared across sections and genotype.

To compare astrocyte and microglial activation across groups, the proportional area of glial fibrillary acidic protein (GFAP) and Mac-1 (Cd11b) staining in the lumbar enlargement was analyzed at 40x magnification using boxes (47,000 μm^2) encompassing medial and lateral portions of the superficial dorsal horn as well as the deep dorsal horn (Detloff et al., 2008). Four sections (800 μm apart) centered in the middle of the lumbar enlargement of the spinal cord were quantified using MCID. Since there was no difference between medial and lateral areas of staining, these values were summed.

Statistical Analyses—Two-way ANOVA was used to compare histological outcomes over distance and between groups, with Bonferroni's correction post-hoc analyses to examine differences at each distance. Values for missing or torn sections were obtained from the immediately adjacent section. Two-tailed T-tests were used to compare average staining areas between uninjured and injured WT mice or injured WT and injured KO mice. Two-way ANOVA with repeated measures was used to compare behavioral outcomes over time between groups. Two-tailed T-tests were used to compare baseline and endpoint scores between groups, with Welch's corrections for unequal variances where appropriate. Differences were considered significant at $p < 0.05$. Graphs represent mean $\pm$ standard error of the mean. Graphing and analyses were performed with Graphpad Prism 4 (Graphpad Software, San Diego, CA, USA).

Results

Behavioral Recovery

KO and WT mice have similar locomotor recovery patterns after SCI—As L1 has previously been shown to play a role in motor function (Jakeman et al., 2006), we first examined the effects of L1 on motor recovery after SCI. Naïve KO mice have mild but reproducible locomotor deficits that are evident in the open field and when crossing a narrow beam (Figure 1A,B). Uninjured WT mice scored 8.9 ± 0.1 on the BMS scale, while KO mice scored 7.8 ± 0.3 with evidence of mild trunk instability, as reported previously (Jakeman et al., 2006) ($p=0.007$). After a moderate contusion injury, both KO and WT mice were completely paralyzed or had only slight ankle movements (BMS 0-1), and then showed a similar partial recovery pattern (Figure 1A). BMS scores reached a plateau by 3 weeks post-injury. All of the mice demonstrated consistent plantar stepping with deficits in forelimb/hindlimb coordination and/or paw rotation (BMS 5-6) throughout the remainder of

the study. Repeated measures two-way ANOVA confirmed a significant recovery effect over time ($p < 0.0001$), but no difference due to genotype. Similarly, there were no differences between groups in subscores over time (not shown).

For animals that can step consistently in the open field, a more difficult task can be used to further separate differences in ability. We used a beam-crossing test to evaluate fine locomotor skills before and after SCI (Figure 1B; for detailed methods, see Supplementary Material). Uninjured WT mice scored 7.8 ± 0.3 (occasional hindlimb foot slips or forepaw gripping) crossing the beam, while KO animals scored 6.3 ± 0.3 (tail carried predominantly down instead of up behind the animal, and occasional stepping deficits) ($p = 0.009$). Beginning at 21 dpi, WT and KO mice performed similarly on the beam. Both WT and KO mice attained scores of 3-4 signifying only occasional plantar steps during crossing (Figure 1B). There was significant recovery over time in both groups ($p < 0.0001$), but no difference between genotypes (2-way ANOVA with repeated measures).

L1-CAM is required for maintaining thermal hyperalgesia after injury—Before injury, WT and KO mice had equivalent hindpaw withdrawal latencies to a radiant heat source (WT 9.0 ± 0.61 s vs. KO 8.7 ± 0.8 s; Figure 2A). The mice were tested again starting at 2 weeks post-injury, when they were able to place the plantar surface of the hindpaws and support weight. By 3 weeks after injury, the withdrawal latency of both groups had decreased to 6-8 seconds, indicating hyperalgesia, or a heightened response to a painful stimulus (Finnerup et al., 2003). The WT withdrawal latencies continued to decrease to 5.2 ± 0.5 seconds at 6 weeks. Interestingly, in KO animals the withdrawal latency began returning to normal after 4 weeks, reaching 8.9 ± 1.4 seconds at 6 weeks after injury ($p = 0.001$, unpaired t-test of WT of KO endpoint latencies). Repeated measures two-way ANOVA confirmed an effect of genotype ($p = 0.02$) and time ($p = 0.005$) on withdrawal latencies. This ameliorated hyperalgesia in KO mice suggests that L1 is crucial for the maintenance of hyperalgesia.

