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Original experimental

Stimulation-induced expression of immediate early gene proteins in the dorsal horn is increased in neuropathy

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HIGHLIGHTS

- We studied the immunoreactivity of Arc, c-Fos and Zif268 after nerve ligation.
- Expression of Arc, Zif and Fos was not elevated in neuropathic animals before stimulation.
- Stimulus-induced immunoreactivity was clearly increased in neuropathy.
- Contralateral dorsal horn showed unchanged immunoreactivity after neuropathic treatment.
- The studied IEGP's may have a role in sensitization in neuropathic conditions.

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ABSTRACT

Background and aims: Peripheral neuropathic pain is described as a pain state caused by an injury or dysfunction of the nervous system, and could have clinical manifestations such as hyperalgesia, allodynia and spontaneous pain. The development of neuropathic pain may depend on long-term forms of neuronal plasticity in the spinal cord (SC). Expression of the immediate early gene proteins (IEGPs) Arc, Zif268, and c-Fos are implicated in establishment of long-term potentiation (LTP) induced by conditioning stimulation (CS) of primary afferent fibres. However, the impact of the neuropathic state (Bennett's model) on CS-induced expression of IEGPs has not been studied. The aim of this study was to compare the levels of Arc, c-Fos and Zif268 immunoreactivity prior to and after conditioning stimulation in animals with developed neuropathic pain, with sham operated, non-ligated controls.

Methods: Twenty-four animals were divided equally into the neuropathic and non-neuropathic groups. Neuropathic pain was induced in all animals by conducting a loose ligation of the sciatic nerve with Chromic Catgut 4.0 sutures 7 days prior to conditioning stimulation or sham operation. The loose ligation was performed by placing sutures around the sciatic nerve compressing the nerve slightly just enough to reduce but not completely diminish the perineural circulation. A state of neuropathy was confirmed by a significant decrease in mechanical withdrawal threshold measured by von Frey's fibres. Immunohistochemical analysis was performed on transverse sections obtained from the L3–L5 segments of the SC at 2 and 6 h post-CS and IEGP positive cells were counted in lamina I and II of the dorsal horn. During statistical analyses, the groups were compared by means of analysis of variance (univariate general linear model). If significant differences were found, each set of animals was compared with the sham group with post hoc Tukey's multiple comparison test.

Results: Strikingly, all IEGPs exhibited a significant increase in immunoreactivity at both time points compared to time-matched, sham operated controls. Maximal IEGP expression was found 2 h after CS in neuropathic rats, and there was a smaller but still significant increase 6 h after CS. The unstimulated side of the dorsal horn in stimulated animals did not show any significant change of the number of IEGP positive cells and was approximately at the same level as sham operated animals. The number of IEGP positive cells in sham operated controls (non-neuropathic and non-stimulated animals) showed same immunoreactivity in 2 and 6 h post sham operation.

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Abbreviations: NS, nociceptive specific neurons; WDR, wide dynamic range neurons; DAB, 3,3′-diaminobenzidine.
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Conclusions and implications: The neurophysiological process of neuropathic pain development is complex and needs to be studied further in order to clarify its nature and components. This present study is meant to reveal a step towards further understanding the role of Arc, c-Fos and Zif268 in neuropathic pain. Moreover, this study might contribute to the knowledge base for further research on better therapeutic possibilities for neuropathic pain.

1. Introduction

Neuropathic pain has been defined as a pain state caused by an injury or dysfunction of the nervous system, and has clinical manifestations such as hyperalgesia, allodynia and spontaneous pain [1,2]. Many etiological factors can lead to neuropathy as nerve compression or damage leading to partial or total nerve ligation. The nerve can be damaged on different locations between the CNS and the peripheral nerve ending [3].

However, there has been limited therapeutic success with drugs such as NSAIDS, opiates [4], anticonvulsants and antidepressants [5] against neuropathic pain. One of the best known animal models of neuropathic pain development is the loose sciatic nerve ligation described by Bennett [6], in which the sciatic nerve gets partially injured by light constriction of the sciatic nerve with a loosely tied thread.

Long-term potentiation (LTP) as a response to conditioning stimulation (CS) of afferent fibres is a common model of synaptic plasticity in the brain and the spinal cord [7]. Many hippocampal studies are used as a source on mechanisms of LTP formation, function and maintenance [8]. LTP has been divided into two phases, early and late, where the late phase has been proven to require de novo protein synthesis [9]. Immediate-early gene proteins (IEGP) such as early growth response protein 1 (Egr-1) (also known as Zif 268 – zinc finger protein 225) and activity regulated cytoskeletal protein (Arc) have been found to be required for the process of late-phase LTP establishment in the hippocampus [9,10]. In the spinal cord, the most effective type of stimulation to induce LTP has been shown to be a series of high-frequency (e.g. 100 Hz) trains of electric stimulation [11]. Previous studies have shown that peripheral stimulation of afferent fibres leads to an increase of expression of c-Fos mRNA in spinal cord neurons and synaptic Arc protein [12,13].

Nociceptive information in the spinal cord is acquired, processed and transmitted mainly by nociceptive specific neurons (NS) and wide dynamic range neurons (WDR). The NS neurons are most abundant in Lamina I and II and respond to intense stimuli [14,15] while in deeper in laminae V and VI, WDR neurons are most abundant. Our research focused on Lamina I and II whose borders were previously defined in the research of Molander et al. [16]. Furthermore, it was shown that WDR neurons react in a graded manner to gentle touch, stronger mechanical and noxious stimulations [17].

