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Chronic morphine treatment enhances sciatic nerve stimulation-induced immediate early gene expression in the rat dorsal horn

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Synaptic plasticity is a property of neurons that can be induced by conditioning electrical stimulation (CS) of afferent fibers in the spinal cord. This is a widely studied property of spinal cord and hippocampal neurons. CS has been shown to trigger enhanced expression of immediate early gene proteins (IEGPs), with peak increases observed 2 hour post stimulation. Chronic morphine treatment has been shown to promoteinduce opioid-induced hyperalgesia, and also to increase CS-induced central sensitization in the dorsal horn. As IEGP expression may contribute to development of chronic pain states, we aimed to determine whether chronic morphine treatment affects the expression of IEGPs following sciatic nerve CS.

Changes in expression of the IEGPs Arc, c-Fos or Zif268 were determined in cells of the lumbar dorsal horn of the spinal cord. Chronic Morphine pretreatment over 7 days led to a significant increase in the number of IEGP positive cells observed at both 2 h and 6 h after CS. The same pattern of immunoreactivity was obtained for all IEGPs, with peak increases occurring at 2 h post CS. In contrast, morphine treatment alone in sham operated animals had no effect on IEGP expression. We conclude that chronic morphine treatment enhances stimulus-induced expression of IEGPs in the lumbar dorsal horn. These data support the notion that morphine alters gene expression responses linked to nociceptive stimulation and plasticity.

Key words: spinal cord, immediate early gene proteins, arc, nociception, pain

INTRODUCTION

Opioids are widely used for the treatment of acute and chronic pain. However, concerns have arisen about the alteration of their efficiency during prolonged use (Haugan et al. 2008a).

Key factors limiting the prolonged use of opioids in pain treatment are opioid induced hyperalgesia (OIH) and tolerance (OIT). Clinical reports indicated that patients under chronic morphine treatment reported elevated pain perception compared with patients in control group when exposed to acute nociceptive stimulation (Haugan et al. 2008a). Pain facilitatory mechanisms in the central nervous system are known to contribute to OIH. The dorsal horn is the location of the synapse between the first and second neuron of pain pathways and therefore is considered as a very important potential target for alteration of nociceptive transmission by both segmental and supraspinal mechanisms. It is physiologically significant that descending facilitation of spinal nociception contributes to a great extent to central sensitization indicating that the balance shifts in favor of facilitation in the transition from acute to chronic pain (Heinricher et al. 2009).

Long-term potentiation (LTP) as a response to conditioning stimulation (CS) of afferent fibers is a common topic of scientific interest for studies of synaptic plasticity in the brain and the spinal cord (Haugan et al. 2008b). Immediate-early gene proteins (IEGPs), such as c-Fos (Harris 1998), Zif268 (aka Egr-1) (Sukhatme et al. 1988) and Arc (activity-regulated cytoskeletal-associated protein) (Haugan et al. 2008b) are previously shown to be induced in neurons in the dorsal horn in response to CS. In the brain, consolidation of LTP at glutamatergic synapses and formation of long-term memory both require activity-induced expression of Zif268 and Arc and other immediate early genes (Guzowski et al. 2000, Kaczmarek 2000,

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Jones et al. 2001, Plath et al. 2006, Messaoudi et al. 2007, Panja and Bramham 2014). Similarly, the induction of Zif268 was shown to be correlated to long term facilitation of neuronal responses in the spinal cord (Rygh et al. 2006). Previous studies from Haugan and others (Haugan et al. 2008a) reported that ketamine blocked morphine-induced enhancement of spinal LTP at 3 h post CS. However, the influence of long-term morphine treatment on the immunoreactivity of Arc, c-Fos and Zif268 at early and late time points after stimulation has not previously been studied.

Arc was first described in 1995 and its mRNA was firstly found to be 'rapidly expressed in principal neurons of rodent forebrain following seizures, learning experience and following induction of LTP by HFS'. After a nociceptive input to a peripheral nerve ending, secondary neurons receiving this input may come inenter a state that leads to an 'enhanced responsiveness to afferent inputs that may last several hours' (Hossaini et al. 2010). This described elevation of the responsiveness level has a similar underlying mechanism as during LTP establishment in the hippocampus (Kullmann and Lamsa 2007). With the fact of having an important role in activity-dependent synaptic plasticity, Arc is involved in the process of LTP, long term depression (LTD), and may 'underlie chronic pain disorders' (Hossaini et al. 2010). Arc was not found expressed non-stimulated spinal cord tissue contrary to stimulated tissue where it was in superficial dorsal horn laminas.