Neither WT nor KO mice develop mechanical allodynia after moderate injury—Before injury, the KO mice had greater variation in withdrawal thresholds, but there was no significant difference between WT and KO mice in the gram force threshold for withdrawal to a tactile stimulus on the plantar surface of the hindpaw. Throughout recovery, the withdrawal threshold did not change from baseline and was similar in both WT and KO mice (Figure 2B).

Histopathology

L1CAM deletion does not affect lesion size or CGRP fiber distribution at the epicenter—L1-CAM was widely distributed in the gray matter of the thoracic and lumbar spinal cord (Figure 3C) and at the lesion epicenter (Figure 3A) in WT mice, but was absent from KO mice (Figure 3B,D).

The moderate contusion injury produced consistent lesions resulting in the loss of gray matter at the lesion epicenter with a rim of spared white matter around the perimeter of the cord, as described previously (Ma et al., 2001) (Figure 4A,B). Analysis of percent cross-sectional area of spared white matter (spared white matter area / total cross-sectional area) at the lesion epicenter revealed no differences in the lesion size between WT and KO animals (WT $8.6 \pm 1.9\%$ vs. KO $10.2 \pm 2.6\%$) (Figure 4C).

L1 is expressed on small diameter CGRP⁺ afferent fibers (Runyan et al., 2005). Surprisingly, a similar distribution and density of CGRP⁺ axons was seen in WT and KO mice at the lesion epicenter (Figure 4D-H). Quantitative analysis of CGRP⁺ fiber staining in the dorsal quadrant at the lesion epicenter revealed no difference between WT and KO mice,

indicating that L1 is not required for growth or sprouting of CGRP⁺ axons at the lesion site (Figure 4F).

CGRP⁺ afferent distribution expands in lumbar spinal cord after mid-thoracic SCI

In the lumbar enlargement of the spinal cord, 5-9 spinal segments caudal to the level of injury, CGRP⁺ staining was restricted to the dorsal horn and ventral motoneurons (Figure 5A-D). The majority of CGRP⁺ fibers in the dorsal horn was concentrated in a band in the superficial layers, while additional fibers extended ventrally to penetrate into the deeper layers of the dorsal horn and a few extended toward lamina X. After moderate contusion injury there was significantly more CGRP staining in both superficial ($p < 0.0001$) and deep ($p = 0.002$, 2-way ANOVA injury effect) laminae in the dorsal horn (Figure 5E,F) compared with uninjured mice. Injured WT and KO mice showed similar staining patterns (Figure 6A,B). There was no difference in the total area of CGRP⁺ staining in the superficial dorsal horn between WT and KO mice (Figure 6C). However, the KO mice had a significantly smaller area of CGRP⁺ fiber staining in the deep dorsal horn of the lumbar cord (2-way ANOVA, genotype effect $p = 0.04$; Figure 6D).

PKC γ ⁺ cells are increased in the lumbar spinal cord after mid-thoracic SCI

PKC γ expression and activation have been associated with neuropathic pain (Mao et al., 1995; Martin et al., 1999; Miletic et al., 2000; Sluka and Audette, 2006; Kerr and David, 2007), including the maintenance of hyperalgesia (Malmberg et al., 1997). PKC γ ⁺ staining was found primarily in cells and processes in the superficial dorsal horn and axons of the dorsal corticospinal tract of uninjured mice. After injury, PKC γ was restricted to the dorsal horn in lumbar spinal cord (Figure 7A). Stained cell soma profiles were concentrated in lamina II of the dorsal horn, with occasional labeled cells scattered through surrounding laminae and near the central canal (Figure 7B,C). There were no differences in the number of PKC γ ⁺ cells with injury or between genotype in laminae I, III-IV, or X (data not shown). However, the number of PKC γ ⁺ cells in lamina II of the dorsal horn in the lumbar spinal cord increased significantly after injury compared with uninjured mice ($p = 0.0006$ injury, $p = 0.001$ by section, 2-way ANOVA; Figure 7D). The difference between the numbers of PKC γ ⁺ cells in lamina II was particularly apparent in the more caudal sections within the lumbar enlargement. The number of PKC γ ⁺ cells found in lamina II after injury was significantly greater in KO mice than in WT mice (genotype $p = 0.003$, sample $p = 0.05$, 2-way ANOVA, Figure 7E), implicating L1 in the regulation of expression of this signaling molecule.

