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Disc degeneration induces a mechano-sensitization of disc afferent nerve fibers that associates with low back pain

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SUMMARY

Objective: We aimed to investigate mechano-sensitivity at the afferent nerve fibers projecting to degenerated intervertebral disc (IVD) and nociceptive behaviour in a rat model of low back pain (LBP).
Design: Animal model with LBP was established by lumbar 4/5 IVD puncture and nucleus pulposus aspiration. *In vivo* single nerve recordings ($n = 121$) were introduced to measure discharge frequency at the afferent nerve fiber innervating the IVD during mechanical stimulations (von Frey filament or intradiscal pressure). Nerve growth factor (NGF) expression levels in the IVD ($n = 20$) were assessed by Western blot. LBP-related behaviour ($n = 22$) was assessed by measuring changes in rearing, mechanical paw-withdrawal threshold, and dynamic weight bearing in a freely walking rat. Inhibitory effect of morphine on the neuronal excitability ($n = 19$) and painful behaviour ($n = 28$) was also assessed.
Results: Compared to those with sham or naïve IVD, animal group with degenerated IVD displayed the sensitized neuronal responses and painful behaviour, with hyperexcitability of the afferent nerve fibers in any range of mechanical stimulations (von Frey filament stimulation; 1, 2, and 26 g; intradiscal pressure, 1,500–3,000 mm Hg), strong upregulation of NGF (200–250 % increase), and LBP-like behaviour such as failure of rearing, front limbs-dependent walking pattern, and hypersensitivity in hind-paws. However, the neuronal hyperexcitability and pain behaviour were attenuated after local (30 μM) or systemic (3 mg kg^{-1}) morphine administration.
Conclusions: Our study suggests that enhanced mechano-sensitivity at the afferent nerve fiber innervating degenerated IVD is deeply correlated with LBP development, which supports the hypothesis that hyperexcited responses at the nerve fibers represent a decisive source of LBP.

Introduction

Intervertebral discs support large spinal loads and enable flexibility between vertebrae[1]. However, with aging and repetitive mechanical stress[2], discs may undergo degeneration that accompany with a loss of disc height, dehydration, fissuring, and disc herniation/bulging. This degenerated disc can lead to compress the adjacent spinal nerves or spinal cord, and abnormally transfer the stressful pressure to the facet joints and ligaments, thus producing mechanical pain.

It has been also hypothesized that in inflamed or degenerated discs neural and vascular ingrowth occurs and the discs themselves transmit nociceptive information to the central nervous system (CNS) as a source of low back pain (LBP)[3]. In this regard, a human study offers an evidence coinciding with the hypothesis, stating that neuronal survival and vascular ingrowth within degenerated discs are highly correlated with β-nerve growth factor (NGF) facilitating angiogenesis around inflamed or degenerated tissues[4]. Another human study revealed that most of the patients with herniated discs and spinal stenosis undergoing decompression surgery experienced LBP when mechanical or electrical stimulations were applied to multiple tissues around the painful area such as annulus fibrosus and vertebral end plate[5]. Moreover, in animal

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studies, disc injuries upregulate pain-related cation channels in the dorsal root ganglion (DRG) neurons via NGF/Tropomyosin receptor kinase A (TrkA) or BDNF signalling[6,7], and mechanical or electrical stimulation to the annulus fibrosus evokes action potentials in the neurons[8,9]. Collectively, these studies provide an important clue that the extensive innervation of afferent nerve fibers to degenerated IVDs with neovascularization, upregulation of NGF and pain-related receptors or cation channels, and the excited responses at the afferent nerve fibers may be strongly linked with nociceptive signalling in LBP. However, it is not well understood whether the afferent nerve fiber innervating degenerated IVD is able to mediate disc pain processing.

The animal models have been developed to resemble features of discogenic LBP patients. In our study, disc puncture was mainly introduced for the formation of painful disc. Animals treated with annulus pulposus (AF) puncture with NP elimination using mechanical (microsurgical drill, microscalpel)[6,10] or chemical (phosphate buffered saline, tumor necrosis factor-α, NGF)[11,12] methods showed a more consistent nociceptive behaviour pattern, compared to those treated with AF puncture only[13]. In this context, we performed a disc puncturing/nucleus pulposus aspiration (PUNCT) operation upon rats, targeting their lumbar 4/5 (L4/5) IVDs as a critical region of LBP, to develop the animal models with severely damaged and rapidly degenerated disc.

Accordingly, in the present study, we aimed to investigate whether the sensitization of afferent nerve fibers innervating degenerated L4/5 IVD has a critical role in relaying nociceptive signals, using *in vivo* electrophysiology and LBP-related behavioural paradigms. As a result, we observed clear mechanosensitive responses in our LBP model rats. Distinct mechanosensitive discharges were generated at the afferent nerve fibers innervating the PUNCT-operated (degenerated) IVD, NGF was upregulated in degenerated IVD tissues, PUNCT-operated animals displayed LBP-like behaviours, and morphine administration to the animals lessened the sensitized neural responses and painful behaviour.

Method

Animals

Male Sprague–Dawley rats (Orient Bio, Korea; for electrophysiology, 3-month-old: 350–400 g, 9-month-old: 470–550 g, 15-month-old: 510–730 g; for other experiments, 3-months-old: 350–400 g) were housed in a temperature- and light-controlled room (22–25°C, 12 h light/dark cycle). Food and water were available *ad libitum*. All animal use procedures were conducted according to the guidelines of the Institutional Animal Care and Use Committee at Korea University (approval number: KUIACUC -2013-234 and KOREA-2018-0064).

Drugs

Morphine hydrochloride (Myungmoon Pharm, Seoul, Korea) was dissolved in saline. Intradiscal (30 uM) or intraperitoneal (0.3 or 3 mg/kg) morphine administration were performed for *in vivo* single nerve recordings or dynamic weight bearing tests, respectively.

