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# Focal Lysolecithin-Induced Demyelination of Peripheral Afferents Results in Neuropathic Pain Behavior That Is Attenuated by Cannabinoids

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Demyelinating diseases can be associated with painful sensory phenomena such as tactile allodynia and hyperalgesia. To study the mechanisms underlying demyelination-induced pain, we have characterized a novel model of demyelination of the sciatic or saphenous nerve. Topical lysolecithin application causes focal demyelination of afferent nerve A-fibers without axonal loss, as assessed either by electron and light microscopy or by immunohistochemical analysis of dorsal root ganglia (DRG) for a neuronal injury marker, activating transcription factor 3. Focal demyelination is accompanied by spontaneous action potentials in afferents and increased expression of neuropeptide Y and  $\text{Na}_v1.3$  sodium channels specifically in DRG neurons that coexpress a specific marker of myelinated afferents. In contrast, expression of tetrodotoxin-resistant,  $\text{Na}_v1.8$  sodium channels is specifically decreased in the same subgroup of DRG cells. Central sensitization of somatosensory processing is also induced, with increased behavioral reflex responsiveness to thermal and mechanical stimuli. These changes are reversed by intrathecal administration of an NMDA receptor antagonist or cannabinoid (CB) receptor agonist, but not by a  $\mu$ -opioid receptor agonist. Recovery of behavioral reflexes occurred  $\sim 3$  weeks after lysolecithin treatment. This is the first time that demyelination of afferent A-fibers has been shown to specifically induce neuropathic pain and indicates that axonal damage is not a prerequisite for development of the pain state. The profile of phenotypic changes in DRG is distinct from other pain models and displays a sensitivity to NMDA and CB receptor agents that may be exploitable therapeutically.

**Key words:** afferents; demyelination; neuropathic pain; cannabinoids; sodium channels; DRG

## Introduction

Both the CNS and the peripheral nervous system (PNS) are susceptible to demyelinating disorders in humans. In the PNS, Charcot-Marie-Tooth disease types 1 and 4 (CMT1 and CMT4) and Guillain-Barré syndrome (GBS) are accompanied not only by segmental demyelination with reduced rates of nerve impulse transmission and impaired motor function, but also by abnormal sensory phenomena such as tactile allodynia (the perception of normally innocuous stimuli such as touching and brushing as painful), hyperalgesia (a heightened response to painful stimuli), and spontaneous pain (Carter et al., 1998). However, the mechanisms underlying neuropathic pain in demyelinating disease are poorly understood (Rasminsky, 1981).

Recently, we showed that mice lacking a functional *Periaxin* (*Prx*) gene develop a late-onset peripheral demyelination, which is associated with reflex behaviors corresponding to allodynia and hyperalgesia (Gillespie et al., 2000). These enhanced sensory responses are reversible by spinal administration of an NMDA receptor antagonist, implying a critical role for central sensitization in the pain behavior. Recently, mutations in the human *PRX* gene have been shown to underlie CMT4. Consistent with the mouse model, there is a particularly large sensory involvement in patients with CMT4F,

who display various distal abnormalities including pain in both upper and lower extremities (Delague et al., 2000; Boerkoel et al., 2001; Guilbot et al., 2001; Takashima et al., 2002).

To determine how peripheral demyelination might contribute to the development of the ensuing neuropathic pain state, we have characterized a model of focal, reversible peripheral nerve demyelination of the sciatic or saphenous nerve using the selective demyelinating agent lysolecithin (lysophosphatidyl choline) (Hall and Gregson, 1971). Using this model we have studied the effects of demyelination on peripheral nerve function and on the phenotype of cells in the dorsal root ganglia (DRG), including changes in the expression of calcitonin gene-related peptide (CGRP), galanin, neuropeptide Y (NPY), and the  $\text{Na}_v1.8$  (sensory neuron-specific type 1) and  $\text{Na}_v1.3$  (brain type III) sodium channels. Furthermore, we have investigated key changes in somatosensory processing within the spinal cord. We show for the first time that transient demyelination causes a distinctive profile of phenotypic changes in DRG. These changes are accompanied by spontaneous activity in afferents and central sensitization of sensory processing, all in the apparent absence of damage to the afferent axons. The sensitivity of the behavioral reflex changes to NMDA receptor antagonists and cannabinoid (CB) receptor agonists suggests therapeutic strategies for pain induced by demyelination.

## Materials and Methods

**Animals and surgical methods.** C57BL/6 mice over the age of 6 weeks were used for all experiments. Animals were anesthetized with 0.3 ml 25% Sagatal (Rhone Merieux, Hertfordshire, UK) in sterile physiological sa-

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line (intraperitoneal) and maintained on 1–2% halothane (Zeneca, Cheshire, UK) in O<sub>2</sub>. For all demyelination experiments, lysolecithin (Sigma, St. Louis, MO) was dissolved in physiological saline to give a final concentration of 15 mg/ml.

**Saphenous and sciatic nerve demyelination.** The saphenous branch of the femoral nerve was exposed at mid thigh level, and 5–10  $\mu$ l lysolecithin solution was applied topically via a 25 gauge needle (Terumo) over a length of 5–6 mm, ensuring that the nerve remained coated with the solution for 10 min after which the reagent was washed off with sterile saline and the incision site was closed with 4.0 suture thread, marking the application site. For sciatic nerve demyelination experiments, once the nerve was exposed at mid thigh level, the two larger fascicles (tibial and peroneal) were separated by blunt dissection from the smallest sural fascicle. Lysolecithin was applied to the tibial and peroneal fascicles for 15 min. Sham control animals were prepared for both nerve preparations by omitting lysolecithin from the solution so that the nerve was exposed as above and sterile saline was applied topically for the relevant time.

**Behavioral reflex testing.** The threshold for hindpaw withdrawal in response to graded mechanical stimulation was measured in conscious animals with von Frey filaments (Stoelting, Wood Dale, IL), which provide a calibrated indentation pressure against the hairless skin of the hindpaws. The threshold response was defined by the filament that caused foot withdrawal at least 5 times in every 10 applications (Meyer et al., 1979; Chaplan et al., 1997). The time for hindpaw withdrawal in response to a quantified noxious thermal stimulus was measured using the Hargreaves-Plantar apparatus (Ugo Basile). The thermal stimulus was of infrared intensity setting 30 (>50°C) applied to the mid-plantar surface of each hindpaw (Hargreaves et al., 1988). The withdrawal was characterized as a brief paw flick, and the withdrawal latency was recorded in seconds.

Baseline measurements were obtained for all animals over the course of 1 week before surgery. Behavioral reflex tests were performed after surgery to identify the development of any reflex sensitivity in treated animals. The threshold value at each time point tested was calculated as the mean  $\pm$  SEM. Any statistically significant differences in paw withdrawal threshold from mechanical stimulation between ipsilateral and contralateral paws were determined by a Mann–Whitney rank sum test. Likewise, any statistically significant differences in paw withdrawal latency from thermal stimulation were determined by a Student's paired *t* test.

**Morphological investigations.** Nerve morphology was examined in animals that previously had focal demyelination induced in either the saphenous or sciatic nerve. Sections of nerve from the region of lysolecithin application were dissected 13 d later, fixed for 4 hr in 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M sodium cacodylate buffer, pH 7.3, postfixed in OsO<sub>4</sub>, and embedded in Araldite.

For light microscopy, 1  $\mu$ m resin sections of the demyelinated section of the nerve (~7–10 mm) were stained with Toluidine blue. Once the area of maximal demyelination was established by bright-field microscopy, ultrathin (80 nm) sections were stained with uranyl acetate and lead citrate and examined on a Phillips BioTwin electron microscope (FEI, UK Ltd., Cambridge, UK). Nerve counts were performed at the electron microscope (EM) level so that the amount of demyelination and integrity of large-diameter and small-diameter, unmyelinated axons could be determined. C-fibers were identified as small-diameter unmyelinated fibers present in bundles, surrounded by Schwann cell cytoplasm. The number of intact C-fibers was calculated from EM photographs of cross sections of the entire nerve. To minimize error, the images from all nerves (*n* = 4 per group) were randomly counted three times (while the source of each image was concealed), and the average for each nerve was calculated. To count the number of myelinated A-fibers, axons with all levels of myelination, including those that were completely demyelinated, must be considered. Therefore, A-fibers were identified as not only fibers with a myelin sheath, but also solitary fibers with a smaller overall diameter attributable to loss of myelin. This allowed distinction of demyelinated fibers from unmyelinated fibers, which usually occur in bundles. The statistical significance of any difference between treated and naive or sham nerves was determined using a Kruskal–Wallis one-way ANOVA on ranks with an all pair-wise multiple comparisons procedure (Dunn's method).