L1-CAM deletion does not effect changes in the distribution of large diameter fibers or glial reactivity after moderate SCI

Staining of CTB-labeled large-diameter primary sensory afferents conveying touch, vibration and limb position (Krenz and Weaver, 1998) was strong as these fibers entered the dorsal horn and traveled to the dorsal columns (Figure 8A,B). Fibers then ascended until they reached the lesion site, where CTB labeling dissipated, with no further labeling in or rostral to the injury site in any specimen. In the lumbar cord, we found no difference between groups in qualitative observations of labeled fiber distribution or total area of labeled fibers (Figure 8C).

Neuropathic pain has been associated with increased astrocyte (Nesic et al., 2005; Hald et al., 2008) and microglial (Tsuda et al., 2003; Narita et al., 2006; Hains and Waxman, 2006; Detloff et al., 2008) activation in both peripheral nerve ligation and SCI models. Glial reactivity was examined as a potential contributor to differences in sensory responses between WT and KO mice. Astrocytes were present throughout the lumbar cord six weeks after a moderate contusion injury. Microglia were present, but were much less prominent (Figure 9). There were no differences between WT or KO mice after injury in either MAC-1 or GFAP immunoreactivity in superficial or deep dorsal horn regions (Figure 9 C,D).

Discussion

L1 is an important contributor to axonal sprouting and guidance. Deletion of L1 in mice did not affect lesion size, motor recovery, or the distribution or density of CGRP containing axons within the lesion site after moderate contusion injury. However, L1 deletion had significant, specific effects on hindlimb sensory response latency after SCI and corresponding effects in possible anatomical substrates in the lumbar spinal cord. Neither KO nor WT mice demonstrated evidence of hindlimb allodynia after SCI, but both developed heat hyperalgesia, and L1 was required to maintain this for the duration of the study. Differences in heat sensitivity corresponded to the distribution of CGRP fibers in the lumbar spinal cord. CGRP fiber sprouting in this region was increased after contusion injury, but was attenuated in mice lacking L1. The number of PKC γ expressing interneurons also increased after injury, but surprisingly, these cells were more numerous in the KO mice that recovered to normal withdrawal latencies to noxious heat than in WT mice that maintained the shorter latencies caused by SCI. These results demonstrate that L1 plays an important role in modulating functional and anatomical plasticity in the denervated lumbar spinal cord after contusion injury.

The absence of L1 CAM does not impede locomotor recovery after moderate SCI

Uninjured L1 KO mice have been reported to have functional motor deficits (Dahme et al., 1997; Fransen et al., 1998; Jakeman et al., 2006), which were further confirmed by the beam and BMS testing performed in this study. A previous study from this laboratory showed that after a mild contusion injury there were smaller differences in locomotor function between groups than before injury (Jakeman et al., 2006). The moderate contusion injury used in the present study highlighted an even more striking similarity in post-injury locomotor recovery, with no differences between groups after injury. The similarity in motor recovery between WT and KO mice implies that L1 is not required for normal recovery of function after SCI.

L1 is not essential for normal hindlimb sensory function in mice

In our evaluation of the effect of L1 on sensory function, we conducted baseline testing comparing sensory responses to hindlimb stimuli in naïve WT and KO littermates. Using well established and accepted methods of measuring sensation, we found no difference in latency to withdrawal from thermal stimulation, and only a trend toward increased withdrawal threshold from mechanical stimulation in KO mice. Our results differ from past reports of reduced mechanical and thermal sensitivity in L1 KO mice (Haney et al., 1999; Thelin et al., 2003). Differences in background strains or testing methods used could contribute to the discrepancy. Over the last decade, L1 KO mice have been maintained on two different genetic backgrounds including the hybrid B6:129S7 used here and by Haney et al., or backcrossed to a C57BL/6 background as used by Thelin et al. (2003). Differences in thermal and mechanical sensitivity have been reported for these background mouse strains, with C57/B6 mice showing greater sensitivity to heat and 129 substrains having a decreased mechanical withdrawal threshold (Mogil et al., 1999; Leo et al., 2008). While the underlying genetic differences are still not fully elucidated, it is possible that the effects of L1 deletion on sensory responses are greater on the C57BL/6 background. In addition, quite different testing paradigms were used to establish mechanical sensitivity in Haney et al. (1999), suggesting that the trends we observed using a standardized mechanical threshold method could become more robust in an alternate testing paradigm. Another important variable in sensory response time in L1 KO mice may be age of the mice at the time of testing. Thelin et al. (2003) used mice 6-12 months old, whereas our mice were 8-11 weeks old at the beginning of the study. Haney et al. described an advancing degeneration of small diameter sensory fibers with defective Schwann cell ensheathment. If peripheral axon degeneration is progressive, then the sensory function in L1 KO mice may deteriorate over time, leaving the