Fluorescent imaging of DiI-labelled neurons from lumbar intervertebral discs

Rats ($n = 4$) were anesthetized with 2% isoflurane in oxygen, and a midline ventral longitudinal incision was made in the supine position. The L4/5 disc was exposed under a microscope, DiI (1,1′-dioctadecyl-3,3,3′3′-tetramethylindocarbocyanine perchlorate, 2 μL) was injected into the intradiscal area using a Hamilton syringe (27 G needle, Wohlen, Switzerland), and the hole was sealed with cyanoacrylate glue to prevent DiI leakage. Fourteen days after the surgery, rats were sacrificed. Autonomic afferent nerve fibers (located in paravertebral sympathetic trunks) were carefully extracted, and post-fixed overnight in 4% PFA at 4°C. Tissues of interest were whole-mounted onto microscope slides with a few drops of mounting medium optimally placed between the slides and coverglass. DiI-labelled dorsal root ganglia (DRGs; T13 to L4) neurons and autonomic afferent nerve fibers traced from the lumbar disc were identified under a confocal microscope (LSM 700, Carl-Zeiss, Oberkochen, Germany, excitation/emission wavelength: 555/585 nm).

Randomization and blinding in experiments

We randomly assigned the animals to PUNCT/sham/naïve groups using simple randomization. And outcome assessment of all the experiments was performed under a blinded condition upon the group assignment.

Disc puncture/nucleus pulposus aspiration

Rats were anesthetized with 1.5–2% isoflurane (Hana Pharm, Seoul, Korea) in oxygen, and a midline ventral longitudinal incision was made. The L4/5 disc (L4/5 and L5/6 for pain behaviour testing, see Table II) was exposed under a microscope. The disc(s) was punctured, and the nucleus pulposus of the disc was completely aspirated via a 22-gauge needle and tubing connected to a suction pump (CW-300, CHANGWOO, Seoul, Korea), which generated negative pressure (≥-760 mm Hg). Muscles and skin margins were sutured with 6–0 and 5-0 silk. Sham control group underwent the same surgical procedures, except for the puncture step following disc exposure. Naïve control group did not undergo any surgery procedures.

Western blotting

Lumbar 4/5 disc tissues were ground into a powder and were homogenized in RIPA buffer (50mM Tris–HCl pH 7.4, 150mM NaCl, 1% NP-40, 0.5% Sodium deoxycholate and 0.1% SDS) supplemented with 50X protease inhibitor cocktail. The supernatants were collected after centrifugation at 13,000 rpm for 10-min at 4°C. Protein concentration was determined using Bradford Assay Kit (Bio-Rad, Hercules, CA, USA). Each protein samples were denatured by boiling at 100°C and separated on a 10% SDS-polyacrylamide gel electrophoresis. Proteins were transferred electrophoretically to nitrocellulose membranes (Millipore, Burlinton, MA, USA) which were then blocked in TBS-T (20mM Tris–HCl (pH 7.6), containing 0.8% NaCl, 0.1% Tween 20) with 5% skim milk solution for 1-h at RT. After blocking, the membranes were incubated with antibodies against NGF (1:500, Abcam, Cambridge, MA, USA), BDNF (1:500, Abcam) or β-actin (1:1,000, Abcam) for overnight. This western blotting was performed in 1-day sham (animals/tissues; $n = 5/5$) and 1- ($n = 5/5$), 14- ($n = 5/5$), 35-day ($n = 5/5$) post-PUNCT.

In vivo single nerve recording

After the animal was anesthetized with urethane (250 mg/kg; Sigma–Aldrich, St Louis, MO, USA) the left jugular vein, carotid artery, and trachea were cannulated to inject drugs and monitor blood pressure (80–120 mm Hg)/the end-tidal CO_2 (3.0–4.0%). A midline ventral longitudinal incision was made, and the abdominal viscera were gently retracted and covered with warm saline-soaked gauze. Autonomic afferent nerve fibers (located in paravertebral

Table I
Electrical stimulation and conduction velocity profiles of afferent nerve fibers recorded in the naïve groups. Afferent nerves fibers with conduction velocities of Aδ or C fiber were evoked by electrical stimulation into the annulus fibrosus of lumbar disc (L4/5). Theses fibres also responded to mechanical stimulation (10-g von Frey filament) applied to the annulus fibrosus of lumbar disc

Group (Months)	Electrical Stimulation		Conduction Velocity (m sec^{-1})	
	Duration (ms)	Voltage (V)	C fiber	Aδ fiber
NAIVE (3)	0.6 ± 0.09	13.0 ± 1.00	1.2 ± 0.18 ($n = 6$)	3.5 ± 0.33 ($n = 3$)
NAIVE (9)	0.5 ± 0.34	15.2 ± 1.59	1.0 ± 0.18 ($n = 6$)	2.5 ± 0.06 ($n = 4$)
NAIVE (15)	0.6 ± 1.42	12.8 ± 0.92	1.9 ± 0.11 ($n = 4$)	4.5 ± 0.86 ($n = 5$)

Table II
Experiments profile of naïve, sham, and punctured IVD groups

Experiments	IVD Group	Age of animals	Disc level	No. of animals	No. of nerves	No. of tissues
Electrophysiology (Intradiscal pressure)	Naïve	3 months	L4-5	9	9	
	Naïve	9 months	L4-5	10	10	
	Naïve	15 months	L4-5	9	9	
Electrophysiology (Intradiscal pressure)	Naïve	3 months	L4-5	13	13	
	Sham (14d or 35d)	3 months	L4-5	12	12	
	Puncture (14d)	3 months	L4-5	13	13	
	Puncture (35d)	3 months	L4-5	14	14	
Electrophysiology (von Frey filaments)	Naïve	3 months	L4-5	13	13	
	Sham (14d or 35d)	3 months	L4-5	11	11	
	Puncture (14d)	3 months	L4-5	7	7	
	Puncture (35d)	3 months	L4-5	10	10	
Electrophysiology (Intradiscal pressure)	Sham (14d, saline first/saline second)	3 months	L4-5	7	7	
	Puncture (14d, saline first/saline second)	3 months	L4-5	6	6	
	Puncture (14d, saline first/30 μM morphine second)	3 months	L4-5	6	6	
Western blotting	Sham (1d)	3 months	L4-5	5		5
	Puncture (1d)	3 months	L4-5	5		5
	Puncture (14d)	3 months	L4-5	5		5
	Puncture (35d)	3 months	L4-5	5		5
Behavior	Naïve	3 months		6		
(Dynamic weight bearing, rearing	Sham	3 months	L4-5, L5-6	8		
, withdrawal threshold)	Puncture	3 months	L4-5, L5-6	8		
Behavior (Dynamic weight bearing)	Sham (14d, saline)	3 months	L4-5, L5-6	7		
	Puncture (14d, saline)	3 months	L4-5, L5-6	7		
	Puncture (14d, 0.3 mg kg^{-1} morphine)	3 months	L4-5, L5-6	7		
	Puncture (14d, 3 mg kg^{-1} morphine)	3 months	L4-5, L5-6	7		