C-Fos was firstly described by Hunt and others as a IEGP rapidly expressed in dorsal horn of the spinal cord 2 hours after CS in L3 and L4 spinal segments (Hunt et al. 1987). C-Fos is one of the factors that have a function in triggering the synthesis of preprodynorphin. Dynorphin is the product of the predynorphin gene (Corvello et al. 1995). Intrathecally injected dynorphin was shown to produce analgesia followed by allodynia (Vanderah et al. 1996, Laughlin et al. 1997). It was has been suggested that, dynorphin might be one of the main responsive factors of allodynia and hyperalgesia development (Caudle and Mannes 2000).

It has been proposed that Zif268 together with c-Fos could have a role in 'converting extracellular events such as noxious stimulation into long-term intracellular changes' (Otahara et al. 2003). Zif268 (aka Egr1) is a zinc-k finger transcription factor that becomes expressed in the dorsal horn spinal cord after peripheral mechanical stimuli, and in states with neuropathic and inflammatory pain (Delander et al. 1997). Furthermore, Zif268 is became expressed in the hippocampus similarly as in the spinal cord after CS (Cole et al. 1989). Application of NMDA antagonists diminished the formation of Zif268 revealing that NMDA receptor activation s could be responsible for Zif268 synthesis after CS (Wang et al. 1994).

Here, we examined the effects of a series of 100 Hz stimulation trains of primary afferent fibers in the sciatic nerve on the immunoreactivity of Arc, c-Fos and Zif268 (Egr-1) in the lumbar spinal cord of rats after seven days of systemic treatment with morphine or saline. This study is motivated by the possibility that IEGPs contribute to increased and prolonged nociceptive facilitation after chronic opioid treatment.

METHODS

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 morphine and saline groups. All animals received continuous 7-day subcutaneous infusion with morphine or saline, respectively, with osmotic pumps implanted under brief Isoflurane anesthesia. The implanted osmotic pumps (Alzet mini-osmotic pumps model 2ML1) had a mean pumping rate of 10 µl/h.

The morphine and saline groups, both consisted of 12 animals, were each divided in two sets of 6 animals. Each set received conditioning stimulation and the other set was only sham operated. In each set, 3 animals were perfused 2 h after stimulation or sham operation, and 3 animals after 6 h. consisted of animals that had a time period of 2 hours before perfusion and the other set with animals 6 hours before perfusion. Both sets (the 2 and 6 hour set) consisted of 3 animals that were stimulated prior to perfusion and the other 3 animals that were only sham operated.

On the 7th day after osmotic pump implantation animals were anesthetized with 1.7–2.2 mg/kg urethane (250 mg/ml in sterile water) injected intra-peritoneal. Absence of pedal and corneal reflexes indicated appropriate level of anesthesia. After shaving of the surgery areas, the rats were transferred to a heating pad where

the animal body temperature was kept at 37±1ºC during the whole experimental procedure.

The left sciatic nerve was 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, the nerve was placed in a bipolar silver hook electrode (2 mm distance between hooks). The hooks were isolated from the surrounding tissue with elastic plastic film (Parafilm, American Can Company, USA). All animals were perfused, dependent of the group, two or six hours after CS or after the sham operation.

Sciatic nerve stimulation

All animals except the sham-operated groups received 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. This amplitude has previously been shown to be approximately four times the threshold for C-fiber evoked neuronal firing (Haugan et al. 2008b) in the dorsal horn. Stimulation with high frequency bursts of electrical pulses are shown to induce LTP in the dorsal horn of intact animals (Liu and Sandkuhler 1997, Svendsen et al. 1997). 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. In the sham-operated groups animals underwent surgery including mounting of the silver hook electrodes without actually delivering CS, with a two or six hour post-surgical idle time period prior to perfusion. After surgery with or without stimulation, the animals were transferred to and kept on heating blankets with feedback regulation of core temperature during the post stimulation period until the time of perfusion.