much older mice used by Thelin and colleagues with more pronounced sensory deficits. The possible interaction of background strain and age in the L1 KO mouse has not been investigated to date.

L1 CAM is required for maintenance of thermal hyperalgesia but not allodynia after SCI

After SCI, we saw a robust development of hindlimb thermal hyperalgesia in all of the mice. The hyperalgesic response was maintained for the duration of the study in WT mice, but began to resolve by 4 weeks in KO mice, and was completely ameliorated by six weeks post-injury. These results demonstrate that L1 plays an essential role in the maintenance of thermal hyperalgesia after thoracic injury, a type of neuropathic pain present in humans clinically, and that we have seen consistently after contusion SCI in mice in our lab (unpublished data). In contrast, there was no change in the threshold for withdrawal from a mechanical stimulus after SCI in either KO or WT mice. The lack of evidence of allodynia is particularly interesting in light of the robust hyperalgesia, supporting recent findings and suggesting separate mechanisms of action for these two modes of sensory dysfunction (Obata et al., 2008; Terayama et al., 2008).

Anatomical substrates of L1-dependant hyperalgesia: CGRP

To investigate the mechanisms of L1-mediated maintenance of thermal hyperalgesia, we compared expression of afferent distribution in the lumbar spinal cord using several histological markers, including the area of CGRP⁺ fibers. Previous studies have shown that these small diameter peptidergic afferents sprout and increase the area of innervation in the lumbar cord after thoracic SCI, and suggest that this sprouting is one cause of hindlimb neuropathic pain symptoms (Christensen and Hulsebosch, 1997; Krenz and Weaver, 1998; Romero et al., 2000; Ondarza et al., 2003; Hofstetter et al., 2005). In accordance with this, we saw significantly increased CGRP⁺ fiber sprouting in the lumbar dorsal horn after injury in WT mice when compared with uninjured WT controls. L1 and CGRP are co-expressed in these afferents (Runyan et al., 2005), and the influence of L1 as a growth molecule on CGRP⁺ fiber sprouting has been shown and discussed previously (Chaudhry et al., 2006; Runyan et al., 2007; Deumens et al., 2007; Yamanaka et al., 2007). Notably, L1 has also been shown to contribute to synapse formation and stabilization (Godenschwege et al., 2006; Triana-Baltzer et al., 2008) as well as the survival of small-diameter unmyelinated afferent axons in the periphery (Haney et al., 1999). Thus, a decrease in CGRP⁺ fiber distribution and/or sprouting would be expected in mice lacking L1.

L1 did not contribute significantly to afferent growth at the lesion site. Deumens et al (2007) have reported a qualitative decrease in CGRP⁺ fiber sprouting at the site of injury after a mid-thoracic dorsal spinal cord hemisection. However, Runyan et al (2007) found no difference between L1 KO and WT mice in CGRP fiber sprouting after a dorsal rhizotomy, and we found no significant differences in CGRP⁺ immunoreactive areas in the lesion site or the surrounding dorsal horn regions. This is consistent with our previous evidence of no effect of L1 deletion on the density of neurofilament positive axons in the lesion site (Jakeman et al., 2006). Blocking semaphorin-mediated L1 inhibition also has no effect on sprouting at the site of injury, indicating that L1 plays a minor role in regulation of afferent fiber growth at the complex injury site (Mire et al., 2008).