sympathetic fibers) were identified and dissected free from connective tissue. Nerve fibers were teased into a single fibre and then placed over a platinum bipolar electrode to record neuronal activity. Electrical stimulation (a concentric bipolar stimulating electrode, NEX-100X, Rhodes Medical Instruments, Summerland, CA, USA) into disc tissue was used for measuring conduction velocity (CV). Conduction distances were identified using the length of the paravertebral sympathetic nerve between the stimulation and recording sites, and conduction latencies were calculated from the time difference between the electrical stimulus and the onset of the evoked action potential. We targeted mechanosensitive Aδ- (CVs > 2 and < 20 m s^{-1}) and C- (CVs < 2 m s^{-1}) fibers. The firing frequency (maximal spikes sec^{-1}) of mechanosensitive afferents (MSA) was recorded and compiled into a post-stimulus time histogram (PSTH, bin width of 1 s). The MSA threshold was determined as at least two firings over a 2-s stimulation. Two types of mechanical stimulation were applied to lumbar 4/5 disc as follows (see also Table II): 1) von Frey filaments (0.2- to 26-g, inter-trial interval [ITI]: 40-sec; animals/fibers, naïve, $n = 13/13$, sham, $n = 11/11$, 14-day post-PUNCT, $n = 7/7$, 35-day post-PUNCT, $n = 10/10$); 2) intradiscal pressure with saline (100–2,500 (3,000) mm Hg, ITI: 70-sec; animals/fibers, 3-month-old, $n = 9/9$, 9-month-old, $n = 10/10$, 15-month-old, $n = 9/9$, naïve, $n = 13/13$, sham, $n = 12/12$, 14-day post-PUNCT, $n = 13/13$, 35-day post-PUNCT, $n = 14/14$) or intradiscal pressure during first (base)/second drug sessions (100–2000 mm Hg, ITI: 70-sec; animals/fibers, PUNCT with saline/saline, $n = 6/6$, PUNCT with saline/30 μM morphine, $n = 6/6$, sham with saline/saline, $n = 7/7$). With a pressure transducer (BLPR2, WPI, Sarasota, FL, USA) connected to a pressure monitor (BP-1, WPI, Sarasota, FL, USA) and calibrated beforehand, intradiscal pressure was generated through expansion of drug-injected volumes and delivered via tubing with a 23-gauge needle.

Dynamic weight bearing

Using a weight-bearing device made by our laboratory, we measured the weight load carried by the four limbs of freely walking rats. The bottom of this device was equipped with a load cell sensor (CB1–K2, DACELL, Cheongju, Korea), and output signals were fed to a digital amplifier (DN-AM 300, DACELL, Cheongju, Korea) for appropriate amplification and filtering. The signal was digitized via an analogue-digital converter (1716, DACELL, Cheongju, Korea) and plotted as a time–weight curve on a personal computer. The test was repeated three or four times to obtain at least eight time–weight curves for a given limb. The weight load difference between the averaged hind and fore limbs (HIND-FORE), averaged right and left limbs (RIGHT-LEFT), and averaged crossing limbs (CROSSING) were used for analysis. Different testing schedules were applied as follows; 1) One- and 3-day before surgery and 7- to 80-day after surgery (naïve, $n = 6$; sham, $n = 8$; PUNCT, $n = 8$); 2) One- and 3-day before surgery and 7- to 14-day after surgery, and 0.5- to 24-h after drug application (i.p. injection; sham with

saline, $n = 7$; PUNCT with saline, $n = 7$; PUNCT with 0.3 mg kg^{-1} morphine, $n = 7$; PUNCT with 3 mg kg^{-1} morphine, $n = 7$). Behavioural experiments were conducted with the experimenter blind to drug type.

Paw withdrawal threshold

To measure the mechanical threshold for hind paw withdrawal, a series of von Frey filaments (0.41–15.10 g, Stoelting, Wood Dale, IL, USA) were applied. Under a transparent plastic dome (28 × 28 × 10 cm) on a metal mesh floor, a von Frey filament was applied to the plantar surface of the right or left hind paw. The 50 % withdrawal threshold was determined using the up-down procedure, and stimuli were presented at intervals of several seconds. A brisk foot withdrawal to the von Frey application was regarded as a positive response. Interpolation of the 50 % threshold was performed according to the method of Dixon[14]. Behavioural testing was applied 1 and 3 days before surgery and 7–80 days after surgery (naïve, $n = 6$; sham, $n = 8$; PUNCT, $n = 8$).

Rearing in an open arena

After habituation in the test room, animals were placed in the centre of an open transparent arena (inner diameter, 80 cm) and permitted to freely explore the arena during the test session (10-min). Rearing behaviour was recorded with a video camera, and the total number and duration of each rearing instance was manually measured. This testing was performed 7–80 days after surgery (naïve, $n = 6$; sham, $n = 8$, PUNCT; $n = 8$).

Statistical analysis

Data were expressed as means with 95% confidence interval (CI) and evaluated using SPSS software version 18 (IBM corp., Armonk, NY, USA). Parametric or non-parametric statistics analysis of data was used depending on the pass of normal distribution testing. For the statistical analysis with independent observation, we analysed one neuronal activity per one animal. Altered firing rates of MSA fibers by intradiscal pressures and von Frey filament stimulations, dynamic weight load, rearing, and withdrawal threshold to hindlimbs were analysed using a two-way repeated measures ANOVA with a post hoc Tukey test. Thresholds of MSA fibers by intradiscal pressures and von Frey filament stimulations were analysed using a Kruskal–Wallis test with a post hoc Bonferroni test. NGF expression levels were analysed using a one-way ANOVA test with a post hoc Tukey test. Firing rates of MSA fibers increased by intradiscal pressures during first/second drugs session were analysed using a Wilcoxon Signed Ranks test. Dynamic weight load behaviour during the drugs session were analysed using a two-way repeated measures ANOVA with a post hoc Tukey test. Differences between experimental conditions were considered statistically significant when $P < 0.05$.