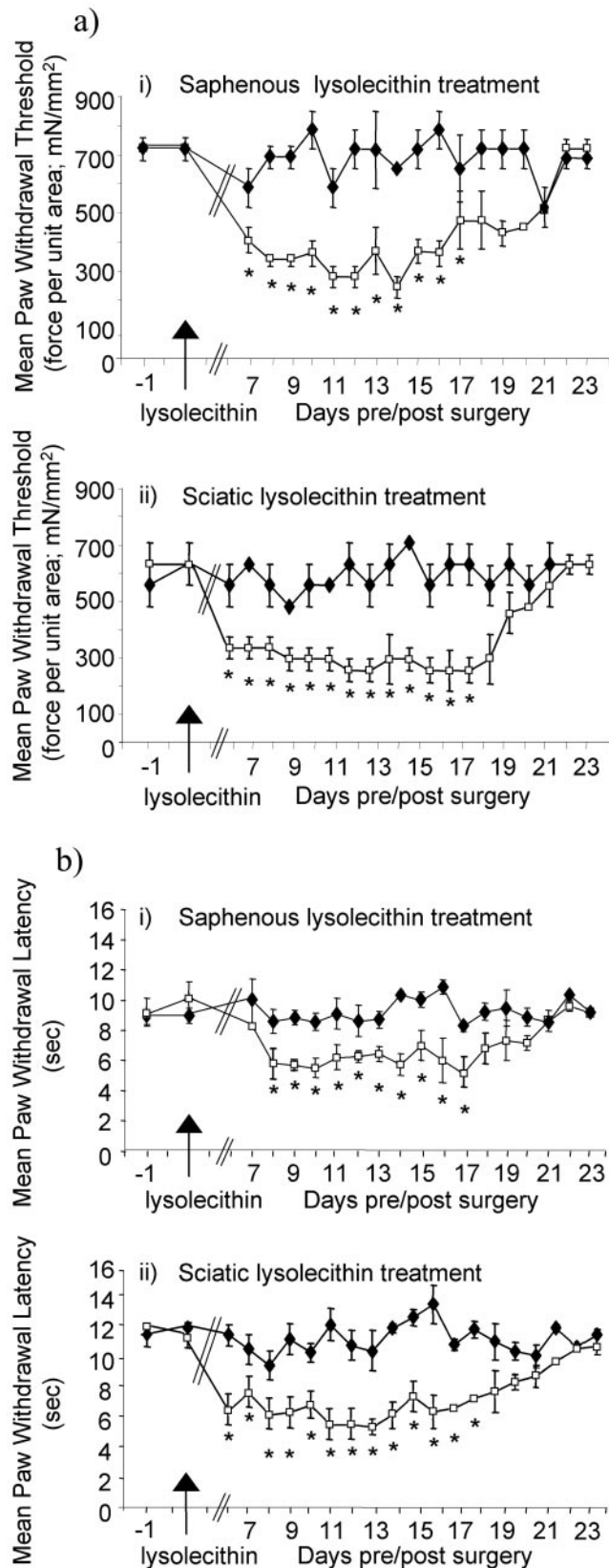
**G-ratio.** The g-ratio of an axon is a measure of myelin thickness calculated by dividing the axonal diameter by the total diameter of axon plus myelin sheath. This gives an indication of the level of demyelination in axons that are not completely demyelinated by lysolecithin treatment. The g-ratios of myelinated fibers were calculated from EM photographs of the whole cross-sectional area of each nerve using the IP Lab Spectrum P program (Scanalytics, Fairfax, VA). This also calculates the number of myelinated axons in the sample (292  $\mu$ m<sup>2</sup>) and enables the proportion of completely demyelinated axons per nerve to be determined. The statistical significance of any difference among treated, naive, or sham nerves was determined by Kruskal–Wallis one-way ANOVA on ranks with an all pair-wise multiple comparisons procedure (Dunn's method).

**Electrophysiology.** Electrophysiological recording of activity and function in afferent fiber units of peripheral nerve was performed on animals that previously had focal demyelination induced in the saphenous nerve. Recording experiments were performed on days 3 and 5 after surgery, times at which behavioral reflex sensitization had not developed, and also on days 11, 12, and 13 after surgery, corresponding to the peak time of development of behavioral reflex sensitization.

Mice were anesthetized with 5% urethane (1.25 gm/kg, i.p.) and maintained at 36–37°C with a radiant heat lamp. The saphenous nerve was exposed in the mid thigh and dissected from its associated vein and artery. Further dissection under liquid paraffin enabled the identification of afferent preparations comprising a small number of units. The mechanosensitivity of sensory receptors with low thresholds was measured using calibrated von Frey filaments, and the thermal threshold was measured with a calibrated radiant heat source. The mechanical threshold was defined as the pressure required to cause units to fire in response to direct application of von Frey filaments to the central zone of the identified receptive field, and the thermal threshold was defined as the temperature required to cause units to fire in response to direct application of the radiant heat source to the central zone of the identified receptive field. The conduction velocity of single identified afferent fibers was determined using bipolar electrodes and the peripheral stimulus technique (Iggo, 1958). The percentage of axons in each conduction velocity range, from 0–3 m/sec up to 18–21 m/sec, was calculated, and the statistical significance of any difference between each treatment group was determined by a Kruskal–Wallis one-way ANOVA on ranks with all pair-wise multiple comparisons procedure (Dunn's method). All recording traces of spike activity were obtained via a direct link of the oscilloscope to the Powerlab/MacLab Chart v3.6/s program (AD Instruments, Oxford, UK). Data were subsequently analyzed on the Claris Works 4.0 illustrator program (Claris Corporation, Smart Computing, Lincoln, NE).

**Immunohistochemistry.** For fixed-tissue immunohistochemistry, animals that 13 d previously had lysolecithin treatment of the right sciatic nerve or saphenous nerve were deeply anesthetized with halothane and perfused through the heart with heparinized saline followed by 4% paraformaldehyde (Sigma) in 0.1 M phosphate buffer (Sigma). The DRG ipsilateral and contralateral to the lysolecithin-treated nerve or sham-operated nerve were removed, postfixed with the same fixative for 2 hr, and transferred through increasing concentrations of sucrose in 0.1 M phosphate buffer for 1 hr and left overnight in 25% sucrose in 0.1 M phosphate buffer. The DRG were then embedded in OCT mounting medium (Tissue Tek) and frozen over liquid nitrogen. For nonperfused tissue immunohistochemistry, animals were killed by CO<sub>2</sub> inhalation 13 d after lysolecithin treatment. DRG ipsilateral and contralateral to the lysolecithin-treated nerve or sham-operated nerve were removed, snap frozen, and embedded in OCT mounting medium. Cryostat sections of all DRG (10  $\mu$ m) were thaw-mounted on glass slides precoated with poly-L-lysine (Merck-BDH). For saphenous nerve-treated animals, L3, L4, and L5 DRG were analyzed, and for sciatic-treated animals, L4, L5, and L6 DRG were analyzed (all *n* = 4).

For colocalization of Na<sub>v</sub>1.8 or the peptides CGRP or NPY with the A-fiber cell body marker neurofilament 200 kDa (NF-200) (Lawson and Waddell, 1991; Michael et al., 1999), or the C-fiber cell body marker peripherin (Goldstein et al., 1991), nonperfused DRG sections were immersion-fixed for 15 min in 4% paraformaldehyde in PBS. Sections were preincubated in buffer (0.1 M PBS, pH 7.4, containing 0.2% Triton X-100 and 2% gelatin) containing 10% normal goat serum or 10% nor-



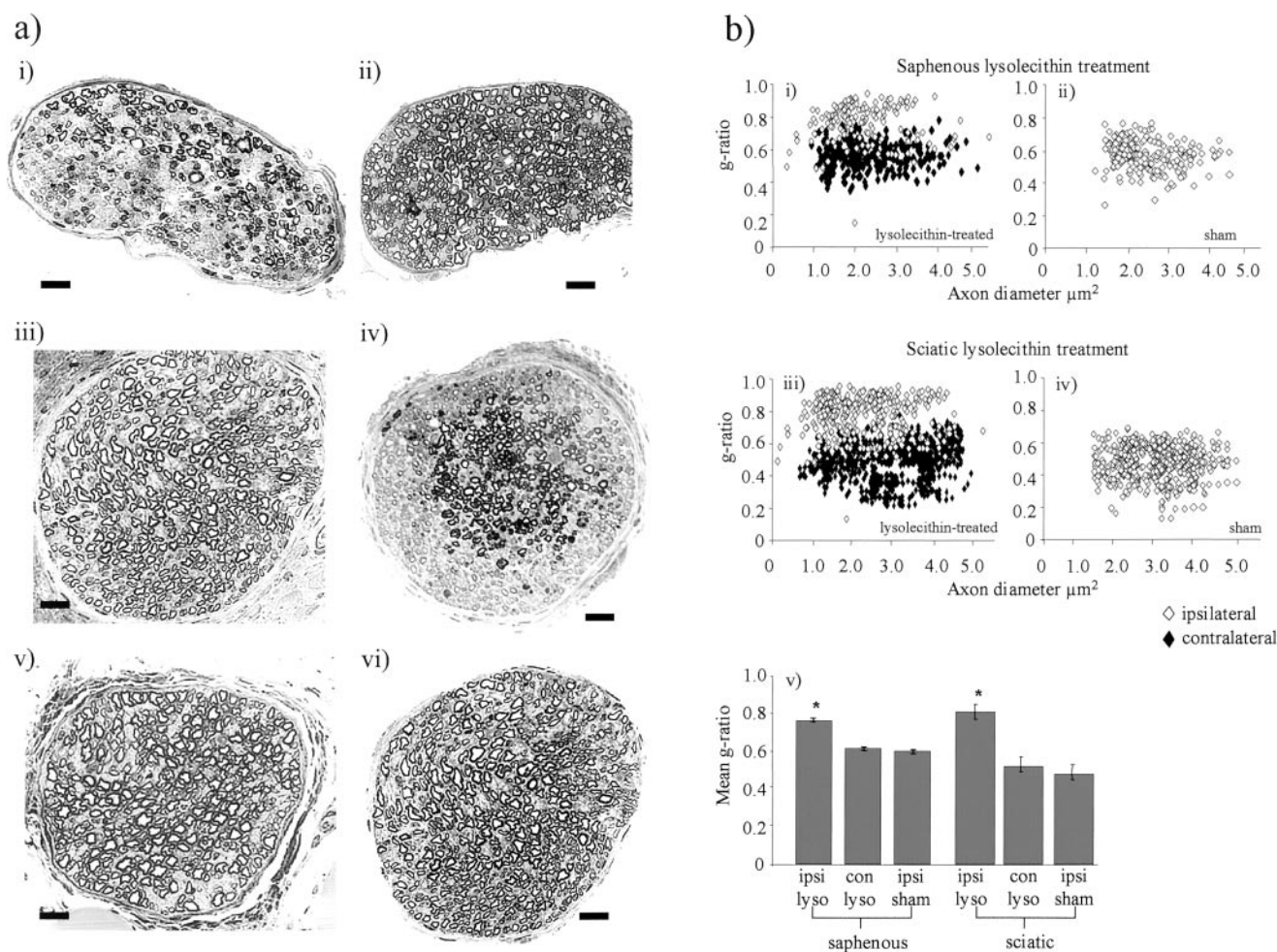
**Figure 1.** Development of mechanical allodynia and thermal hyperalgesia ipsilateral to lyssolecithin treatment of the saphenous or sciatic nerve. *a*, Filament indentation pressure thresholds for paw withdrawal responses to von Frey filaments were measured after lyssolecithin treatment ( $\uparrow$ ) to one saphenous ( $n = 6$ ) (*i*) or one sciatic nerve ( $n = 6$ ) (*ii*). Statistical significance of differences between ipsilateral ( $\square$ ) and contralateral ( $\blacklozenge$ ) paws ( $*p < 0.05$ ) was determined by a Wilcoxon test. Each value is the mean  $\pm$  SEM. *b*, The time taken for

hindpaw withdrawal from a noxious thermal stimulus was measured after lyssolecithin treatment ( $\uparrow$ ) to one saphenous ( $n = 6$ ) (*i*) or one sciatic nerve ( $n = 6$ ) (*ii*). Statistical significance between ipsilateral ( $\square$ ) and contralateral ( $\blacklozenge$ ) paws ( $*p < 0.05$ ) was determined by a paired Student's *t* test. Each value is the mean  $\pm$  SEM.