Previous studies have shown elevated c-Fos, Zif268 and Arc immunoreactivity in neuropathic rats where neuropathy was induced by means of various methods such as the Bennett protocol [18] and Kim and Chung protocol [19]. However, it is not known whether CS-induced expression of IEGPs in the spinal cord dorsal horn differs between neuropathic and non-neuropathic states. Here, we compared CS-induced of IEGPs in the Bennett model of neuropathic pain relative to shame operated, non-ligated controls. The present study contributes to the clarification of the highly complex process of neuropathic pain formation. The protocol of sciatic nerve ligation represents a model of constriction nerve injury, such as a nerve injury present during spinal nerve root constriction caused by disc prolapse, or nerve constriction in the carpal tunnel syndrome.

2. Materials and methods

2.1. Animals and surgery

Female Sprague-Dawley rats, 2–3 months of age, weighing 240–300 g were used (NTac:SD, Taconic Europe, Ejby, Denmark). The animals had free access to food and water and were held on a 12/12-h light/dark cycle.

Twenty-four animals were divided in two groups of twelve animals, the neuropathic and non-neuropathic groups. All animals underwent a surgical intervention where the non-neuropathic animals were sham operated while the sciatic nerve of the neuropathic rats was ligated. All animals were operated under brief Isoflurane anaesthesia. Neuropathic pain was induced by means of surgical procedures previously described [6]. During this intervention, the common sciatic nerve was revealed by a blunt dissection through the biceps femoris muscle. Around 10 mm of the nerve was freed of adhering tissue and 4 ligatures with 4/0 chromic catgut (Chromic Catgut 1/2 circle, 4.0 round bodied; from KRUUSE Norge) were tied loosely around it with 1– 2 mm spacing between them and constricted to a degree previously described [6] to reduce the diameter of the nerve by a just noticeable amount [5]. The sutures were placed on the caudal part of the sciatic nerve, leaving the upper, cranial part free from sutures. The cranial part will further on be the location for stimulation electrode placement.

To check the level of allodynia as a clinical indication of neuropathic pain, the mechanical withdrawal thresholds were tested first at baseline before surgery and afterwards from the 4th post-surgery day with von Frey's filaments of varying thickness. Before testing, the animal was placed in an elevated Plexiglas cage with a wire mesh floor and allowed to adapt for 10 min. The mid-plantar surface of the rats' hind paws was stimulated with von Frey's filaments through the wire mesh floor until the filament bent slightly. During allodynia testing we used 14 von Frey filaments, numbers 1–20 (Somedic Sverige) with a calibrated stiffness corresponding to 0.008, 0.02, 0.04, 0.07, 0.16, 0.4, 0.6, 1.0, 1.4, 2, 4, 6, 8, 10, 15, 26, 60, 100, 180 and 200 g. The filaments are presumed to roughly represent a logarithmic scale of applied force and a linear scale of perceived force (information provided by the manufacturer: North Coast Medical Inc.). As previously described [20] the filaments were applied to each hind paw in an order of increasing stiffness and the withdrawal threshold of each individual hind paw was defined as the force (in grams) of the filament that induced three of five positive responses (brisk withdrawal). The non-lesioned side served as a control, displaying no effect of the contralateral loose nerve ligation [20].

The baseline response thresholds before surgical intervention in the non-affected side were >60–70 g pressure [20]. Allodynia was defined as severe if the response was positive to the filaments with a stiffness corresponding to 0.16–1.0 (g) (four filaments 0.16; 0.40, 0.60 and 1.0 g). Allodynia was defined as moderate if the animals responded to filaments with a stiffness corresponding to 1.4–6.0 (g) (four filaments 1.4, 2.0, 4.0 and 6.0). Mild allodynia was positive if the animals responded to the filaments with a stiffness corresponding to 8.0–26 (g) (four filaments 8.0, 10, 15 and 26).

Both the neuropathic and non-neuropathic group was divided in 2 sub-groups, the two and six-hour groups with six animals

per subgroup. Every subgroup was divided into 2 smaller groups, one stimulated and one sham operated small group. Each small group consisted of three animals, where the animals of the stimulated small group received CS while the control animals were sham operated without receiving the actual CS.

On the 7th day after nerve ligation or sham operation the animals were first checked for withdrawal threshold as previously described, and then anaesthetized with 1.7–2.2-mg/kg urethane (250 mg/ml in sterile water) injected intraperitoneally. Animals were then checked for presence of pedal and corneal reflexes whose absence indicated an appropriate level of anaesthesia. After shaving of the surgery areas, the rats were transferred to a heating pad and during the whole process of stimulation and waiting, the animal body temperature was kept at 37 °C.

The left sciatic nerve was re-dissected from the surrounding muscles on the thigh so that a total length of 1–2 cm of the nerve was exposed. Proximal to the nerve division and proximal to the ligatures, the nerve was placed in a bipolar silver hook electrode (2 mm distance between hooks). The hooks were isolated from the surrounding tissue by means of elastic plastic film (Parafilm, American Can Company, USA).

All animals were perfused, dependent of the group, 2 or 6 h after CS or after the sham operation.