Results

Mechanosensitive afferent nerve fibers innervate naive lumbar discs and linearly encode intradiscal pressure

After identifying that DiI-labelling from naïve lumbar (L4/5) discs travelled via autonomic afferent nerve fibers (located in paravertebral sympathetic chain) and multi-segmentally distributed into DRGs (Supplementary Fig. 1(A) and (B)), we undertook *in vivo* electrophysiology in 3-, 9-, and 15-month-old rats to evaluate the recruitment properties of naïve disc afferent nerve fibers. Fig. 1(A) shows an experimental set-up in this study. Electrical stimulation of disc tissue recruited afferents with conduction velocities of Aδ or C fibers [Table I and Fig. 1(B)]. These fibers were also identified as mechanosensitive afferent nerve fibers (MSAs) recruited following 10-g von Frey filament mechanical stimulation of the L4/5 annulus fibrosus lumbar disc.

We examined the response properties of MSAs in naïve discs to changes in intradiscal pressure. While some of these MSAs had slowly-adaptive firing patterns, other firings patterns were also observed [Fig. 1(C)]. The median pressure threshold for MSA was similar for all ages examined (3-month, 1,500 mm Hg, IQR 1,000–2000 mm Hg; 9-month, 1,500 mm Hg, IQR 500–2000 mm Hg, 15-month, 1,000 mm Hg, IQR 500–2,500 mm Hg) [Fig. 1(D)]. Pressure-response curves showed that maximal firing frequency increased linearly with increasing applied intradiscal pressure intensity [Fig. 1(E)].

Degenerated lumbar disc tissue upregulates NGF, and mechanosensitive afferent nerve fibers innervating degenerated lumbar disc tissues become hyper-responsive, supporting the emergence of peripheral sensitization

We performed L4/5 disc puncture/nucleus pulposus aspiration (PUNCT) surgery upon 3-month-old rats to induce disc degeneration and then examined injury-induced changes in nerve growth factor (NGF). Compared to the 1-day sham disc group, NGF expression significantly increased at 14- (2.6, CI 2.1–3.2) or 35-day (2.5, CI 1.7–3.2) but not at 1-day post disc puncture (1.6, CI 0.6–2.7 [Fig. 2(A) and (B)].

We investigated response properties of MSAs to von Frey filaments and intradiscal pressures in naïve, sham, 14- or 35-day post-PUNCT rats [Fig. 3(A)–(F)]. Mechanical thresholds using von Frey filaments changed only in the 14-day post-PUNCT group (median 1.0 g, IQR 0.6–1.0 g) [Fig. 3(B)]. However, 14- or 35-day post-PUNCT groups fired at higher frequencies, which led to left-shifted stimulus–response curves [Fig. 3(C)]. Post hoc test showed that the MSAs of PUNCT groups fired at higher frequencies in response to 1-, 2-, and 26-g von Frey filaments compared to control disc groups.

Intradiscal pressure thresholds (median) were significantly reduced in the 14-day post-PUNCT populations (naïve, 2000 mm Hg, IQR 1,250–2000 mm Hg; sham, 1,500 mm Hg, IQR 1,125–2000 mm Hg; 14-day post PUNCT, 1,000 mm Hg, IQR 500–1,250 mm Hg; 35-day post PUNCT, 1,000 mm Hg, IQR 500–1,500 mm Hg) [Fig. 3(E)]. Degenerated disc groups fired at higher frequencies in response to saline-induced intradiscal pressure; this led to a leftward shift in the pressure-response curve [Fig (3(F)]. Post hoc test revealed that MSA firing frequencies of puncture groups were significantly greater at intradiscal pressures ranging from 1,500 to 3,000 mm Hg compared to the control disc groups.

Degenerated lumbar discs induce low back pain-related behaviour

We performed two-level consecutive lumbar disc (L4/5 and L5/6) injuries to examine their effect on LBP-related behaviour (Fig. 4). We repetitively tested the dynamic weight load distribution between HIND-FORE, rearing behaviour, and mechanical threshold in hind-paws of control (sham or naïve) or disc-punctured animals (7- to 80-day post PUNCT) [Fig. 4(A)].

Normally, weight load is preferentially distributed to the hind limbs, with hind limb load averaging 60–63% of body weight and forelimb load averaging 48–52% of body weight[15]. Fig. 4(B) shows the significant alteration of the PUNCT population after injury, revealing similar forces between HIND-FORE; the normally 10–13% difference between HIND-FORE disappeared at 7- to 42-day after