mal donkey serum (for CGRP detection) for 1 hr at room temperature and incubated with primary antibodies diluted in buffer containing 4% goat or donkey serum overnight at 4°C. Perfused tissue was used for detecting colocalization of Na<sub>v</sub>1.3, galanin, or activating transcription factor 3 (ATF3) (Tsujino et al., 2000) with NF-200 or peripherin. The immunohistochemical procedure used was as for fresh tissue, omitting the immersion-fixation step.

Antisera were used at the following concentrations: rabbit anti-Na<sub>v</sub>1.8 [1:200; K107 supplied by S. Tate, GlaxoSmithKline; specificity of K107 was tested by preincubating primary antibody with dilutions of the relevant antigenic peptide (Amaya et al., 2000)]; rabbit anti-Na<sub>v</sub>1.3 sodium channel [1:200; Alomone Labs, Jerusalem, Israel (Black et al., 1999)]; sheep anti-CGRP [1:1500; Affiniti, Exeter, UK (Todd, 1997)]; rabbit anti-NPY [1:1000; Peninsula Laboratories, Belmont, CA (Polgar et al., 1999)]; rabbit anti-galanin (1:2000; Advanced Chemtech, Cambridge, UK); rabbit anti-ATF3 (1:400; Santa Cruz Biotechnology, Santa Cruz, CA); mouse monoclonal anti-NF-200 kDa [1:2000; clone N52; Sigma (Bennett et al., 1998)]; and mouse monoclonal anti-peripherin [1:1000; Chemicon International, Harlow, UK (Amaya et al., 2000)]. Sections were then washed in buffer and incubated with the appropriate secondary antibodies linked to either tetramethyl rhodamine isothiocyanate (TRITC) (goat anti-mouse-TRITC, 1:200, to detect all mouse primary antisera; Southern Biotechnology, Birmingham, AL) or fluorescein isothiocyanate (FITC) (goat anti-rabbit-FITC, 1:200, to detect all goat primary antisera; Cappel; donkey anti-sheep-FITC, 1:100, to detect anti-CGRP primary antiserum; Jackson ImmunoResearch Laboratories, West Grove, PA) for 2 hr at room temperature. Three final washes in 0.1 M PBS were conducted before coverslipping with VectaShield (Vector Laboratories, Burlingame, CA) for analysis. Control sections were processed as above omitting the primary antisera. Observations were made and sections were photographed on an Olympus microscope equipped for epifluorescence. Counts of profiles labeled for immunopositive cells were performed on four to five randomly selected 10  $\mu$ m sections of DRG (separation of 100  $\mu$ m) from each of four animals in each group, and only neurons with clear nuclei were counted. Results were expressed either as a proportion of labeled profiles per section or the proportion per total number of single- or double-labeled profiles from all 16–20 sections. The statistical significance between groups was tested by Kruskal–Wallis one-way ANOVA on ranks with all pair-wise multiple comparison procedures (Dunn's method).

*Intrathecal administration of drugs.* Animals with focal demyelination induced in the saphenous nerve were used for all intrathecal injection experiments. Baseline measurements for mechanical allodynia and thermal hyperalgesia were recorded over a period of up to 2 hr before injection. The mice were briefly anesthetized with halothane and O<sub>2</sub> and injected intrathecally at the level of the L4 spinal vertebra using a 25 gauge needle with the NMDA receptor antagonist 3-((*R*)-2-carboxypiperazine-4-yl)-propyl-1-phosphonic acid [(*R*)-CPP; Tocris Cookson, Bristol, UK; 100 pmol/10  $\mu$ l saline], the selective  $\mu$  opioid receptor agonist D-Ala<sub>2</sub>, MePhe<sub>4</sub>, Gly-ol enkephalin (DAMGO; Tocris Cookson; 10 pmol/10  $\mu$ l saline), the mixed CB<sub>1</sub>/CB<sub>2</sub> cannabinoid receptor agonist mesylate (*R*)-(+)-(2,3-dihydro-5-methyl-3-(4-morpholinylmethyl) pyrrolo (1,2,3-de)-1,4-benzoxazin-6-yl)-1-naphthalenylmethanone [WIN 55,212-2; Tocris; 60 pmol/10  $\mu$ l saline with 0.02% dimethylformamide (DMF)], the selective CB<sub>1</sub> receptor antagonist *N*-(piperidin-1-yl)-5-(4-iodophenyl)-1-(2,3-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide (AM 251; Tocris; 100 pmol/10  $\mu$ l saline with 0.3% DMF), or a combination of WIN 55,212-2 plus AM 251 (100 pmol/10  $\mu$ l saline with 0.3% DMF). To determine the effects of each drug on both mechanical allodynia and thermal hyperalgesia, behavioral reflex testing commenced 15 min after injection to allow recovery from anesthesia and continued every 5 min thereafter until readings returned to baseline levels. For all drugs tested, any statistically significant differ-



**Figure 2.** Effects of lysolecithin treatment on the myelination state of the saphenous and sciatic nerve. *a*, Toluidine blue-stained resin sections ( $1\ \mu\text{m}$ ) from the ipsilateral (*i*) and contralateral (*ii*) saphenous nerves of lysolecithin-treated animals on day 13 after treatment, ipsilateral saphenous nerve of sham-treated animals on day 13 after treatment (*iii*), ipsilateral (*iv*) and contralateral (*v*) sciatic nerves of lysolecithin-treated animals on day 13 after treatment, and ipsilateral sciatic nerve of sham-treated animals (*vi*) on day 13 after treatment were analyzed by light microscopy ( $n = 4$  in each case). Contralateral nerves on day 13 after lysolecithin treatment and nerves ipsilateral to sham treatment all appeared to be morphologically normal with consistent levels of myelination across the nerve. Scale bar,  $10\ \mu\text{m}$ . *b*, Quantification of the degree of myelination of A-fibers after lysolecithin treatment to the saphenous nerve was determined as a g-ratio from electron microscope images of the ipsilateral and contralateral saphenous nerves of lysolecithin-treated (*i*) and sham animals (*ii*) on day 13 after treatment ( $n = 4$  in each case). Results are shown as the mean g-ratio of each fiber as a function of the axon diameter (*i–iv*), and the data are summarized as bar charts below (*v*). The data show a general decrease in myelin thickness, i.e., increased g-ratio after lysolecithin treatment but not after sham surgery. Statistical significance ( $*p < 0.05$ ) between conditions was determined by Kruskal–Wallis one-way ANOVA on ranks with an all pair-wise multiple comparisons procedure (Dunn’s method).

ence of the postinjection paw withdrawal from baseline paw withdrawal thresholds was determined by a one-way repeated measures ANOVA with Dunnett’s multiple comparisons versus control group *post hoc* analysis. Extensive control studies have shown that intrathecal injections of the vehicles, saline, or 0.1–0.5% DMF in saline have no effect on these behavioral reflex measures. Dye injections using Pontamine Sky Blue demonstrated that over various time periods up to 60 min after injection there was no spread of dye to the proximal spinal nerve or DRG.

## Results

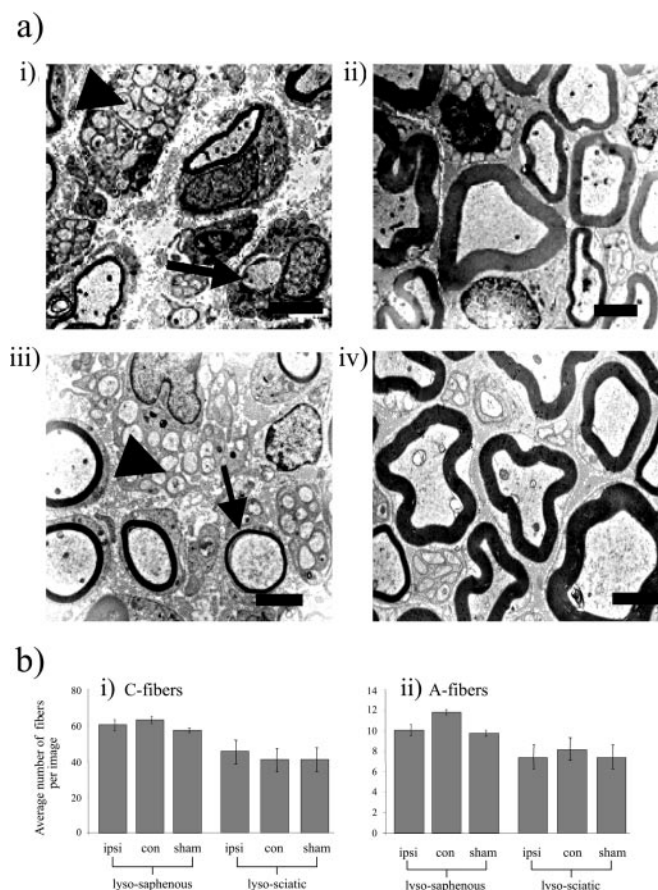
### Lysolecithin treatment causes behavioral allodynia and hyperalgesia

Lysolecithin-treated mice displayed a markedly lowered threshold in reflex tests both of cutaneous mechanical sensitization using calibrated von Frey filaments and of thermal nociceptive sensitivity (Fig. 1). The heightened sensitivity to both tests was apparent from day 5 after lysolecithin treatment of saphenous or sciatic nerve, but reached peak values from day 9 until day 15 after surgery. Typically, after lysolecithin treatment of the saphenous

nerve, von Frey thresholds were reduced from  $\sim 700$  to  $\sim 300$  mN/mm<sup>2</sup>, and thermal withdrawal latencies were reduced from  $\sim 9$  to 5 sec at peak behavioral change. Similarly, after lysolecithin treatment of the sciatic nerve, von Frey thresholds were reduced from  $\sim 600$  to  $\sim 250$  mN/mm<sup>2</sup>, and thermal withdrawal latencies were reduced from  $\sim 11$  to 5 sec at peak behavioral change. In each case the changes recovered to baseline levels by 23 d after treatment. There was no evidence for overt motor deficit after lysolecithin treatment of either nerve. Furthermore, mice that had undergone surgery and saline treatment of the saphenous or sciatic nerve showed no change from baseline von Frey threshold withdrawal values of between 600 and 700 mN/mm<sup>2</sup> or baseline thermal paw withdrawal latencies of  $\sim 10$  sec ( $n = 4$  in each case).