2.2. Sciatic nerve stimulation

A conditioning stimulation (CS) consisting of 10 stimulus trains, with stimulus duration of 0.5 ms, amplitude of 7.2 mA, a frequency of 100 Hz, train duration of 2 s and 8 s intervals between trains was used to stimulate all animals except the sham-operated groups. These characteristics of CS have shown to be approximately four times the threshold for C-fibre evoked neuronal firing [7] and have been shown to induce LTP in the dorsal horn of intact animals [21]. CS was given via a PC with Spike2 software coupled via a Digitimer 1401 interface to a stimulator (Neurolog Systems with Stimulus isolator NL800) connected to the bipolar silver hook. The sham-operated animals underwent surgery and sciatic nerve mounting on the silver hook electrodes without actually CS delivery.

2.3. Immunohistochemistry

After reflex testing that showed abolishment of corneal and pedal reflexes the animals were transcardially perfused with a 4 °C, 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer with pH 7.4. After fixing the animal's scull in a stereotaxic frame, one cranial-caudal incision was made from the medial occipital region to the top of the inter-ilium bone line on the skin to reveal the processi spinosi and the paravertebral musculature [22].

Six segments of the spinal cord (Th13–L5 segments) were dissected out. Thereafter, the caudal pieces that contained the L3–L5 segments of the spinal cord were cut from the residual trunk frozen in custom made aluminium foil wells (Ø8 mm) in dry ice and kept at −80 °C. A technician at this point marked the spinal cord samples so that the following procedures continued without knowledge of the time post CS or treatment of animal groups. The information of the pretreatment and the time point was sealed, and the seal was broken when all results were obtained.

From this tissue part, the cranial 3.2 mm of the frozen L3-L5 trunk was transverse sectioned in 20 μm thick sections. In this manner, the sections were taken from the region L3/L4 which was in our previous research found to be the area of the spinal cord with maximal cellular IEGP response after sciatic nerve stimulation. The sections were mounted on Superfrost GOLD slides (Braunschweig, Germany) so that each slide contained a number of sections for each time point and control sections.

Spinal cord sections were first washed in PBST (0.1% Triton X in PBS) and blocked in blocking buffer for 1 h (3% horse serum, 0.3% Triton X in PBS). Afterwards they were incubated overnight in primary antibody diluted in PBST (0.1% Triton X), 3% normal horse serum at 4 °C. After three 5 min washes in PBST, the section were incubated with biotinylated secondary antibody in PBST for 2 h at room temperature. Sections were then washed 3 times 5 min in PBST and incubated in streptavidin-HRP diluted in PBST for 1 h, washed again in the same way, and 3,3′-diaminobenzidine (DAB) stained for 8 min approximately at room temperature under a microscope to control the colour development. The slides were then washed in Milli-Q water three times and stained for 5 min with 0.1% Cresyl Violet (pre heated to 50 °C). The slides were then washed 3 times with Milli-Q water and subsequently immersed 3 min in each of four baths with increasing ethanol concentration (75%, 90%, 96%, and 96%) and 3 min in two 100% xylene baths. The sections were cover-slipped with DPX mounting medium, and were dried in RT 24 h before imaging.

Mouse anti-Arc monoclonal antibody (1:300 dilution) was purchased from Santa Cruz Biotechnology (cat. #sc-17839), rabbit anti-c-Fos (1:1000 dilution) polyclonal antibody was from Calbiochem (cat. #PC38 T) and rabbit anti-zif 268 (Egr-1) (1:300 dilution) was from Santa Cruz Biotechnology (cat. # sc-110). Secondary antibodies were biotin-conjugated anti-mouse IgG (cat. #PK-4002 VECTASTAIN® ABC Kit, Vector laboratories) or biotin-conjugated anti-rabbit IgG (cat. #PK-6101 VECTASTAIN® ABC Kit, Vector Laboratories).

The same person (OB) analyzed all sections. For each stimulated animal, time point and treatment, 32 sections were analyzed for every antibody used. For all three IEGPs the analyzed sections were distributed evenly over the entire area of interest explained previously. As controls, sham operated animals were used and every stimulated animal had as its own control a sham operated animal, that had received exactly the same treatment except the conditioning stimulus. IEGP positive cells were counted in lamina I and II using lamina border differentiation previously defined in the research of Molander et al. [16]. Cells were identified as positive if they showed clear staining of the nucleus with or without staining of the cytoplasm or cellular extensions as defined by Haugan et al. [7].

The stimulated and non-stimulated sides from all dorsal horn sections were imaged (Nikon Eclipse E-600; NIS elements software; resolution 2560 × 1920; colour depth 24-bit RGB) with 400× magnification. All neurons were manually counted in digital pictures and during counting the neurons were labelled to avoid double-counting.

We performed negative controls for all three antibodies (Arc, c-Fos and Zif268) by incubating the sections as previously described but without the primary antibody. These procedures led to elimination of all specific staining.

2.4. Ethical considerations

The experiments were approved by the Norwegian Committee on Research in Animals, and were carried out in accordance with the European Communities Council directive of 24 November 1986 (86/609/EEC). Efforts were made to minimize the number of animals used, and experiments were designed to minimize suffering.

2.5. Statistics

Graphpad Prism 6.0 and SPSS 22.0 were used for graphical representation and statistical evaluation of our results.