In contrast to the absence of effect at the injury site, our data reveal a clear and significant difference in sprouting in the lumbar cord between KO and WT littermates six weeks after mid-thoracic SCI. While the mechanisms for the effects of L1 are not clear, there are three plausible contributing factors. First, the difference may be due to L1 adhesion enhancing the ability of CGRP⁺ fibers to sprout in the denervated lumbar dorsal horn. Secondly, L1 may increase the stability or maintain the integrity of synaptic contacts of sprouted CGRP⁺

fibers, maintaining the increased sprouting apparent in WT mice. This possibility is consistent with effects of L1 deletion on the maintenance of LTP, which involves a similar establishment of strengthened excitatory synapses (Luthi et al., 1994). A third possibility is that over the time course of the study, the small diameter afferents lacking L1 may sprout, but may be susceptible to axonal degeneration and/or neuronal death after a spinal cord injury. Dahme et al. (1997) and Haney et al. (1999) both noted that L1 KO mice had impaired Schwann cell ensheathment, and the latter found that this impaired ensheathment may cause progressive degeneration. However, Runyan et al. (2007) show that after dorsal rhizotomy the enhanced sprouting in that injury model is maintained for up to three months, suggesting that L1 KO mice maintain sufficient innervation of the dorsal horn for at least three months after adulthood, which is within the age window of the mice in the current study. Additional studies are needed to determine if short-lived changes in sensory withdrawal responses correspond to transient anatomical sprouting or reorganization. The diminished sprouting is only apparent in the deep dorsal horn of the lumbar enlargement, where a difference in sprouting between WT and KO mice would be expected to cause the behavioral difference manifested as loss of hyperalgesia in the hindlimbs of KO mice.

Anatomical substrates of L1-dependent hyperalgesia: PKC γ

PKC γ -expressing neurons have been identified mainly as excitatory interneurons that receive input from myelinated, non-noxious afferents (Neumann et al., 2008). Increased PKC γ activation is generally associated with neuropathic pain behavior (Mao et al., 1995; Malmberg et al., 1997; Miletic et al., 2000; Sluka and Audette, 2006). PKC γ regulates synaptic activity by phosphorylating essential signaling molecules and receptors, and receptor activation in turn influences PKC γ expression (Mao et al., 1995; Brenner et al., 2004). Here, we show that the number of PKC γ^+ cells increases in lamina II of the dorsal horn after a moderate contusion injury, concurrent with hyperalgesic behavior (Kerr and David, 2007). Surprisingly, we also found that the post-injury number of PKC γ^+ cells is increased in injured L1 KO mice above the numbers of PKC γ^+ cells in injured WT mice. The precise relationship between L1 and PKC γ is unknown. However, the increase in cells expressing PKC γ in L1 KO mice suggests that L1 modulates PKC γ expression in the novel post-injury environment induced by trauma and deafferentation. We and others have shown (Krenz and Weaver, 1998; Romero et al., 2000; Hofstetter et al., 2005) that the small diameter afferents on which L1 is present sprout after injury, and that PKC γ is expressed in an increased number of cells, likely bringing L1-expressing afferents in proximity with PKC γ^+ neurons and allowing L1 to interact with the PKC γ pathway. We postulate that L1 is an upstream mediator of PKC γ -mediated chronic hyperalgesia. The unexpected further increase in PKC γ in hyperalgesia-deficient, L1-lacking mice may be due to restricted cytosolic expression of PKC γ rather than translocation to the cell membrane, where it is most correlated with pain-like behavior (Martin et al., 1999; Miletic et al., 2000). We speculate that the novel post-injury distribution of L1 and CGRP $^+$ fibers allows L1 to contribute to PKC γ translocation to and action at the cell membrane, resulting in hyperalgesia. We propose this unexplored relationship between L1 and PKC γ as an important component of post-injury hyperalgesia in mice.

Moderate SCI does not produce hindlimb allodynia in KO or WT mice

An important finding in this study is that while injured mice developed robust thermal hyperalgesia, we saw no indication of allodynia after SCI in either genotype. This reiterates the recent trend in the literature demonstrating tactile allodynia and thermal hyperalgesia as distinct symptoms caused by separate mechanisms (Bennett et al., 2000; Obata et al., 2008; Terayama et al., 2008). In light of the absence of allodynia contrasting robust hyperalgesia, the inflammatory and anatomical changes reported to contribute to neuropathic pain that were not affected by the lack of L1 are also of particular interest. Microglial activation has

received much attention lately in the literature (Tsuda et al., 2003; Narita et al., 2006; Beggs and Salter, 2007; Detloff et al., 2008), and perhaps surprisingly was not different between groups. Similarly, GFAP expression, which we used as an indicator of astrocyte activation (Hald et al., 2008), was also equivalent between groups. We also saw no evidence of differences in sprouting or redistribution of CTB-labeled large-diameter myelinated sensory afferents. While these may be contributors to allodynia and/or hyperalgesia in other models of neuropathic pain, they were not sufficient to maintain thermal hyperalgesia after a moderate contusion SCI in mice in the absence of L1.