### Lysolecithin treatment causes demyelination without overt axonal damage or sensory loss

To determine the extent of demyelination as a result of lysolecithin treatment, we examined demyelinated, sham, and naive pe-



**Figure 3.** Effects of lysolecithin treatment on morphology of saphenous and sciatic nerve fibers at the EM level. *a*, Electron microscopy images of either the lysolecithin-treated saphenous (*i*) or sciatic (*iii*) nerves, compared with the equivalent sham-operated nerves [saphenous (*ii*) and sciatic (*iv*) nerves] on day 13 after treatment ( $n = 4$  in each case). Thirteen days after lysolecithin treatment, ipsilateral nerves display complete demyelination in  $\sim 40\%$  of A-fibers with partial demyelination of many remaining myelinated fibers. The images demonstrate marked lysolecithin-induced reduction in the myelination of A-fibers (arrow), whereas C-fibers (arrowhead) remain intact ( $5000\times$ ). Scale bar,  $2\ \mu\text{m}$ . *b*, *i* and *ii* show the mean number of C- and A-fibers recorded per image area in different conditions. Unmyelinated axons were identified as small-diameter fibers devoid of any myelin sheath (which were present in bundles, surrounded by Schwann cell cytoplasm) and were individually counted for all groups. A-fibers (with or without lysolecithin treatment) were identified as the larger-diameter axons with myelin sheaths or smaller-diameter thinly myelinated fibers or occasionally those with no myelin that were solitary. Data for both fiber types are presented as average numbers of fibers per  $292\ \mu\text{m}^2$  image  $\pm$  SEM. A Kruskal-Wallis one-way ANOVA on ranks showed no statistically significant differences between treatment groups.

ripheral nerves by light and electron microscopy at the time of peak behavioral change. A maximum of 40% of A-fibers in the saphenous nerve was completely demyelinated 11–13 d after lysolecithin treatment, and many remaining myelinated fibers had myelin of reduced thickness. This was observed by light microscopy (Fig. 2*a*) and calculated from the number of myelinated fibers or formerly myelinated fibers present in each EM image (representing  $292\ \mu\text{m}^2$  of the nerve). Lysolecithin-treated saphenous nerve ( $n = 4$ ) contained  $4.9 \pm 0.9$  myelinated axonal profiles per area sampled (155 profiles over 33 images analyzed), and control nerve ( $n = 4$ ) contained  $8.2 \pm 1.7$  profiles per area sampled (261 myelinated axonal profiles over 32 images analyzed). After topical application of lysolecithin, a similar degree of demyelination was observed in the sciatic nerve as in the saphenous nerve (Fig. 2*a*). The g-ratios were calculated for the remaining myelinated A-fibers in each nerve after treatment with lysoleci-

**Table 1. Effects of topical lysolecithin treatment on afferent fiber numbers**

Nerve	Treatment	Side	Average number of axons per $292\ \mu\text{m}^2$ EM image	
			A-fibers	C-fibers
Sciatic	Lysolecithin	Ipsi	$6.8 \pm 1.5$	$45.1 \pm 9.0$
	Lysolecithin	Contra	$7.9 \pm 2.2$	$42.9 \pm 8.9$
	Sham	Ipsi	$6.7 \pm 3.3$	$39.3 \pm 7.3$
Saphenous	Lysolecithin	Ipsi	$10.1 \pm 0.5$	$60.1 \pm 2.0$
	Lysolecithin	Contra	$11.8 \pm 0.2$	$62.9 \pm 1.9$
	Sham	Ipsi	$9.7 \pm 0.3$	$57.3 \pm 1.3$

EM images were analyzed to count profiles characteristic of C-fiber axons or A-fiber axons that were either still myelinated or had been demyelinated to varying degrees. No differences were apparent in the number of A-fiber or C-fiber axons between lysolecithin-treated and control animals. Ipsi, Ipsilateral; Contra, contralateral.

**Table 2. Effects of topical lysolecithin treatment of the saphenous nerve on afferent fiber characteristics**

Treatment	Receptive field	Spontaneous activity (imp/sec)	von Frey filament threshold ( $\text{mN}/\text{mm}^2$ )	Thermal threshold ( $^{\circ}\text{C}$ )	Conduction velocity range ( $\text{msec}^{-1}$ )
Naive	Normal	0	8.41–16.70	$>48$	1.4–20.0
Sham (day 11–13)	Normal	0	8.41–16.70	$>48$	0.9–18.2
Lysolecithin-treated (day 3–5)	Normal	0	8.41–16.70	$>48$	0.5–16.6
Lysolecithin-treated (day 11–13)	Normal	0	8.41–16.70	$>48$	1.3–16.0

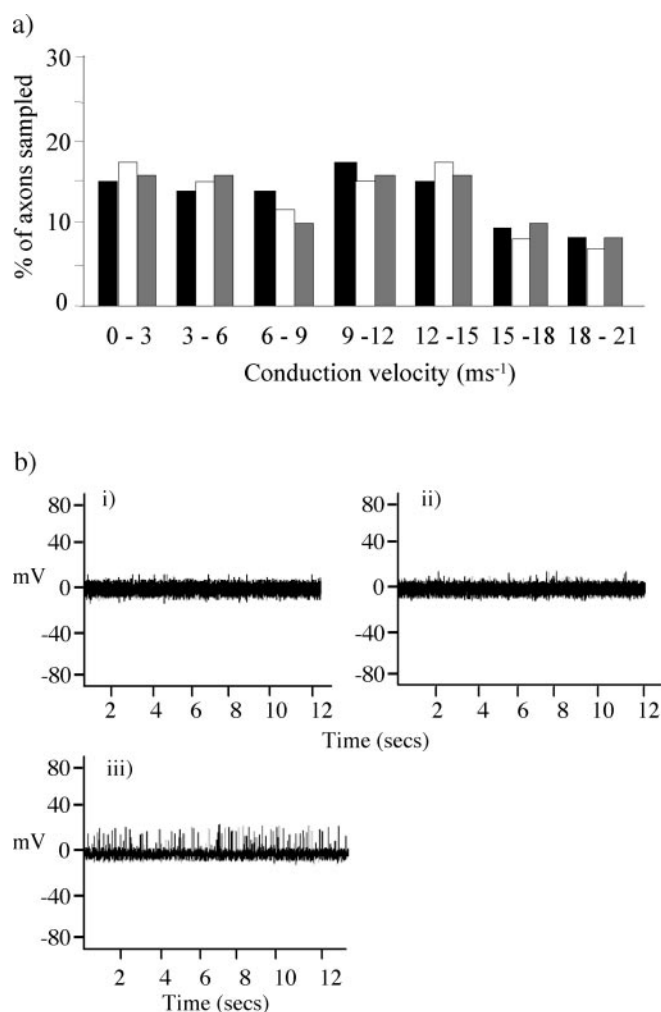
Electrophysiological recordings of afferent fiber unit activity and cutaneous responsiveness were performed on animals 3–5 d ( $n = 4$ ) and 11–13 d ( $n = 6$ ) after lysolecithin treatment to the saphenous nerve, 11–13 d after sham treatment ( $n = 4$ ), and on naive animals ( $n = 4$ ). The total numbers of afferent units recorded in each condition were 21, 66, 25, and 49, respectively. Any sensitization of mechanical sensory receptors with high thresholds was measured using calibrated von Frey filaments, and the presence of any sensitization of thermally responsive receptors was measured with a calibrated radiant heat lamp. The conduction velocity range of afferent fiber units was determined using the peripheral stimulus technique via bipolar electrodes. There was no difference in threshold for unit firing in response to mechanical or thermal stimulation or conduction velocity range among any of the groups. Afferent fiber units of the lysolecithin-treated nerves (11–13 d after treatment) displayed spontaneous activity. imp/sec, Impulses per second.

thin. The g-ratio was significantly increased in the demyelinated saphenous nerve from  $0.61 \pm 0.01$  in contralateral control nerves to  $0.76 \pm 0.01$  in demyelinated nerves and similarly in the demyelinated sciatic nerve from  $0.56 \pm 0.02$  in contralateral control nerves to  $0.81 \pm 0.03$  in demyelinated nerves (Fig. 2*b*), indicating a general decrease in myelin thickness. There was no significant difference in the level of myelination in sham-operated animals compared with control naive animals.

Electron microscopy demonstrated that after lysolecithin treatment unmyelinated C-fiber bundles appeared to be morphologically normal, and fiber counts revealed no statistically significant change in the number of unmyelinated C-fibers per EM image of either saphenous or sciatic nerve (Fig. 3*a,b*, Table 1). Furthermore, the total number of larger A-fiber axons that were either myelinated or demyelinated was also unchanged in saphenous or sciatic nerve after lysolecithin (Fig. 3*a,b*, Table 1). Hence, there was no evidence that treatment with lysolecithin under the conditions used in these experiments affects the integrity of axons.