The effect of Bennett's loose sciatic nerve ligation was evaluated by the means ± S.E.M. of unpaired Student's *t*-test used to compare mean mechanical response thresholds (in grams) between subjects

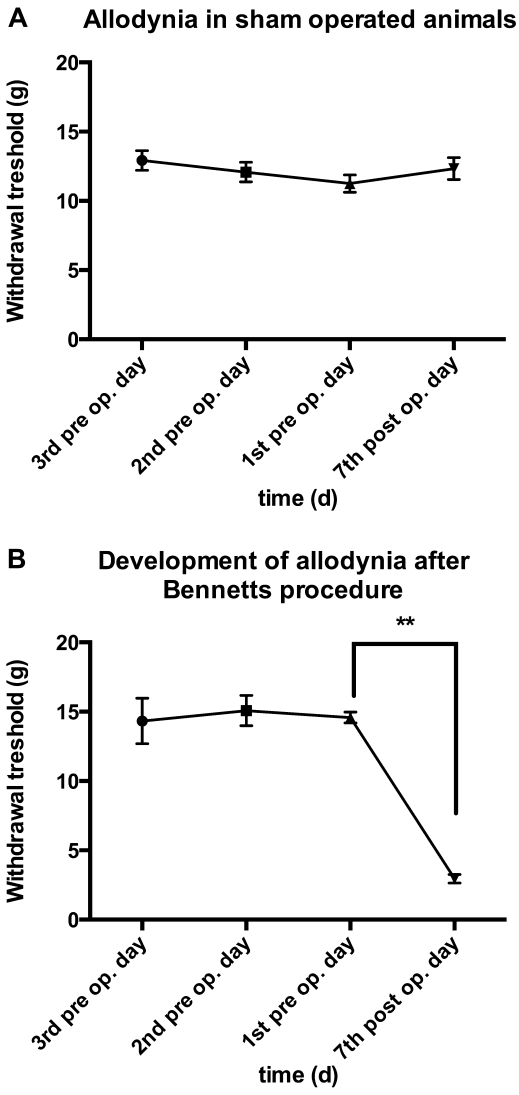

Fig. 1. (A and B) Change in foot withdrawal to von Frey fibres in sham operated and neuropathic animals. (A) Changes in foot withdrawal threshold to von Frey hair stimulation measured daily, 3 days before surgery and 7th day after surgery ($N = 12$) on operated side of animal, after unilateral sciatic nerve sham operation. Data given as mean withdrawal threshold in grams (g). (B) Changes in foot withdrawal threshold to von Frey hair stimulation measured daily, 3 days before surgery and 7th day after surgery ($N = 12$) on operated side of animal, after unilateral sciatic nerve loose ligation. Data given as mean withdrawal threshold in grams (g).

before and after the operation calculated with use of von Frey's fibres (Fig. 1A and B).

To compare the quantitative difference for all IEGPs, a univariate general linear model of statistical analyzes was applied. Here we conducted comparisons between the 2 and 6 h post-stimulation groups and the control (sham-operated) groups. The stimulation/non-stimulation, neuropathic/non-neuropathic and time point were used as independent variables while the number of positive cells was used as the dependent variable. Statistical significance was accepted at the 1% level (Fig. 2A–C). If a significant effect of stimulation was found, each set of animals was compared with the sham group with post hoc Tukey's multiple comparison test. Separate controls (for non-neuropathic and neuropathic) were used for each treatment group, but data for different time points within the same treatment group were merged since the number of positive cells within same treatment groups, as expected, did not show significant variation with time.