This study further supports the role of L1 in the motor and sensory function of the nervous system. We confirm that L1 plays an important role in the development of normal motor function. Of perhaps greater importance, particularly to the SCI population and the SCI research community, we show that L1 is essential in maintenance of thermal hyperalgesia after contusive SCI on mice. In contrast to robust hyperalgesia, we saw no indication of allodynia. We propose that interactions between L1 and PKC γ , as well as L1 and CGRP⁺ afferent fibers contribute to the maintenance of thermal hyperalgesia in this model.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

WT and KO mice showed no difference in locomotor recovery after injury. **A.** Prior to injury, deficits were observed in the open field according to the Basso Mouse Scale (BMS) ($p=0.007$, t-test of baseline scores), but there were no differences in the time course or extent of recovery after injury. Dotted line represents SCI. **B.** Uninjured KO mice showed deficits while crossing a narrow beam compared with WT controls ($p=0.009$), but there were no differences between KO and WT in the extent of recovery after contusion injury.

Figure 2.

KO mice recovered from hindlimb heat hyperalgesia after SCI. KO and WT mice had no differences in either thermal nociceptive responses (**A**) or mechanical responses (**B**) to stimuli applied to the hindlimbs before injury. Dotted line represents SCI. **A**. After injury mice of both genotypes developed a hyperalgesic response to thermal stimuli. WT mice sustained this response for the duration of the study, but thermal withdrawal latencies completely recovered to baseline values in KO mice. 2-way ANOVA showed differences due to genotype, $p=0.02$; over time, $p=0.005$. T-test of endpoint latencies $p=0.01$. **B** Neither KO or WT mice show any change in withdrawal threshold to mechanical stimuli after injury.

Figure 3.

L1 CAM staining in KO and WT mice. **A,B** Distribution of L1-expression in transverse sections through the lesion epicenter from a WT mouse (**A**) and KO littermate (**B**). **C,D** L1-stained transverse sections obtained from below the level of the injury site (L1 level) in WT (**C**) and KO (**D**) specimens. Scale bars are 100 μm (**A,B**) and 200 μm (**C,D**).

Figure 4.

Differences in hyperalgesia were not due to differences in lesion size or sprouting at the epicenter. **A,B.** Representative sections stained with eriochrome cyanine (EC, a myelin stain) of the lesion epicenter from WT (**A**) and KO (**B**) mice. Outlining represents tissue designated as spared white matter. **C.** There was no difference between groups in percent spared white matter at the lesion center. **D,E,G,H** CGRP staining at the lesion site. **D** Representative section from a WT mouse at the injury epicenter. **G** Representative section from a KO mouse at the injury epicenter. **E,H** Higher magnification of box from WT (**E**) and KO (**H**) animals. **F** There were no significant differences between groups in the density of CGRP⁺ staining in the dorsal quadrant of the lesion site. **A,B,D,G** Scale bar is 200 μm . **E,H** Scale bar is 50 μm .

Figure 5.

After SCI, CGRP⁺ fiber staining is increased in the lumbar enlargement of the spinal cord, 5-9 spinal segments caudal to the lesion, in WT mice. **A** A CGRP-stained section from an uninjured lumbar spinal cord at low magnification. **B** A schematic of the paradigm used for superficial (Sup.) and deep dorsal horn (DH) sampling. **C,D** Higher power images of the dorsal horn of the lumbar spinal cord comparing sections from **(C)** uninjured mouse and **(D)** injured mice. **E,F** Graphs comparing the rostro-caudal distribution of CGRP⁺ fibers in sections spaced 600 μm apart in injured and uninjured mice in the **(E)** superficial and **(F)** deep dorsal horn of the lumbar spinal cord. The rostral end of the lumbar enlargement is oriented to the left on the graph (section 1), and the caudal end on the right (section 10). Injured mice had significantly more CGRP labeling in both superficial ($p < 0.0001$) and deep ($p = 0.002$) dorsal horn across the length of the enlargement. Scale bar in **A** is 200 μm , bars in **B,C,D** are 40 μm .

Figure 6.