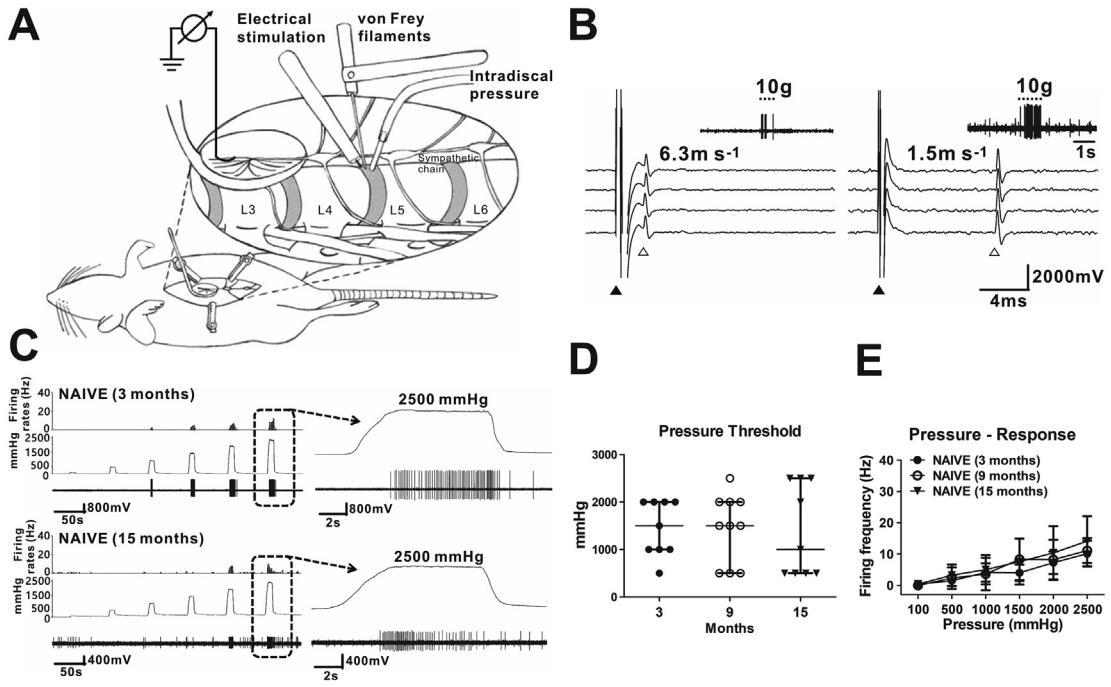

Fig. 1. Afferent nerve fibers innervating naive lumbar discs elicit mechanosensitive firings. (A) *In vivo* electrophysiology approach within disc primary afferent pathways. Electrophysiological recordings were conducted at the autonomic afferent nerve fibers traveling up the paravertebral sympathetic chain. (B) The activities of afferent nerve fibers were evoked by the application of electrical stimulation and von Frey filament (10 g) into the annulus pulposus of naive disc (L4/5). The disc nerve fibers showed the conduction velocity of Aδ- and C-fibers. (C) Representative mechanosensitive afferent firing induced by intradiscal pressure in the naïve group. (D and E) There was no significant difference in the pressure thresholds of 3- (animals/fibers; $n = 9/9$), 9- ($n = 10/10$), and 15-month old ($n = 9/9$) rats and the pressure-response curves. Values are expressed as median + IQR (D) or mean + 95% CI (E).

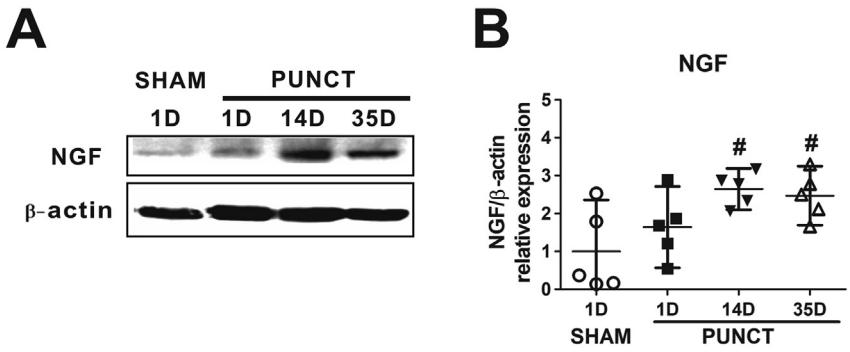

Fig. 2. The degenerated lumbar disc tissue upregulates NGF. (A) Western blot results for nerve growth factor (NGF) expression were determined 1 day post-sham surgery or 1, 14, and 35 days post-PUNCT. (B) Compared to 1 day post-sham (animals/tissues, $n = 5/5$) disc group, expression levels of NGF were significantly increased 14 ($n = 5/5$) and 35 ($n = 5/5$) days, but not 1 ($n = 5/5$) day, post-PUNCT. Values are expressed as mean + 95% CI (post hoc Tukey test, $^{\#}P < 0.05$ vs sham disc).

injury, with a gradual recovery that returned to control values by 60-day post PUNCT [Fig. 4(C)]. In comparison, no injury-induced changes in weight distribution between right and left limbs (RIGHT-LEFT) or CROSSING were detected during walking.

Disc puncture impaired rearing behaviour. There were significant effects of disc puncture on the total number and total duration of rearing, but not on the average duration of each rearing. Compared to those of control disc groups, post hoc comparisons showed that total rearing number and duration of puncture groups significantly decreased at most post-surgical days sampled (7-, 14-, 21-, 35-, 60-, and 80-day) [Fig. 4(D)].

Disc puncture also induced hypersensitivity in hind-paws. There were significant effects of disc puncture on withdrawal threshold of both left and right hind-paws. Compared to control disc groups, post hoc test showed that disc puncture groups significantly decreased in withdrawal threshold of hind-paws at 7- to 42-day after PUNCT [Fig. 4(E)], with a gradual recovery by 60- to 80-day after PUNCT.

The hyper-responsiveness of mechanosensitive afferent nerve fibers and pain-related behaviour induced by disc degeneration are nullified after the application of morphine

We investigated whether the application of intradiscal or intraperitoneal (i.p.) morphine attenuated MSA activity or LBP-related behaviour 14-day post PUNCT, respectively [Fig. 5(A)]. In the first (base) session, intradiscal application of saline (SAL) was used to increase pressures in sham and PUNCT population. Subsequently, a second injection that included 30μM morphine was administered 10-min later to one of the PUNCT populations. As