### Lysolecithin treatment induces ectopic firing in saphenous afferents in the absence of changes in conduction velocity or peripheral thresholds

At 3 and 5 d after lysolecithin treatment, reflex testing showed no detectable change in either mechanically or thermally evoked responses, indicating that a sensitized state had not yet developed. Neither at this time nor at the time of peak behavioral change (11–13 d after treatment) was there any significant difference among normal, sham, and demyelinated afferent fibers in the



**Figure 4.** Effects of lysolecithin treatment on electrophysiological properties of saphenous afferents. *a*, Afferent fiber units from the lysolecithin-treated saphenous nerve of animals on days 11–13 after treatment ( $n = 6$ ) displayed a range of conduction velocities that were not significantly different from those of fibers from naive nerves ( $n = 4$ ). The range included slow conduction velocities of  $<1 \text{ msec}^{-1}$  up to the fastest in the range of  $20 \text{ msec}^{-1}$ . The number of units recorded for each conduction velocity did not differ significantly among the lysolecithin-treated (black column), naive (white column), and sham (gray column) nerves. *b*, Electrophysiological recordings of saphenous afferent fiber unit activity 11, 12, and 13 d after lysolecithin treatment to the saphenous nerve ( $n = 6$ ), 11–13 d after sham treatment ( $n = 4$ ), and in naive animals ( $n = 4$ ). Spontaneous activity in saphenous afferents was recorded with a frequency of 2–3 Hz. Examples of electrophysiological recordings of naive (*i*), sham-treated (*ii*), and lysolecithin-treated (*iii*) nerves are displayed.

threshold for their firing response to von Frey filaments or to thermal nociceptive stimuli (Table 2). Thus there is no evidence that changes in peripheral transduction mechanisms might be responsible for the increased behavioral reflex sensitivity that was observed (Table 2). The focal nature of this demyelination together with the integrity of the axons was reflected in the normal range of overall conduction velocities compared with sham and control nerves. The range included slow conduction velocities of  $<1 \text{ msec}^{-1}$  up to the fastest in the range of  $20 \text{ msec}^{-1}$ . Further-

more, the number of units recorded for each conduction velocity did not differ significantly among the lysolecithin-treated, sham, and naive nerves (Fig. 4*a*, Table 2). This provided further support for the view that there had been no significant axon damage.

A common feature of lysolecithin-demyelinated nerves over a range of 11–13 d after application, however, has been the presence of a spontaneous low-frequency discharge (2–3 impulses per second), reminiscent of a similar discharge that we have observed in the sciatic nerve of *Prx*-null mice (Gillespie et al., 2000) (Fig. 4*b*).

#### Expression of NF-200, peripherin, and the axonal damage marker ATF3 in DRG is unaffected by lysolecithin treatment

To assess the integrity of the cell bodies 13 d after lysolecithin treatment of the saphenous or sciatic nerve, we analyzed the expression of NF-200, which labels the larger-diameter, myelinated DRG cell population (Lawson and Waddell, 1991; Michael et al., 1999), and peripherin, a type III intermediate filament protein, which is normally expressed selectively by unmyelinated sensory neurons (Amaya et al., 2000). No significant difference was observed in the number of either NF-200 or peripherin-positive DRG neurons ipsilateral to lysolecithin compared with contralateral or sham treatment of the sciatic or saphenous nerve (Table 3).

We also analyzed the expression of ATF3, a member of the ATF/cAMP response element-binding protein (ATF/CREB) family (Hai et al., 1999), which is induced in DRG neurons after peripheral nerve injury and thus considered to be a nerve injury marker, identifying axotomized neurons (Tsujino et al., 2000). As a positive control, we analyzed the expression of ATF3 in the DRG of mice, which were at the peak of neuropathic sensitization after chronic constriction injury (CCI) to the sciatic nerve. In CCI mice we found expression of ATF3 in  $69 \pm 16\%$  of ipsilateral DRG neurons. Under control (sham) conditions we found a very small number of ATF3-positive cells, all of which were also positive for NF-200. However, there was no significant increase in the proportion of ATF3-immunopositive cells in the DRG ipsilateral to lysolecithin treatment of the sciatic nerve as compared with contralateral or sham-treated animals (Fig. 5*a*, Table 4).

#### Lysolecithin treatment causes a distinctive pattern of neuropeptide expression in sensory neurons

After traumatic injury to the peripheral nerve, there are a number of neurochemical and morphological changes, both in peripheral nerve fibers and centrally in the spinal cord (Dray et al., 1994; Hokfelt et al., 1994), that can contribute to the altered sensory transmission associated with chronic pain states. Phenotypic changes occur in many primary afferent DRG neurons, resulting in the altered expression of neuropeptides including CGRP, ga-

**Table 3. Immunohistochemical assessment of structural markers and the neuropeptide CGRP in DRG cells after lysolecithin treatment**

Nerve	Treatment	Side	Number of positive cells per DRG		
			NF-200	Peripherin	CGRP
Sciatic	Lysolecithin	Ipsi	$78.5 \pm 3.2$	$129.7 \pm 19.6$	$145.9 \pm 19.7$
	Lysolecithin	Contra	$77.3 \pm 2.2$	$119.7 \pm 4.6$	$154.5 \pm 9.1$
	Sham	Ipsi	$68.1 \pm 9.6$	$109.4 \pm 9.6$	$149.6 \pm 5.8$
	Sham	Contra	$73.4 \pm 2.7$	$103.9 \pm 1.9$	$129.9 \pm 12.9$
Saphenous	Lysolecithin	Ipsi	$67.1 \pm 6.2$	$120.4 \pm 11.6$	$152.8 \pm 8.7$
	Lysolecithin	Contra	$74.3 \pm 13.9$	$124.7 \pm 8.6$	$135.3 \pm 7.4$
	Sham	Ipsi	$69.9 \pm 9.7$	$119.5 \pm 9.1$	$139.6 \pm 5.8$
	Sham	Contra	$71.7 \pm 9.6$	$121.7 \pm 4.6$	$132.4 \pm 9.6$

The expression of the neuronal structural markers peripherin and NF-200 and the peptide CGRP in DRG cells ipsilateral and contralateral to lysolecithin treatment of the saphenous or sciatic nerve on day 13 after treatment and from sham animals ( $n = 4$  in each case). There was no difference in the number of cells expressing either structural marker after treatment with lysolecithin as determined by a Kruskal–Wallis one-way ANOVA on ranks or paired Student's *t* test. Data are presented as average number of immunopositive cells per DRG  $\pm$  SEM. Ipsi, Ipsilateral; Contra, contralateral.

lanin, and NPY (Villar et al., 1991; Hokfelt et al., 1994). We therefore investigated whether such changes occur in the demyelination-induced pain state to compare the changes with those occurring in other models of neuropathic pain in which axonal damage is a major factor. CGRP is normally expressed by approximately one-half of unmyelinated DRG neurons as well as one-fifth of those with A $\delta$  myelinated neurons (Rosenfeld et al., 1983; Gibson et al., 1984; McCarthy and Lawson, 1990; Hokfelt et al., 1994) and is downregulated after peripheral nerve injury such as axotomy and CCI (Noguchi et al., 1990; Dumoulin et al., 1992). However, there was no significant difference in the numbers of ipsilateral versus contralateral DRG cells expressing immunoreactive CGRP after lysolecithin treatment or sham treatment of one sciatic nerve or of one saphenous nerve (Table 3), suggesting that lysolecithin treatment caused no extensive damage to the axons of CGRP-expressing cells.

Further immunohistochemical investigations were restricted to the sciatic model of lysolecithin-induced demyelination, because it is a larger nerve and would contribute a larger proportion of cell bodies in relevant DRGs, thereby facilitating detection of changes.

Galanin is normally expressed at very low levels in sensory and sympathetic neurons. However, after peripheral nerve lesions, it is strongly upregulated mainly in small and medium-sized neurons (Hokfelt et al., 1987; Villar et al., 1989), especially those that normally contain substance P and CGRP (Xu et al., 1990; Doughty et al., 1991; Kashiba et al., 1992), the majority of which are unmyelinated. However, we observed no significant galanin immunoreactivity in DRG cells ipsilateral or contralateral to focal demyelination of one sciatic nerve. As a positive control to test the specificity of the antibody, we used DRG from mice at peak neuropathic sensitization after sciatic CCI (Garry et al., 2003). These sections showed a significant proportion of mainly small diameter cells positive for galanin (ipsilateral  $9.3 \pm 2.4$  cells per section; contralateral 0), verifying that the antibody could successfully detect the presence of galanin when expressed after traumatic nerve injury and that there is, in contrast, no significant upregulation after lysolecithin treatment.

NPY is not normally expressed in DRG cells (Lundberg et al., 1983). However, the expression of NPY is strongly upregulated in DRG neurons after axotomy (Wakisaka et al., 1991, 1992; Noguchi et al., 1993; Kashiba et al., 1994), partial sciatic injury (Ma and Bisby, 1998), CCI of the sciatic nerve (Nahin et al., 1994; Munglani et al., 1995), and streptozotocin-induced diabetic neuropathy (Rittenhouse et al., 1996). This upregulation occurs mainly in large primary sensory neurons containing NF-200 (Kashiba et al., 1994; Marchand et al., 1999). We also observed significant NPY immunoreactivity in  $\sim 16\%$  cells after demyelination of one sciatic nerve (185 NPY-positive cells from 1164 NF-200-positive cells counted over 26 sections;  $n = 4$ ) as opposed to no detectable immunoreactivity in contralateral or sham nerves (Table 4). All NPY-immunopositive cells were also immunoreactive for NF-200, indicating that they were cells with myelinated fibers (Fig. 5b).