L1 KO mice have reduced CGRP⁺ fiber sprouting in the deep dorsal horn of the lumbar spinal cord compared with WT mice. **A, B.** CGRP staining in the dorsal horn of the lumbar cord from a WT (**A**) and a KO (**B**) specimen. Scale bars are 100 μm. **C, D** Quantification of staining in superficial (**C**) and deep (**D**) laminae from the lumbar enlargement. There was no effect of genotype on staining area of fibers in the superficial dorsal horn, but a significant reduction in staining in the deep dorsal horn of KO mice ($p=0.04$).

Figure 7.

PKC γ ⁺ cells are increased in lamina II of the lumbar spinal cord after injury. **A** A low power image of a PKC γ -stained section from the lumbar enlargement of the spinal cord. **B,C** Higher magnification of the box from the lateral portion of lamina II from injured (**B**) WT and (**C**) KO animals. **D,E** Quantification of PKC γ ⁺ cells in lamina II of the lumbar spinal cord from (**D**) injured vs uninjured mice and (**E**) injured WT vs KO mice. Injured mice had significantly more PKC γ ⁺ cells than uninjured mice ($p=0.001$). KO mice had significantly more PKC γ ⁺ cells than WT mice ($p=0.003$). Star indicates post-hoc value of $p < 0.05$. Scale bar in **A** is 200 μm , bars in **C,D** are 20 μm .

Figure 8. Injured KO and WT mice had no difference in distribution area of large-diameter sensory afferents in lumbar spinal cord. **A** A low power image of CTB (cholera toxin subunit β)-labeling in the lumbar spinal cord. **B** CTB-labeled sensory fibers at a higher magnification. **C** Average area of CTB-labeled fibers in the dorsal horn of the lumbar spinal cord in KO and WT mice. Scale bar is 20 μm in **A**. Scale bar is 200 μm in **B**.

Figure 9.

Glial activation in lumbar spinal cord of injured L1 knockout and wild type mice. Astrocytes were identified with GFAP staining (**A**) and microglia were identified using a Mac-1 (CD11b) antibody (**B**). **C** Average area of GFAP⁺ staining per section in the dorsal horn of the lumbar spinal cord in WT and KO mice. **D** Average area of Mac-1 staining in the dorsal horn of the lumbar spinal cord in WT and KO mice. There was no difference between wild type and knockout animals in Mac-1 or GFAP staining in the lumbar dorsal horn. Scale bars are 20 μm.

Table 1

Antibodies and Stains

Stain / Antibody	Target	Sampling Method	Primary Antibody	Secondary Antibody	References
EC (Eriochrome Cyanine)	Myelinated axons	Epicenter, every 100 μm	--	--	Jakeman et al., 2006
L1	L1 cell adhesion molecule	Epicenter, every 100 μm	1:4,000 rabbit anti-L1 (Dr. Rathjen)	1:1600 goat anti rabbit (Jackson Labs)	Rathjen and Schachner, 1984
CGRP (calcitonin gene-related peptide)	small-diameter peptidergic sensory afferents	Lumbar, every 600 μm ; Epicenter, -200, 0, +200 μm from epicenter	1:20,000 rabbit anti-CGRP (Peninsula Laboratories)	1:5000 goat anti rabbit (Jackson Labs)	Krenz and Weaver 1998; Christensen and Hulsebosch 1997
CTB (cholera toxin subunit B)	large-diameter sensory afferents	Lumbar, every 600 μm	1:40,000 goat anti-CTB (List Biological Labs)	1:400 horse anti-goat (Vector Labs)	Bradbury et al. 1999; Krenz and Weaver 1998
PKC γ (protein kinase C)	lamina II excitatory interneurons	Lumbar, every 800 μm	1:4000 rabbit anti-cPKC γ (Santa Cruz Biotechnology)	1:2000 goat anti rabbit (Jackson Labs)	Kerr and David 2007; Polgar et al 1999
GFAP (glial fibrillary acidic protein)	astrocytes	Lumbar, every 800 μm	1:2000 Rabbit anti GFAP (DAKO)	1:1000 Alexafluor 488 goat anti rabbit (Molecular Probes)	Vega-Avelaira et al. 2007
Mac-1 (CD11b)	microglia	Lumbar, every 800 μm	1:100 Rat Mac-1 monoclonal anti-CD11b (Serotec Ltd)	1:200 Alexafluor 546 goat anti-rat (Molecular Probes)	Terayama et al. 2008