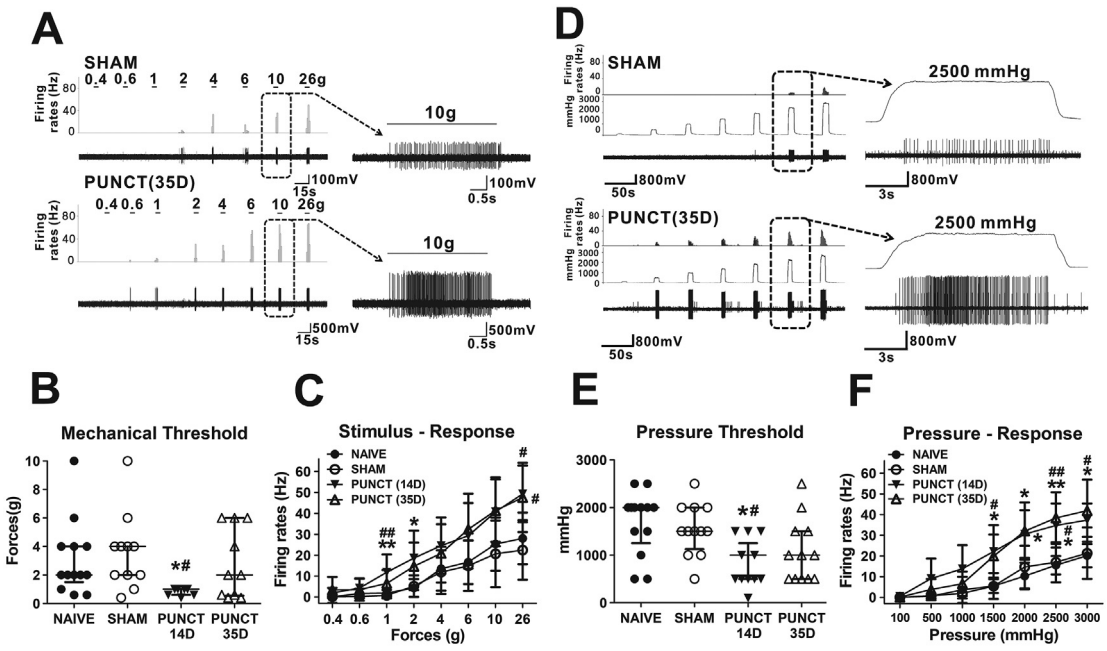

Fig. 3. Mechanosensitive afferents innervating degenerated lumbar discs become hyper-responsive. (A and D) Representative MSA firing evoked by mechanical stimulations applied to lumbar disc. (B and C) Compared to control (sham or naïve) discs, the thresholds after von Frey filament application were significantly reduced 14 days post-PUNCT. However, 14- or 35-day post-PUNCT MSAs fired at higher frequencies. (E and F) Intradiscal pressure thresholds were significantly reduced 14 days post-PUNCT, compared to those of the control groups, and MSA firing frequencies in the PUNCT groups were significantly greater when 1,500–3,000 mm Hg pressures were applied. Naïve (animals/fibers; von Frey filament, $n = 13/13$; intradiscal pressure, $n = 13/13$), sham ($n = 11/11$; $n = 12/12$), 14 days ($n = 7/7$; $n = 13/13$) or 35 days ($n = 10/10$; $n = 14/14$) post-PUNCT. Values are expressed as median + IQR (B and E; post hoc Bonferroni test) or mean + 95% CI (C and F; post hoc Tukey test). $^{*}P < 0.05$ and $^{**}P < 0.01$ vs naïve disc, $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ vs sham disc.

shown in Fig. 5(B) and (C), morphine reduced firing frequencies relative to what was recorded after saline injection in the PUNCT population. Differences were observed at pressures of 1,500 and 2000 mm Hg [Fig. 5(C)].

We repetitively tested weight load alterations between HIND-FORE among animals with sham or PUNCT, followed by i.p. injection of saline or morphine (0.3 or 3.0 mg kg^{-1} morphine). We examined behavioural changes 0.5- to 24-h post-drugs application. Post-drug data (3.0 mg kg^{-1} morphine) for the PUNCT group are shown in Fig. 5(D). Morphine was shown to have a dose-dependent ability to restore force differences between HIND-FORE [Fig. 5(E)]. Specifically, the application of 3 mg kg^{-1} morphine restored HIND-FORE force differences to near-normal levels, and these actions were seen from 0.5- to 1.5-h post-drug delivery (post hoc comparisons). At 0.3 mg kg^{-1}, morphine actions were transient (0.5 h post-drug). As expected, drug/injury-induced changes in weight distribution between right and left or CROSSING were observed during walking.

Discussion

In this study, we found that afferent nerve fibers innervating L4/5 IVDs were mechanosensitive regardless of animal group. Following application of two mechanical stimuli including von Frey hair testing and intradical pressure into the IVD, mechanosensitive afferent nerve fibers (MSAs) fired in a stimulus intensity-dependent manner and were shown to linearly response to them. The afferent nerve fibers were also identified as an Aδ- or C-fiber after CV examination. In addition, there was a substantial increase in NGF expression within the IVDs on the 14- and 35-day post-disc puncture, which corresponded to a period when the MSAs became hyper-responsive, thus implicating the emergence of peripheral sensitization. During this period, the animals treated with punctured IVD also displayed LBP-like behavior, which began to subside by the 60-day post-disc puncture. Both the MSA hyper-responsiveness and painful behavior were attenuated after intradiscal or intraperitoneal administration of morphine.

Normal (healthy) IVDs have not been considered as innervated tissue, but we found that normal lumbar IVDs are connected to afferent nerve fibers originated from multi-segmental DRGs (a confocal imaging study; Supplementary Fig. 1(A)–(C)). Moreover, in our study, the responses to mechanical stimuli applied to the IVDs were recorded at the nerve fibers (Fig. 3) and most of DiI-labelled DRG neurons expressed CGRP and/or TPRV1 (Supplementary Fig. 1(D)), indicating that MSAs are strongly engaged in nociceptive information transmission. Accumulating evidences are indeed in line with our results, stating that human normal lumbar IVDs are innervated by proprioceptive fibers (Ruffini/Golgi type-mechanoreceptors), free nerve endings, and putative sympathetic fibers (neuropeptide Y)[16,17]. Similarly, animal studies reported CGRP- or Substance P-immunopositive afferent nerve fibers and autonomic nerve terminals projecting to disc tissues[18–20].

We identified mechanosensitive afferent nerve fibers (MSA) of disc, which in effect responded to mechanical stimuli and showed increased neuronal discharge frequency with an increase in stimulation strength. Importantly, the MSAs became hyper-responsive after disc-punctured injury. In detail, we applied 100–3,000 mm Hg pressure (≈0.01–0.39 MPa) into the L4/5 IVD. The applied pressure intensity was comparable to measurements obtained from human and other animal studies. Specifically, human intradiscal L4/5 pressure ranges from 0.1 to 0.12, 0.5–1.1, and 0.53–0.65 MPa for lying, standing, and walking, respectively[21]. In sheep (cows), intradiscal L4/5 pressure ranges from 0.49 to 0.54, 0.64–1.05 (0.84 ± 0.24), and 1.2–3.22 (2.3) MPa for sleeping, standing, and walking, respectively[22,23]. Notably, the reduced thresholds or