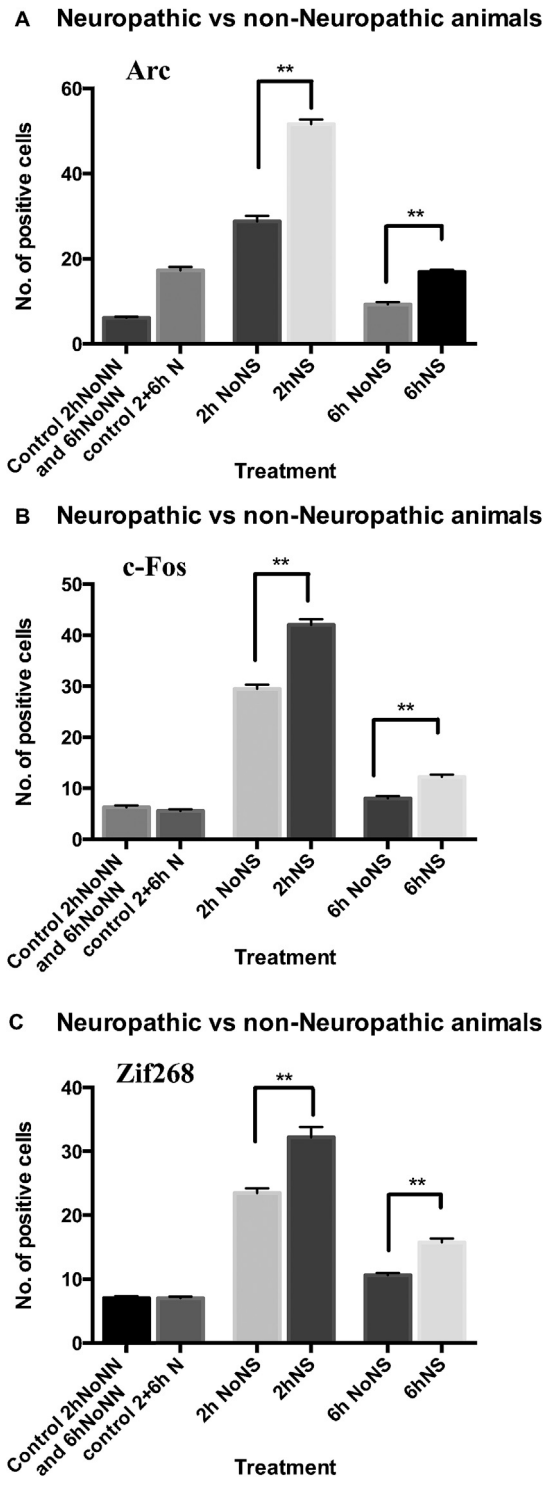

Fig. 2. (A–C) Number of IEGP positive cells in the dorsal spinal cord in stimulated and sham operated animals 2 and 6 h after conditioning stimulation. A (Arc), B (c-Fos), C (Zif268). For each time point, for each IEGP, the mean number of positive cells of the 6 sections with highest numbers of cells ($N = 3$ animals) was calculated, and the graphs show the means ± S.E.M. of these values. ('NoNS' – non-neuropathic stimulated animals; 'NS' – neuropathic stimulated; 'NoNN' – non-neuropathic non-stimulated; 'N' – neuropathic animals.) During statistical analyzes, the univariate general linear model was used and significance accepted at the 1% level (**$p < 0.01$). The control bars consist of one control bar for NoNN (2 h and 6 h post sham operation) and one for N animals.

The statistical results (Fig. 2A–C) were represented with bar graphs, where the height of graphs was calculated as mean values ± S.E.M. ($N = 18$ sections, the six sections with the highest numbers of positive cells per animal SC (three animals in each group)).

2.6. Inclusion criteria

We used modified criteria for inclusion of animals, previously defined by Smits et al. [20]. The criteria were modified so that animals with a pre-nerve lesion baseline of repeated von Frey withdrawal thresholds below 10 g should not be included. The withdrawal threshold exclusion point was decreased from 60 g to 10 g due to a significant percentage of animals that responded to von-Frey fibres calibrated to 10 g pressure. In addition, it was decided that animals without any decrease in von Frey withdrawal threshold below pre-ligation threshold should be defined as non-responders ($N = 0$) to the stimulation protocol and therefore should be excluded from the analysis. In our study, all animals were included as the inclusion criteria were met in all subjects.

3. Results

3.1. Response thresholds were markedly decreased after loose ligation of the sciatic nerve

As previously described, animals were divided into groups, consisting of 12 sham operated animals and 12 animals with sciatic loose ligation. Before sham operation the withdrawal thresholds (Fig. 1A) were 12.021 ± 0.391 g (mean ± standard error of mean, S.E.M.). At day 7 post sham operation, the mean withdrawal thresholds did not change significantly (t-test $p > 0.05$; $N = 12$; unpaired Student's t-tests) and stayed approximately the same, as expected. Hence, sham operated rats are referred to as non-neuropathic rats.

Prior to ligation withdrawal thresholds (Fig. 1B) were 13.47 ± 0.455 g (mean ± standard error of mean, S.E.M.). At day 7 post nerve ligation, the mean withdrawal thresholds decreased significantly (t-test $^{**}p < 0.0001$; $N = 12$; unpaired Student's t-tests) to 2.95 ± 0.306 g.

3.2. Neuropathy increases stimulus-induces expression of Arc, Zif268 and c-Fos in the dorsal horn

According to the allodynia classification proposed by Smits et al. [20], all animals in our study that were subjected to sciatic loose ligation showed moderate allodynia (withdrawal threshold <6.0 g) 7 days after surgery. Neuropathy significantly increased the number of IEGP positive cells at both 2 h and 6 h post CS. Both neuropathic and sham operated animals showed a higher number of positive cells 2 h after CS than after 6 h (Fig. 2A–C).

Statistical analysis (univariate general linear model) was performed comparing the 2 and 6 h post-stimulation groups with the control (sham-operated) groups. Stimulation vs. non-stimulation, neuropathy vs. non-neuropathy and time point were used as independent variables while the number of IEGP positive cells was used as the dependent variable (Fig. 2A–C).

Bennett's procedure led to a maximal IEGP expression 2 h after CS in neuropathic animals. The increase in the number of IEGP positive cells compared to non-neuropathic stimulated animals was 83.0% for Arc, 42.4% for c-Fos and 37.2% for Zif268 (Fig. 2A–C). After 6 h the increase compared to non-neuropathic animals was 80.4% for Arc, 52.2% for c-Fos and 48.3% for Zif268 (Fig. 2A–C).

The unstimulated side of the dorsal horn in stimulated animals did not show any significant change of the number of IEGP positive cells and was approximately at the same level as sham operated animals. The control (non-neuropathic and non-stimulated animals, no stimulation) values in non-neuropathic and neuropathic groups separately showed same immunoreactivity in 2 and 6 h post sham operation and therefore were considered with their data combined as one group (separately for non-neuropathic and neuropathic animals) (Fig. 3).