#### Afferent expression of Na $_v$ 1.8 and Na $_v$ 1.3 is altered by lysolecithin treatment

Normally, DRG neurons express a complex repertoire of sodium channel transcripts (Waxman et al., 1999) distinguished by their differential sensitivity to tetrodotoxin (TTX) (Black and Waxman, 1996). The TTX-resistant sodium channel Na $_v$ 1.8 is expressed in  $\sim 50\%$  of small-diameter, unmyelinated DRG cells (Amaya et al., 2000) and in  $\sim 20\%$  of medium- to large-diameter, myelinated DRG cells. Our results from the DRG of contralateral untreated nerve

confirm that result, showing expression of Na $_v$ 1.8 in 1078 of the 2130 (51%) peripherin-immunopositive cells (C-fibers) and 500 of the 2086 (24%) NF-200-immunopositive cells (myelinated fibers). Likewise, in sham controls we saw Na $_v$ 1.8 expressed in 51% of peripherin-immunopositive cells ipsilaterally and 48% contralaterally and in 23% of NF-200-immunopositive cells ipsilaterally and 22% contralaterally. It has been shown previously that after axotomy, CCI, or spinal nerve ligation, expression of Na $_v$ 1.8 is downregulated in neurons in the DRG, a change that has been proposed to contribute to the production of a neuropathic pain state (Dib-Hajj et al., 1999; Waxman, 1999; Decosterd et al., 2002). In the L4, L5, and L6 DRG taken from animals 13 d after lysolecithin treatment to the sciatic nerve, there was a  $34.1 \pm 4.2\%$  reduction of Na $_v$ 1.8 expression in the ipsilateral DRG ( $62.4 \pm 3.4$  Na $_v$ 1.8-immunopositive cells per section) versus the contralateral and sham DRG (with corresponding values of  $94.7 \pm 4.7$  Na $_v$ 1.8 and  $95.7 \pm 6.0$ , respectively). To address the question of whether these changes in sodium channel expression were restricted to a subset of neurons, we performed dual labeling experiments to reveal the colocalization of Na $_v$ 1.8 with NF-200/peripherin. The reduction in Na $_v$ 1.8 expression appeared to be restricted to NF-200-immunopositive cells, i.e., those presumed to have formerly myelinated axons (Fig. 5c, Table 4). No change in the number of cells coexpressing Na $_v$ 1.8 and NF-200 was observed between the ipsilateral or contralateral DRG of sham control animals, and we found no decrease in the proportion of peripherin-immunoreactive cells expressing Na $_v$ 1.8 after lysolecithin treatment (Table 4).

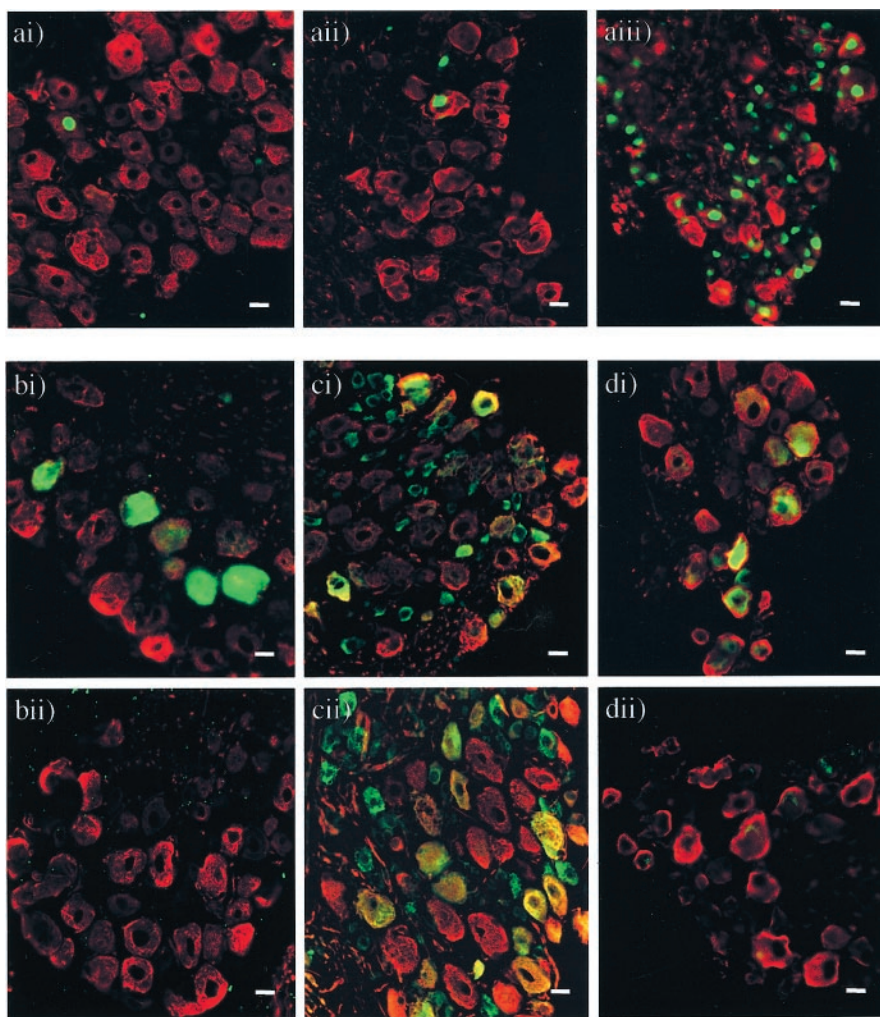
The Na $_v$ 1.3 channel is normally found in DRG only during development, and consistent with this we could not detect expression of this channel in the DRG before treatment (Fig. 5d, Table 4). However, expression of this channel has been reported to increase in sensory neurons after axotomy (Black et al., 1999), CCI, and spinal nerve ligation (Kim et al., 2001). After lysolecithin-induced demyelination of the sciatic nerve, we found a significant increase in the number of NF-200-immunoreactive cells per ipsilateral DRG that now expressed Na $_v$ 1.3 ( $18.5 \pm 2.4\%$ ) (Fig. 5d, Table 4). No Na $_v$ 1.3 positive cells were also immunopositive for peripherin, showing that expression of the channel was not increased in C-fibers, and no Na $_v$ 1.3 immunoreactivity was detected in contralateral or sham tissue.

#### Lysolecithin treatment causes NMDA receptor-dependent central sensitization that can be reversed by cannabinoids

Sensitization of cells in the dorsal horn of the spinal cord is a key change in other neuropathic pain states that is thought to involve activation of the NMDA receptor (Wilcox, 1991; Codderre and Melzack, 1992; Chaplan et al., 1997). We found that spinal NMDA receptors also play a role in the mechanical allodynia seen in animals with focal demyelination of the saphenous nerve, because behavioral sensitization was reversed after spinal administration of the selective NMDA receptor antagonist (*R*)-CPP (Lehmann et al., 1987) (Fig. 6a). This parallels observations that we made previously in a demyelination model based on the *periaxin*-null mouse (Gillespie et al., 2000). Similarly, the reduction in thermal nociceptive response latency ipsilateral to lysolecithin treatment was reversed by (*R*)-CPP (Fig. 6e). There was no effect of (*R*)-CPP on thermal or mechanical responses contralateral to demyelination. Equivalent injections of the saline vehicle had no effect on mechanical or thermal reflex responses either ipsilateral or contralateral to lysolecithin (data not shown;  $n = 4$ ).

Because neuropathic pain states often show a relative insensitivity to spinal opioid analgesia compared with acute or peripheral inflammatory pain states (Arner and Meyerson, 1988; Fleetwood-Walker





**Figure 5.** Immunohistochemical colocalization of neuronal subtype markers with ATF3, NPY, and the sodium channel subtypes  $Na_v1.8$  and  $Na_v1.3$  in DRG cells after lyssolecithin treatment of the sciatic nerve. *a*, Expression of ATF3 in ipsilateral and contralateral DRG sections from lyssolecithin-treated animals on day 13 after treatment ( $n = 4$ ). There was no significant increase in expression of ATF3 from that seen in control DRG after lyssolecithin treatment or sham treatment. The statistical significance ( $*p < 0.05$ ) of any difference was determined by a Kruskal–Wallis one-way ANOVA on ranks with all pair-wise multiple comparison procedures (Dunn’s method). Images show nuclei of ATF3-immunopositive cells labeled with FITC (green) and NF-200-immunopositive cells labeled with TRITC (red), in ipsilateral (*i*) and contralateral (*ii*) DRG from lyssolecithin-treated animals and ipsilateral DRG (*iii*) from CCI animals. Scale bar, 20  $\mu$ m. *b*, Expression of the neuropeptide NPY in ipsilateral and contralateral DRG sections from lyssolecithin-treated animals at 13 d after treatment ( $n = 4$ ). Images show NF-200-immunopositive cells labeled with TRITC (red) and NF-200/NPY-colocalized, immunopositive cells labeled with FITC (green) in DRG ipsilateral to lyssolecithin treatment (*i*) as opposed to no positive cells in DRG contralateral to lyssolecithin treatment (*ii*). Scale bar, 20  $\mu$ m. *c*, The expression of the  $Na_v1.8$  channel in ipsilateral and contralateral DRG sections from lyssolecithin-treated animals on day 13 after treatment ( $n = 4$ ). After lyssolecithin treatment of the sciatic nerve, there was a decrease in the number of cells expressing  $Na_v1.8$  in ipsilateral DRG compared with contralateral DRG, all of which were immunopositive for NF-200. There was no significant decrease in expression after sham treatment. The statistical significance ( $*p < 0.05$ ) of any difference was determined by a Kruskal–Wallis one-way ANOVA on ranks with all pair-wise multiple comparison procedures (Dunn’s method). Images show  $Na_v1.8$ -immunopositive cells labeled with FITC (green) and NF-200-immunopositive cells labeled with TRITC (red), with coexpression appearing yellow in ipsilateral (*i*) and contralateral (*ii*) DRG from lyssolecithin-treated animals. Scale bar, 20  $\mu$ m. *d*, Expression of the  $Na_v1.3$  channel in ipsilateral and contralateral DRG sections from lyssolecithin-treated animals 13 d after treatment and sham-treated animals on the same day after treatment ( $n = 4$  in each case). Images show NF-200-immunopositive cells labeled with TRITC (red) and  $Na_v1.3$ -immunopositive cells labeled with FITC (green) with coexpression appearing yellow in DRG ipsilateral to lyssolecithin treatment (*i*) as opposed to no positive cells in DRG contralateral to lyssolecithin treatment (*ii*). Scale bar, 20  $\mu$ m.

et al., 1988; Yaksh and Harty, 1988), we assessed the effect of the potent and selective  $\mu$ -opioid receptor agonist DAMGO in the lyssolecithin model. Spinal  $\mu$ -opioid receptors exert powerful analgesic effects in inflammatory models of chronic pain (Malmberg and Yaksh, 1993; Chaplan et al., 1997). At the highest dose of intrathecal DAMGO that could be administered before overt

psychomotor incoordination was seen (10 pmol/10  $\mu$ l), there was no specific reversal of the mechanical allodynia or thermal hyperalgesia seen ipsilateral to lyssolecithin treatment (data not shown;  $n = 4$ ). The same intrathecal dose of DAMGO produces significant analgesia in tail pressure and thermal paw withdrawal tests in mice (Sakurada et al., 1999, 2001).