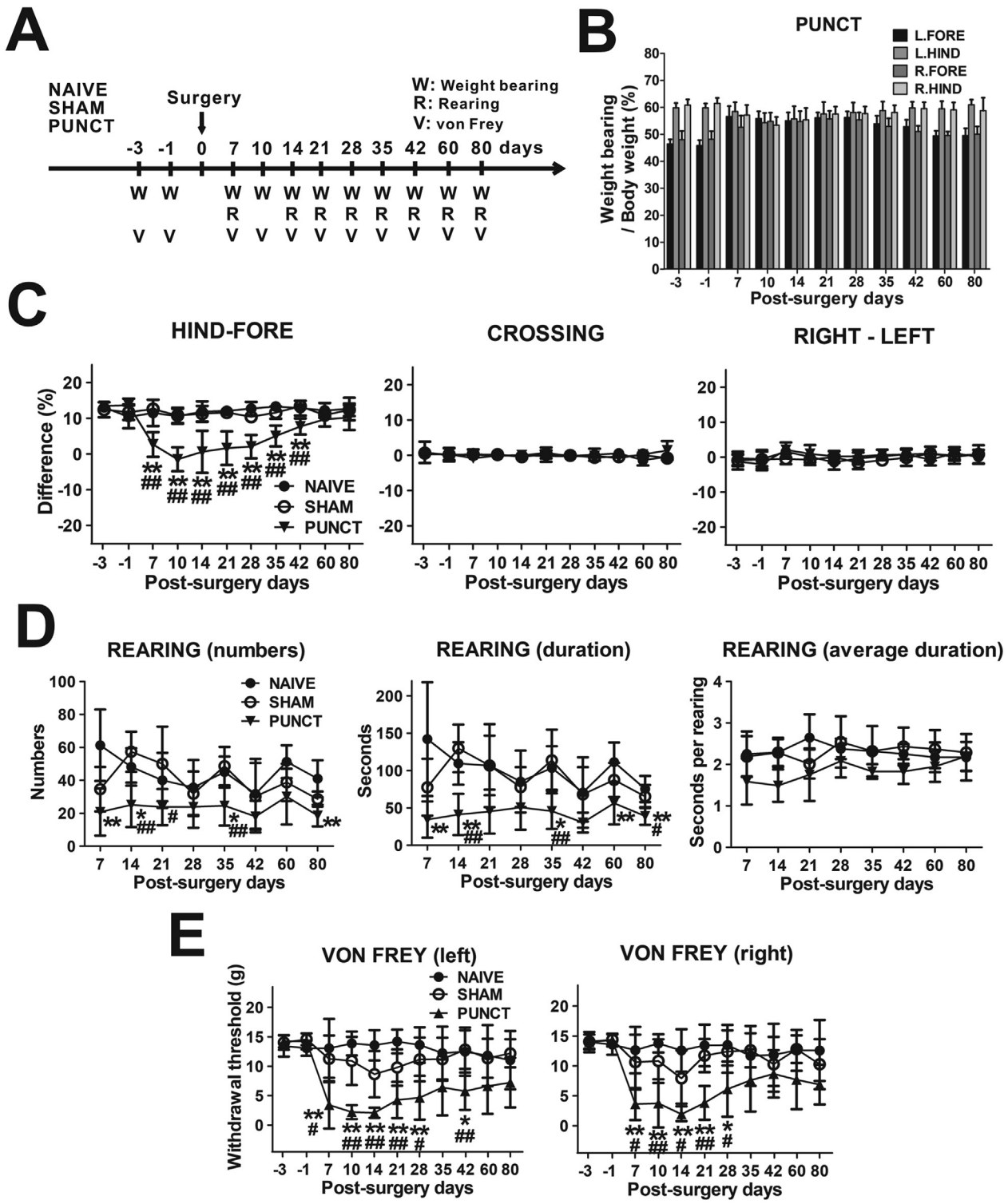

Fig. 4. Degenerated lumbar disc induces low back pain-related behavior. (A) Behaviour test schedules of the punctured (PUNCT, $n = 8$), sham ($n = 8$), and naïve ($n = 6$) disc group. (B) Representative weight-bearing data for the PUNCT group. (C) Normal weight bearing during freely walking was preferentially distributed to hind limbs, but a 10–13% difference in HIND-FORE measure disappeared 7–42 days post-PUNCT. There were no PUNCT-induced changes in weight distribution in RIGHT-LEFT or CROSSING. (D) The PUNCT group showed significant reductions in rearing 7, 14, 21, 35, 60, and 80 days post-PUNCT. (E) The PUNCT group showed significant decreases in withdrawal thresholds of the hind paws 7, 10, 14, 21, 28, and 42 days post-PUNCT. Values are expressed as mean + 95% CI (C-E; post hoc Tukey test). $^{*}P < 0.05$ and $^{**}P < 0.01$ vs naïve disc, $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ vs sham disc.

sensitized responses of the afferent nerve fiber to intradiscal pressure after disc puncture were associated with behavioural changes (reductions in hind limb weight loading and rearing), implicating the presence of LBP.

Inflammatory responses in degenerated discs can be essential for developing discogenic pain. Increased levels of proinflammatory cytokines (IL-6, TNF-α, and IL-1β) are observed in serum and disc tissues of LBP patients[24–27]. Similar result has been