4. Discussion

In this study we have evaluated the difference of stimulus-induced immunoreactivity of Arc, c-Fos and Zif268 in the dorsal horn between neuropathic rats and sham-operated rats after sciatic nerve CS. We used a series of 100 Hz stimulation trains on primary afferent fibres of the sciatic nerve. Our focus of interest was the L3–L5 segment of lumbar spinal cord that probably receives the highest density of afferent nerve fibre input from the sciatic nerve [22,23] and shows the highest immunoreactivity of IEGPs as a response to CS. Sham-operated animals without CS were used as controls. We used the Bennett's sciatic nerve loose ligation model as the most commonly used model of neuropathic pain. However Bennett's nerve ligation involves a slight variability in the degree of damaged fibres leading to some variability in the number of responders and their behaviour [3]. Our SC sections showed a slight variability of positive cells between animals receiving same CS at same levels of the SC that could be explained by the previous fact mentioned.

The loose sciatic nerve ligation in Bennett's model of neuropathy features a 'co-mingling' of axons and Schwann cells that become denervated distal to the ligation. Damaged Schwann cells could influence intact axons with a variety of neuroactive cytokines [24] and factors as tumour necrosis factor alpha (TNFα) contributing to the pain state. Properly conducted Bennett's loose ligation results in neuropathic development that can be found the first day after ligation [25] and might last for many weeks after the procedure [26]. A recovery and decrease in neuropathy was reported 8–10 weeks after the ligation [27]. Since our experiments were conducted with a 7-day post operation time period, we cannot comment on the previous report. A variety of neurobiological mechanisms are responsible for the development of the neuropathic state. Some of the factors are collateral spreading of non-damaged fibres into denervated skin regions, increased excitability of damaged axons, leading to central sensitization. Loss of inhibitory mechanisms and inhibitory neurons in the spinal cord could also be responsible for neuropathy [3]. Electrophysiological studies in the spinal cord of neuropathic rats led to a finding that C-fibre evoked responses increased while the threshold for C-fibre activation decreased compared to naïve animals. Studies reported that more neurons were spontaneously active with an increased firing frequency in neuropathic rats. The previous implies that the spinal cord increased excitability in neuropathic rats, compensating for the decrease of peripheral input formed as a consequence of nerve ligation [28].

Development of peripheral neuropathy after loose sciatic nerve ligation is validated in a series of Von Frey fibre pain threshold tests. Similar to our experiments, Decosterd et al., reported a significant decrease of withdrawal threshold after ligation surgery and maintained a stable level afterwards [3,4]. Withdrawal thresholds to von Frey filament stimulation decreased significantly in our data 7 days after loose ligation, from 11.91 ± 0.746 g (mean ± S.E.M.; before Bennett's procedure) to 2.95 ± 0.306 g.

Similar to other studies mentioned later in the discussion, we found a difference in IEGP expression after CS between neuropathic and naïve rats. The stimulated SC side showed the significant increase in IEGP expression in both 2 h and 6 h post CS compared to controls. The control sides and sham operated control animals did not show significant IEGP expression change due to CS. Interestingly, Lee et al. [19] reported an elevation in IEGP expression