An alternative spinal modulatory system that can exert selective antinociceptive effects in both neuropathic and inflammatory hyperalgesia is the endogenous cannabinoid system (Herzberg et al., 1997; Richardson et al., 1998a; Fox et al., 2001). This is of particular interest because there is evidence that cannabinoids may exert analgesic and additional beneficial effects in demyelinating diseases such as multiple sclerosis (Consroe et al., 1997; Williamson and Evans, 2000; Robson, 2001). In this animal model of peripheral nerve demyelination, spinal administration of the cannabinoid receptor agonist WIN 55,212–2 attenuated the sensitization [that is the reduction in mechanical nociceptive thresholds (Fig. 6*b*) and thermal reflex latencies (Fig. 6*f*)] that was displayed ipsilateral to lyssolecithin. In both cases, changes were limited to the lyssolecithin-treated side and were statistically significant up to  $\sim 1$  hr after injection. The  $CB_1$  receptor in particular is implicated, because spinal administration of the selective  $CB_1$  receptor antagonist AM 251 reversed the effects of WIN 55,212–2 on mechanical sensitization (Fig. 6*c*) and thermal sensitization (Fig. 6*g*). However, when administered alone, AM 251 had no significant effect, indicating that endogenous spinal  $CB_1$  receptors had not become tonically activated as a result of the lyssolecithin treatment (Fig. 6*d,h*).

## Discussion

Abnormal sensory phenomena including spontaneous pain, hyperalgesia, and allodynia are associated with human peripheral demyelinating neuropathies such as types of CMT and GBS. In CMT, although sensory deficits are usually less severe than motor problems, pain is a problem in many sufferers and is a clear aspect of the clinical picture in CMT4F (Carter et al., 1998; Boerkoel et al., 2001; Guilbot et al., 2001; Takashima et al., 2002). Similarly, GBS or acute inflammatory polyneuropathy may involve pain in 54–84% of cases (Asbury, 1990; Moulins, 1998). Because the mechanisms underlying neuropathic pain in demyelinating disease are poorly understood (Rasminsky, 1981), we developed and characterized an animal model of focal peripheral nerve demyelination.

### Morphology of the demyelinated nerve

To understand the mechanisms underlying demyelination-induced neuropathic pain, it is important to establish whether axonopathic changes may contribute. The protocol used here results in demyelination of rapid onset confined to the immediate vicinity of the site of injection, causing little damage to the Schwann cell cytoplasm (Love et al., 1986) or the axon (Figs. 2, 3). However, because small-diameter fibers may be damaged by lysolecithin injection into the nerve (Mitchell and Caren, 1982), we applied lysolecithin topically to the saphenous or sciatic nerve, producing a focal demyelination without detectable axon damage or loss as assessed by light and electron microscopy. Correspondingly, in immunohistochemical studies we found neither loss of neuronal somata in the DRG, which were positive for NF-200 or peripherin, nor increased expression of the neuronal injury marker ATF3. This model of peripheral nerve demyelination appears to involve neither axonopathy nor cell loss and in that sense is less severe than models of neuropathic pain such as axotomy, CCI, and spinal nerve ligation (SNL), which involve axonal injury.

### Demyelination-induced allodynia and hyperalgesia: the role of functional changes in peripheral nerve

Damage to sensory nerves as a result of human peripheral demyelinating disease has been linked to pain and heightened sensitivity to touch (Pentland and Donald, 1994; Devor and Seltzer, 1999). After lysolecithin treatment of either the mixed sciatic nerve or the sensory saphenous nerve, we have demonstrated, for the first time, the development of mechanical allodynia and thermal hyperalgesia. Peripheral sensitization may contribute to neuropathic pain behavior in other models (Koltzenburg, 1998; Woolf and Costigan, 1999), but in the demyelination model we could find no significant changes in the cutaneous threshold for action potential generation by mechanical or thermal nociceptive stimuli in either A- or C-fibers. Some forms of mechanical allodynia are considered to be mediated by myelinated A-fibers providing inputs that are inappropriately processed by sensitized dorsal horn neurons (Woolf, 1997; Koltzenburg, 1998). Indeed, in the conditions that occur after section of peripheral or spinal nerves (for example, with phenotypic changes in DRG cells), spontaneous, ectopic activity in A, but not C afferents may play a role in the induction of neuropathic pain behaviors (C. N. Liu et al., 2000; X. Liu et al., 2000). Such activity may be important in the development of neuropathic pain here because repetitive ectopic firing may underlie abnormal sensation in diseases involving segmental demyelination, particularly where axonopathic changes are lacking (Rasminsky, 1981). The presence of low-frequency spontaneous discharge in demyelinated saphenous nerve corresponded to times of peak behavioral reflex sensitivity and was similar to observations in the *Prx*-null mouse (Gillespie et al., 2000). Because inflammatory neuritis can lead to a sensitized neuropathic pain state (Eliav et al., 1999), the possibility should be considered that a neuroimmune response could contribute to generating the allodynia and hyperalgesia observed here. However, in the *Prx*-null mouse, an alternative model of demyelination-induced neuropathic pain, minimal evidence could be found for macrophage infiltration of the degenerating

**Table 4. Immunohistochemical assessment of ATF3, the neuropeptide NPY, and the sodium channel subtypes Na<sub>v</sub>1.8 and Na<sub>v</sub>1.3 in sciatic nerve DRG cells after lysolecithin treatment**

Treatment	Side	Positive cells as a proportion of NF-200/peripherin-positive cells (%)				
		ATF3/ NF-200	NPY/ NF-200	Na <sub>v</sub> 1.8/ NF-200	Na <sub>v</sub> 1.8/ peripherin	Na <sub>v</sub> 1.3/ NF-200
Lysolecithin	Ipsi	7.3 ± 3.2	13.1 ± 2.2*	15.7 ± 1.6*	54.8 ± 3.7	18.5 ± 2.4*
Lysolecithin	Contra	6.1 ± 1.4	0	24.0 ± 2.6	50.6 ± 6.3	0
Sham	Ipsi	8.2 ± 3.4	0	24.0 ± 2.9	51 ± 1.4	0
Sham	Contra	5.1 ± 3.2	0	22.5 ± 2.7	48.1 ± 2.4	0

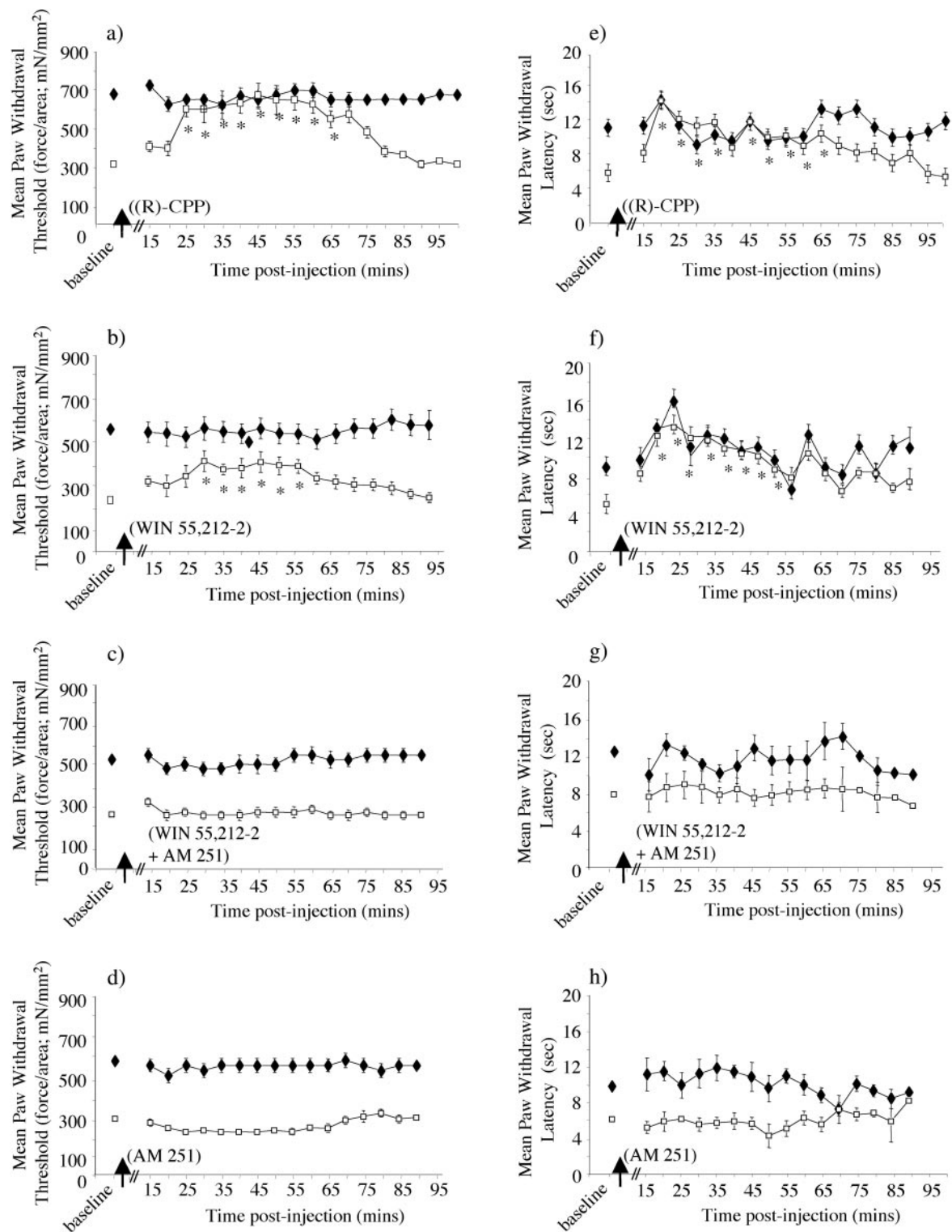
The expression of NPY, ATF3, and the sodium channels Na<sub>v</sub>1.8 and Na<sub>v</sub>1.3 in DRG cells ipsilateral (Ipsi) and contralateral (Contra) to lysolecithin treatment of the sciatic nerve are shown for day 13 after treatment ( $n = 4$ ) and from sham animals ( $n = 4$ ). There was no significant increase in expression of ATF3 from that seen in contralateral DRG after lysolecithin treatment or sham treatment. All ATF3-expressing cells were NF-200 immunopositive, and none were peripherin immunopositive. After lysolecithin treatment of the sciatic nerve there was a significant increase in the number of cells expressing NPY, which were all positive for NF-200 and none of which were immunopositive for peripherin. There was no detectable expression of NPY in the DRG contralateral to lysolecithin treatment or in DRG cells from sham-treated animals. Ipsilateral to lysolecithin treatment, there was a decrease in the number of DRG cells expressing Na<sub>v</sub>1.8 together with NF-200 compared with contralateral DRG or sham controls. In contrast, there was no significant decrease in the proportion of cells coexpressing Na<sub>v</sub>1.8 and peripherin. Expression of the Na<sub>v</sub>1.3 channel became detectable in a subpopulation of NF-200-immunopositive cells after lysolecithin treatment, whereas there was no detectable expression in the contralateral DRG or in DRG from sham-treated animals. No coexpression of Na<sub>v</sub>1.3 with peripherin was detected. The statistical significance (\* $p < 0.05$ ) of any difference between each group was determined by a Kruskal–Wallis one-way ANOVA on ranks with all pair-wise multiple comparison procedures (Dunn's method). Data are presented as the mean ± SEM percentage of total NF-200-positive cells that were positive for the relevant marker.