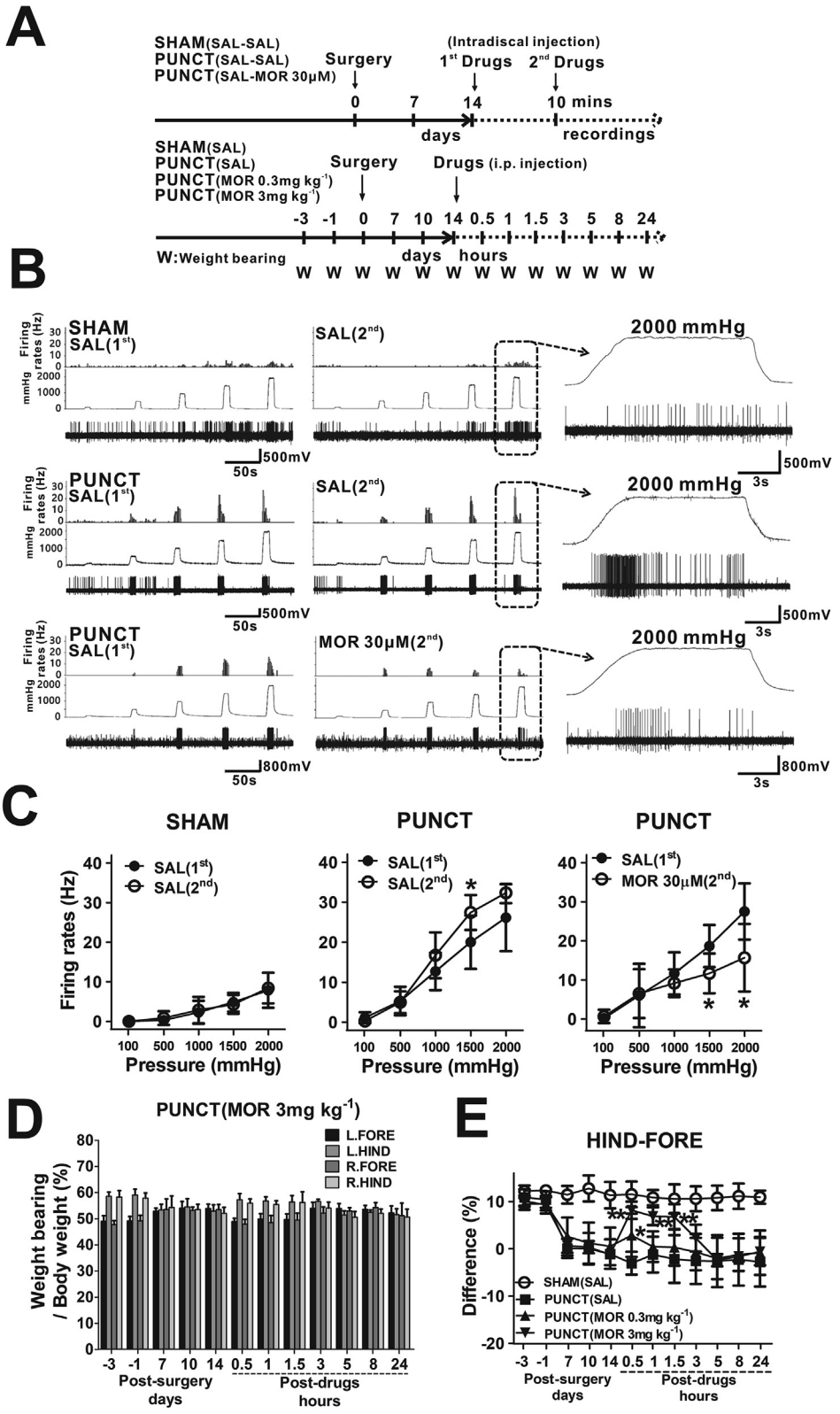

Fig. 5. Both hyper-responsiveness of mechanosensitive afferents and pain-related behaviours induced by disc degeneration are attenuated by application of morphine. (A) Time schedules of drug applications for *in vivo* electrophysiology or dynamic weight bearing test. (B) Representative MSA firing evoked by intradiscal pressure (first/second sessions) applied to PUNCT disc with saline/saline (animals/fibers, $n = 6/6$) or saline/30 μM morphine ($n = 6/6$), and to sham disc with saline/saline ($n = 7/7$). (C) In the second session, the intradiscal pressure with 30μM morphine injection applied to the degenerated disc significantly reduced MSA firing (Wilcoxon Signed Ranks test, $^{*}P < 0.05$ vs intradiscal pressure with saline in the first session). (D) Representative weight-bearing data after i.p. injection of 3 mg kg^{-1} morphine in the PUNCT group. (E) Morphine dose-dependently restored the force differences in the HIND-FORE measure. Contrary to the PUNCT group treated with saline (i.p.), the morphine-administered (3 mg kg^{-1} morphine, i.p.) PUNCT group displayed a recovery behaviour near to normal level 0.5–1.5 h post-drug. With the dosage of morphine, there were transient effects only at the 0.5 h ($n = 7$ per group). Values are expressed mean + 95% CI (E; post hoc Tukey comparison, $^{*}P < 0.05$ and $^{**}P < 0.01$ vs PUNCT disc with i.p. saline).

reported in rodent disc injury model, demonstrating that they include elevated mRNA or protein expression of IL-1β and TNF-α in disc tissues[28]. Importantly, these inflammatory cytokines promote the synthesis of NGF in DRG neurons, which in turn can affect neuronal plasticity by enhancing the density of innervated nerve fibers[29]. In addition, the increased concentration of NGF can regulate the release of CGRP and substance P at nerve endings[30], thus sensitizing peripheral nerves. Therefore, intradiscal increase in NGF expression may be important to cause LBP. This is consistent with clinical data reporting the alleviation of chronic LBP observed in patients treated with anti-NGF[31,32].

LBP patients show altered gait, increased stiffness in pelvis-thorax movements[33], and greater co-activation of superficial abdominal and lumbar muscles[34] (made to avoid painful motion and injuries). Behavioural evaluation of disc-related pain in animal models is challenging and limited by some factors, including those associated with the performance of behavioural testing, animal behavioural strategies, and differences in disc injury models. By using a lumbar dual disc puncture model of degeneration (L4/5 and L5/6), we observed impaired rearing behaviour and mechanical hypersensitivity, consistent with a recent study in mice[6]. We also found reduced hindlimb loading with greater weight transfer to the forelimb in freely walking rats [Fig. 3(B)]. Alterations in weight distribution were observed from 7- to 80-day post-injury, and recovery to pre-injury weight distribution occurred by 60-day post-injury. The fact that morphine could temporarily reverse the injury-induced shift in weight distribution strongly supports that this shift was made to protect against movement-induced pain [Fig. 4(D)]. Other animal studies are also consistent with these results, showing that disc injury leads to motor behaviour changes (long standing time and short stride gait)[11,35]. However, a disc study where complete Freund's adjuvant was injected intradiscally into only one disc (L4/5) did not find alterations in weight load. This discrepancy may be attributed to reduced injury severity and differences in analysis (four limbs vs one limb) or testing schedules (repetitive vs one-day fixed). Indeed, rats having two consecutive disc punctures are more prone to vocalize in response to painful stimulation than those having only one-disc puncture[10].

Despites the increased neuronal excitability at the afferent nerve fiber after disc puncture, it should be noted that we mainly targeted afferents associated with the anterior (ventral) lumbar disc. In effect, we did not record from sinuvertebral nerve afferents innervating the posterior (dorsal) disc adjacent the spinal cord, which may better reflect the afferent nerve fibers encoding LBP in patients with posterior disc herniation.

Taken together, our results provide clear electrophysiological evidence of a disc injury-induced increase in the excitability of morphine-sensitive nociception-encoding MSAs. These results are consistent with disc MSAs which play a critical role in encoding LBP. Whether these hyper-excited mechanosensitivities observed in rats with degenerated disc are linked to an increase in inflammatory mediators and an upregulation of pain-related channels or receptors remain to be determined.

Author contributions

All authors have made substantial contributions to (1) the conception and design of the study, or acquisition of data, or analysis and interpretation of data; (2) drafting the article or revising it critically for important intellectual content; and (3) final approval of the version to be submitted.

Study concept and design: E.H.P, H.C.H.

Data acquisition and assembly: E.H.P, S.W.M., H.R.S.

Data analysis and interpretation: E.H.P, S.W.M., H.R.S. S.H., M.G.L., T.I.K., I.T.J., H.C.H.

Drafting of the article: E.H.P, S.H., H.C.H.

Conflict of interest

The authors declare no conflicts of interest.

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Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.joca.2019.07.010.

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