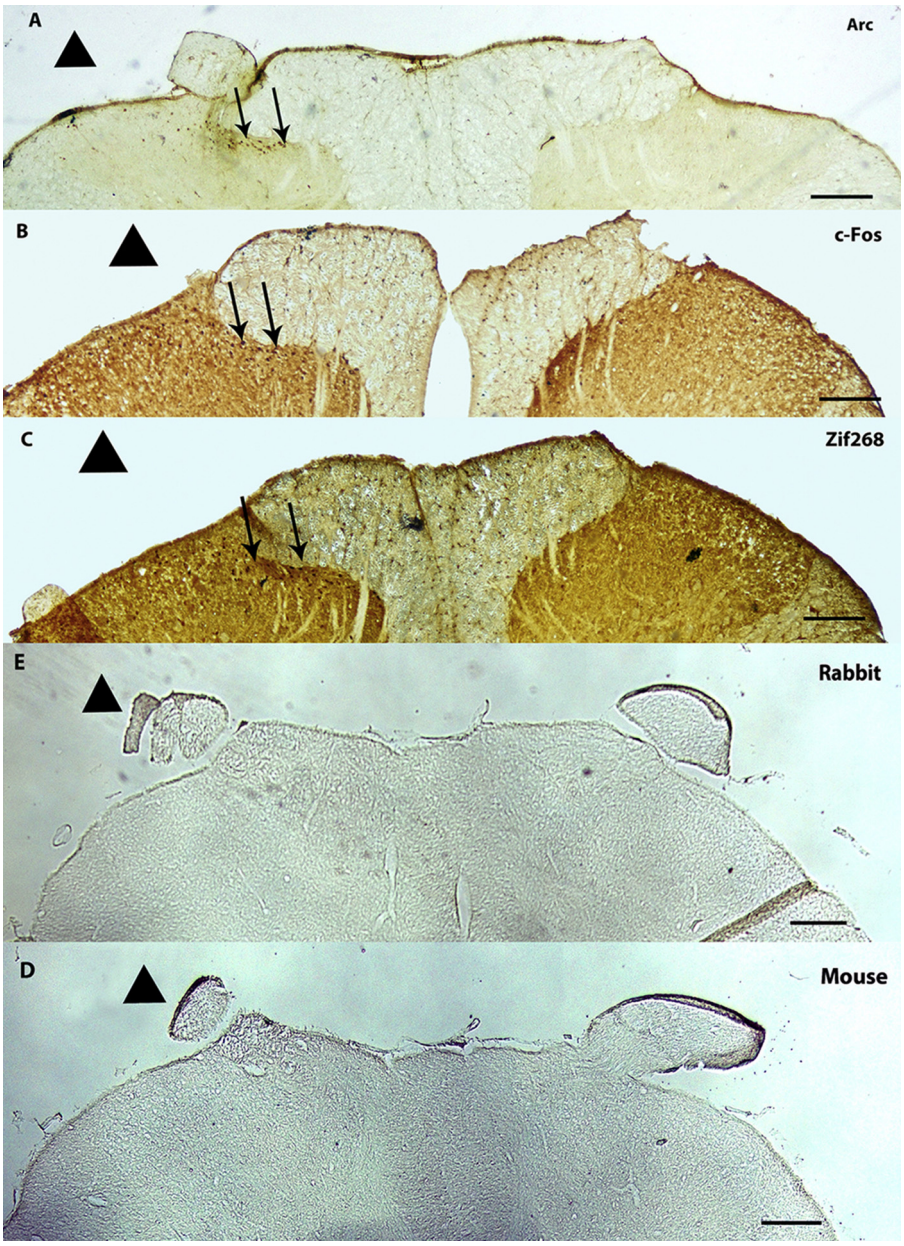

Fig. 3. (A–E) DAB staining photomicrographs of dorsal spinal cord 2 h post stimulation and photomicrograph of staining controls. Photomicrographs of a transverse sectioned SC, immunohistochemical DAB staining of cells expressing Arc (A), c-Fos (B) and Zif268 (C) in superficial SC dorsal horn laminar neurons (10×; 2 h post-CS, 7d morphine treated animals). Arrows indicate positive cells. The number of positive cells is greatest in dorsal horn ipsilateral to stimulation (marked with triangle). The contralateral side shows little or no expression of Arc (A), c-Fos (B) and Zif268 (C). Section D and E are controls of staining without the primary antibody with different secondary antibodies marked on pictures. Scale bars = 100 μm.

on the contralateral, unstimulated side of the SC. Our sections contained IEGP positive cells in lamina I and II of the lumbar spinal cord as previously shown in other studies [7]. However, in contrary to Lee et al. [19] and Williams et al. [29], we did not manage to confirm increased IEGP expression in deeper laminas of the SC. Previous studies reported elevation of the number of c-Fos positive cells was found predominantly in the lamina I and II of the dorsal horn [19,27] while Zif268 was reported to be increased in all the laminas I–IV [2]. Experimental work from Hossaini et al. from 2010 showed that Arc protein was elevated in neuropathic rats at 2 h post ligation [30]. Moreover, stimulation of afferent fibres was shown to lead to enhanced expression of c-Fos mRNA in spinal cord neurons and synaptic Arc protein [12,13].

Strikingly, our study showed an increase in the number of Arc positive cells in neuropathic, non-stimulated rats, observed 7 days after ligation. The expression of c-Fos and Zif268 was not altered significantly in neuropathic rats prior to CS. The influence of neuropathy was reported previously to induce increased immunoreactivity of IEGP positive cells in the dorsal horn [19,29] without CS. Moreover, Arc protein was defined as an important factor for consolidation of memory and it is necessary for the late phase LTP in the hippocampus [10,31]. Arc enables expansion of the cytoskeletal network, which underlies stable morphological changes in dendritic spines during in vivo LTP [10,32,33]. In the SC, Arc dependent long-term synaptic changes in spinal transmission were defined as a feature of anti-nociceptive neurons [30]. Hossaini et al. reported the presence of increased immunoreactivity in Arc protein predominantly located in lamina II of the spinal dorsal horn. He reported elevated Arc expression 2 h after nerve ligature. However, contrary to our results, Arc expression

was reported as diminished one and two weeks after the ligature in non-stimulated animals which simultaneously had developed thermal hyperalgesia and allodynia [30]. The elevation of Arc after loose sciatic nerve ligation can be a sign that Arc might have a role in neuropathic pain development. Studies have yet not determined the effects of selective Arc inhibition on neuropathic pain development. Others have found that Arc/Arg3.1 mRNA was only increased 2 h after ligation but not 1 week nor 2 weeks after the operation when the neuropathic pain symptoms, i.e. mechanical and thermal hyperalgesia and allodynia, had developed [30]. Moreover, in the spinal cord, the central projections of nociceptors synapse with second order neurons. These neurons may enter a state of elevated responsiveness [30] after nociceptive stimulation due a responsible mechanism similar to the mechanism of LTP development in the hippocampus [34]. Arc IEGP plays a role in the processes of LTP establishment, long term depression (LTD), homeostatic scaling of AMPA receptors [31], spinal processing [35] and may 'underlie chronic pain disorders' [30]. Moreover, immunohistochemical studies revealed that about 10% of Arc positive cells in the spinal cord expressed PKC-γ receptors. PKC-γ receptors have an important role in the formation of chronic pain [36].