structures (Gillespie et al., 2000), suggesting that demyelination per se is a critical factor in generating the neuropathic pain state.

### Phenotypic changes in the DRG

After peripheral nerve lesions, changes in the expression of peptides occur in primary sensory neurons that may contribute to mediating central sensitization (Hokfelt et al., 1994). We assessed whether changes in the lysolecithin model are similar to those in other models of neuropathic pain, such as the downregulation of CGRP and the upregulation of galanin and NPY. Because this model appears to cause morphological and functional changes only in myelinated afferents, we asked whether changes in peptide expression are similarly restricted. Alterations in CGRP and galanin expression (generally in small-diameter afferents) have been implicated in the development of neuropathic pain (Hokfelt et al., 1987; Villar et al., 1989; Dumoulin et al., 1992; Kerr et al., 2000; Wynick et al., 2001), but we saw no change in these peptides after peripheral nerve demyelination.

We did find, however, upregulated expression of NPY, restricted to large NF-200-positive cells ipsilateral to focal demyelination. This parallels changes seen in other models of neuropathic pain. Notably, the upregulation of NPY occurred without any alteration in the expression of ATF3, indicating that direct neuronal injury was not responsible for the effect. After axotomy (Waxman et al., 1994), CCI (Dib-Hajj et al., 1999), and SNL (Kim et al., 2001), changes are seen in the expression of sodium channel isoforms, leading to functional changes in afferent sodium currents. For example, Na<sub>v</sub>1.8 [expressed preferentially in small- and medium-diameter DRG and implicated in pathological pain states (Akopian et al., 1996; Sangameswaran et al., 1996; Novakovic et al., 1998)], is downregulated (Dib-Hajj et al., 1996; Waxman, 1999), whereas Na<sub>v</sub>1.3 (normally found only in the DRG during development) is upregulated (Waxman et al., 1994; Waxman, 1999). We found a similar decrease in the expression of Na<sub>v</sub>1.8 channels in the DRG after focal demyelination that was restricted to formerly myelinated cells. In contrast, Na<sub>v</sub>1.3 was upregulated in corresponding cells. This further supports the specificity of A-fiber changes in evoking neuropathic pain after demyelination. Changes in channel type and distribution could contribute to hyperexcitability and ectopic pacemaker activity (Matzner and Devor, 1992, 1994; Devor et al., 1994; Cummins and Waxman, 1997; Omana-Zapata et al., 1997; Waxman, 1999). Na<sup>+</sup> channel-blocking agents may be effective analgesics not only in nerve injury-induced neuropathic pain (Chabal et al.,



**Figure 6.** Effects of intrathecal administration of NMDA and cannabinoid receptor-targeting agents on sensitized behavioral reflex responses after lysolecithin treatment. Paw withdrawal thresholds in response to cutaneous mechanical stimulation with von Frey filaments (*a–d*) and paw withdrawal latencies in response to cutaneous noxious thermal stimulation (*e–h*) were measured in animals at peak behavioral reflex sensitivity after topical application of lysolecithin to one saphenous nerve. Ipsilateral (□) and contralateral (◆) values are displayed before and after intrathecal administration of each pharmacological agent as paw withdrawal threshold in millinewtons per millimeter squared (mN/mm<sup>2</sup>) for mechanical stimulation or paw withdrawal latency (seconds) for thermal stimulation. For all measurements, each value is the mean  $\pm$  SEM, and statistical significance (\**p* < 0.05) of any differences between postinjection threshold values and preinjection baseline values was determined by a one-way repeated measures ANOVA with Dunnett's *post hoc* analysis. *a, e*, The NMDA receptor antagonist (*R*)-CPP (100 pmol) reversed the behavioral reflex sensitivity to mechanical stimulation (*a*) and thermal stimulation (*e*) in lysolecithin-treated animals for up to 65–70 min after intrathecal application (*n* = 8). *b, f*, The mixed CB<sub>1</sub>/CB<sub>2</sub> cannabinoid receptor agonist WIN 55,212–2 (60 pmol) significantly reversed the behavioral reflex sensitivity to mechanical stimulation (*b*) and thermal stimulation (*f*) in lysolecithin-treated animals (*n* = 7) for up to 55–60 min after intrathecal application. *c, g*, When injected in combination, WIN 55,212–2 and AM 251 produced no significant effect on the behavioral reflex sensitivity to mechanical stimulation (*c*) and thermal stimulation (*g*) in lysolecithin-treated animals (*n* = 8). *d, h*, The CB<sub>1</sub> receptor antagonist AM 251 (100 pmol) resulted in no change in the behavioral reflex sensitivity to mechanical stimulation (*d*) and thermal stimulation (*h*) in lysolecithin-treated animals (*n* = 7).

1992; Devor et al., 1992; Omana-Zapata et al., 1997; Rizzo, 1997), but also in demyelination-induced pain.

### Central changes after afferent demyelination and central modulation of sensitization

Afferent hyperexcitability and ectopic firing after lysolecithin treatment provide a basis for the ongoing input that brings about central sensitization. Because a spinal NMDA antagonist reverses sensitization in lysolecithin-treated mice, central changes play a critical role in the phenotype. NMDA receptors are similarly important in the mechanical allodynia and thermal hyperalgesia of other chronic pain models (Dickenson and Sullivan, 1987; Mao et al., 1993; Bennett, 1994; Chaplan et al., 1997; Woolf and Costigan, 1999). The relative insensitivity of neuropathic pain models to spinally administered opioid analgesics (Arner and Meyerson, 1988; Yaksh and Harty, 1988; Lee et al., 1995) also corresponds to our findings in the lysolecithin demyelination model.

Cannabinoid receptor drugs may represent an alternative strategy for the relief of chronic pain. Spinal cannabinoids can effectively modulate nociception (Herzberg et al., 1997; Richardson et al., 1998a; Martin et al., 1999; Bridges et al., 2001; Fox et al., 2001; Kelly and Chaplan, 2001), and the majority of these analgesic effects appear to be CB<sub>1</sub> receptor-mediated (Richardson et al., 1998b). In line with experiments on nerve-injury pain (Herzberg et al., 1997; Bridges et al., 2001; Fox et al., 2001), we found that intrathecal administration of the mixed CB receptor agonist WIN 55,212–2 attenuated mechanical allodynia and thermal hyperalgesia in lysolecithin-treated mice. This effect was reversed by the CB<sub>1</sub> antagonist AM 251, although AM 251 had little effect alone. It is possible that additional, noncannabinoid side effects of the compounds may occur in the immediate vicinity of the intrathecal injections where the local concentrations are higher, but the most likely explanation is that the WIN 55,212–2-induced, AM-251-reversed analgesia is mediated by CB<sub>1</sub> receptors. The efficacy of cannabinoids in the treatment of neuropathic pain may be linked to their actions on myelinated A-fibers. Under normal conditions, spinal cannabinoid receptors may predominantly regulate C-fiber input to the spinal cord, but after nerve injury these effects may be lost, whereas inhibitory influences on A $\delta$ -fiber inputs are retained (Chapman, 2001). This might result in cannabinoid agonists causing a reduction of mechanical allodynia, with more limited effect on thermal hyperalgesia, consistent with evidence from a number of studies (Herzberg et al., 1997; Bridges et al., 2001; Fox et al., 2001). In the present model, in which C-fiber primary afferents appear to be unaffected, both mechanical (A-fiber) and thermal (C-fiber) inputs should be targeted effectively by cannabinoids, as observed experimentally. These findings point further to the potential use of cannabinoids as therapeutic agents in human demyelinating neuropathies.

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