C-fos is known as a neuronal activity marker [5] and found to be increased after spinal [37] and peripheral CS [29] in neuropathic rat models. Similarly, with immunohistochemical staining we found a significant increase of the number of c-Fos positive cells in neuropathic animals compared to non-neuropathic sham operated animals after CS. Here we reported a significant c-Fos expression increase 7 days after loose ligation in animal subjects 2 and 6 h after CS. However, other studies reported that higher expression of c-Fos could be found 3 weeks after the neuropathic intervention. Interestingly, c-Fos was reported to have a bi-phasic expression elevation in deep dorsal SC layers, 2 h and 24 h after loose ligation [38] and that contralateral expression can be found 8 h to 24 h after ligation. Williams et al. reported c-Fos expression elevation in superficial layers 2 h after ligation that was reported normalized 24 h after the ligation [29]. Further on, the expression of c-Fos was reported to be blocked in deep but not superficial layers after morphine and MK-801 (antagonist of NMDA receptors) block. Conditioning stimulation of afferent C-fibres results in the formation of fast synaptic potentials by activation of NMDA receptors, AMPA receptors and kainic acid (KA) [39]. Activation of NMDA and voltage gated Ca2+ channels results in Ca2+ influx activating many protein kinases and nitrous oxide (NO) activating the expression of IEG c-Fos [40]. FOS protein, the product of c-Fos gene activation, together with JUN (protein product of c-Jun gene) inhibits c-Fos transcription [41,42].

FOS and JUN are part of the AP1 (activation protein-1 transcription factor) heterodimer, that binds to a DNA site located in genes coding for preprodynorphin, among many other genes [43]. The product of predynorphin is dynorphin that as an endogenous opioid binds to kappa opioid receptors [44] located on neurons synapsing with local excitatory circuit neurons [45]. Furthermore, studies have shown that dynorphin injected intrathecally produces prolonged allodynia [46,47]. NMDA antagonists have the characteristic of preventing allodynia. It has therefore been concluded that dynorphin and its activity on NMDA receptors, represents one of the main factors of allodynia establishment [48]. Therefore, the previous suggests that dynorphin might be responsible for chronic allodynia and hyperalgesia development [49]. Since c-Fos was found to be expressed in the same areas of the superficial layers of spinal cord after CS as Zif268, it was suggested that those two IEGP together might have a role in 'converting extracellular events such as noxious stimulation into long-term intracellular changes' [50,51]. However, the detailed role of c-Fos in pain regulation has to be further investigated [52].

Zif268 (aka Egr1) is a zink finger transcription factor, a functional marker of neuroplasticity [53], that becomes expressed after noxious stimulation. Studies showed that LTP formation [54] and the development of persistent pain states [55] in the spinal cord as well and memory consolidation [56] investigated in the hippocampus are dependent on Zif268 IEGP expression. Further on, Zif268 has a function as a functional marker of neuroplasticity [53]. The formation of Zif268 can be decreased or stopped by the application of NMDA antagonists. Therefore it has been suggested that NMDA receptors might be responsible for the up-regulation of Zif268 after tissue injury [57]. To conclude, it has therefore been proposed that Zif268 'may contribute to signalling pathways involved in synaptic potentiation' [58].

Zif268 was shown to have an important role in LTP maintenance [59] and it is induced in neurons in response to conditioning stimulation [60]. Similarly to our findings, the results of Ruiz-Torner et al. [61] showed that CS induces an increase of immunoreactivity of Zif268, described as a characteristic of neuronal activation. This increase was found in the superficial laminae, defined as the location of primary terminal nerve endings of A-delta and C noxious fibres [61]. Strikingly, Vadakkan et al. [62] reported bilateral SC dorsal horn Zif268 expression and proposed supraspinal structures of the nervous system as possible mechanisms of this change. Opposite to Vadakkan et al., we found only ipsilateral Zif268 immunoreactivity increase similar to Williams et al. and Lee et al. [19,29]. Moreover, Ko et al. suggested that Zif268 plays a selective role in nociceptive behavioural responses to persistent inflammatory pain but not to acute noxious stimuli [58]. Our results showed a similar trend of Zif268 expression as for Arc and c-Fos. Contrary to the expression of Arc, Zif268 did not show elevated expression after neuropathic pain prior to CS.

All IEGPs showed a significant increase in expression 6 h post-CS. Our results showed a higher increase in expression (compared to controls) at 6 h than at 2 h post-CS while at the same time the expression levels were higher at the 2 h post-CS time point. Unfortunately, we cannot propose a reason for this specific expression pattern that followed all studied IEGPs. As previously mentioned for Arc and c-Fos, before studies include selective IEGP knock-out animals and determine the dependence of neuropathy on specific IEGPs we can argue for that the previous mentioned IEGPs have a potential role in neuropathic formation. However, the specific function has not been yet described.

To conclude, the results of this study showed that conditioning stimulation led to an increased expression of all studied IEGPs. Hence the fact that all studied IEGPs showed significant increase after induction of neuropathic pain, we might suggest a potential cooperative or individual role of Arc, c-Fos and Zif268 in the establishment and/or sustainability of neuropathic pain in rats.

The process of chronic pain development is a complex neurobiological phenomenon. This study adds to the knowledge of the mechanisms of neuropathic pain and improves the basis for further studies of neuropathic pain development and treatment. This study however only involves animal research and neuropathic pain as a consequence of sciatic nerve ligation. The findings of this experiment are similar to neurobiological alterations formed as a consequence of nerve compression during carpal tunnel syndrome or spinal root compression due to a vertebral disc prolapse but have clear limitations since it was conducted on animals. Future investigations should focus on revealing details of IEGP functions, potentially by selective knock out of specific IEGPs.

Conflict of interest

The authors have no affiliations with or involvement in any organization or entity with any financial interest (such as honoraria;

educational grants; participation in speakers' bureaus; membership, employment, consultancies, stock ownership, or other equity interest; and expert testimony or patent-licensing arrangements), or non-financial interest (such as personal or professional relationships, affiliations, knowledge or beliefs) in the subject matter or materials discussed in this manuscript.

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