Immunohistochemistry

Animals with abolished reflexes were trans-cardially perfused with 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer, pH 7.4 kept at 4˚C.

A piece of the spinal cord consisting of the Th13–L5 segments was dissected out and the spinal cord was cut at the lumbar thickening between L2 and L3 segments. The caudal section consisted of the L3–L5 segments of the spinal cord. All caudal trunks were frozen in dry ice and kept at −80ºC. At this point the spinal cord preparations were code marked by a technician so that the next procedures continued without knowledge of the time post CS or the pretreatment of animal groups.

SC sections, 20 µm thick sections (SC length of 3.2 mm), 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 4ºC and the next day incubated with biotinylated secondary antibody in PBST for 2 h at room temperature. Sections were then incubated in streptavidin-HRP diluted in PBST for 1 h, and 3,3'-Diaminobenzidine (DAB) stained for 8 min approximately at room temperature. The slides were thereafter stained for 5 min with 0.1% Cresyl Violet (pre heated to 50ºC). The slides were then washed 3 times with Milli-Q water and subsequently immersed 3 minutes 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 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. #PC38T) and rabbit anti-Zif268 (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).

For all three IEGPs the analyzed sections were distributed evenly over the entire area of interest explained previously. For all IEGPs cells were identified as positive if they showed staining of the nucleus with or without staining of the cytoplasm or cellular extensions.

All neurons were manually counted in digital pictures and during counting the neurons were labeled to avoid double-counting. We performed negative controls for all three antibodies that led to elimination of all specific staining (Figs 1A–1E).

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.

Statistics

Statistics and graphical presentations were done with the Graphpad Prism 6.0 statistical package. Statistical significance was accepted at the 5% level.

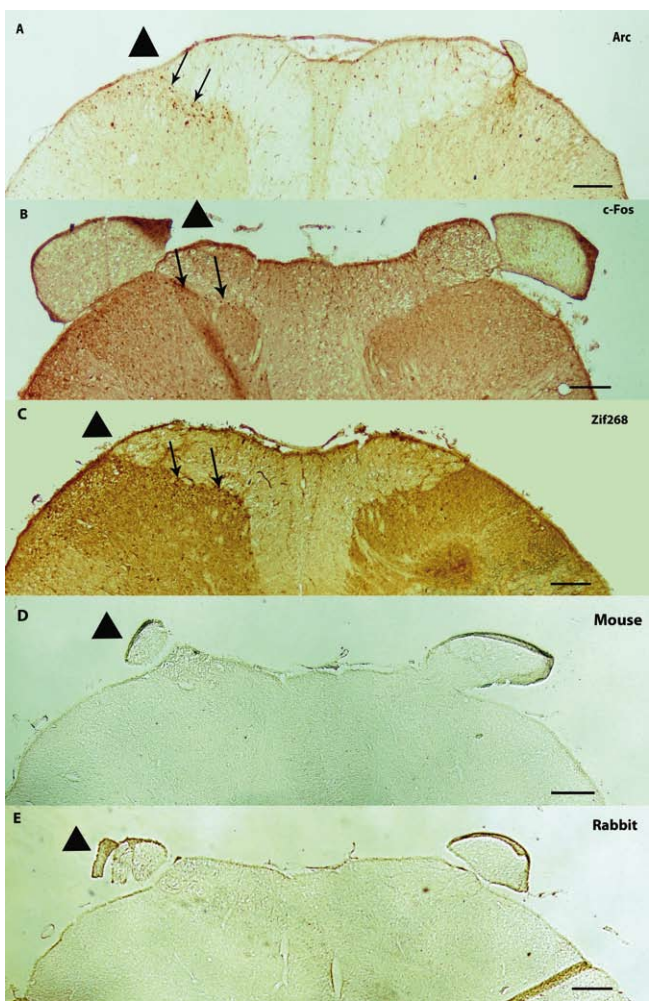

Fig. 1. Immunochistochemical DAB staining of cells expressing Arc (A), c-Fos (B) and Zif268 (C) in superficial SC dorsal horn laminar neurons. Arrows indicate positive cells (10×; 2 h post-CS, 7 days morphine treated animals). Triangle indicates stimulated side of the SC. Left, stimulated side, shows a higher amount of positive cells expressing 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.

For statistical evaluation of cell numbers, the values for each animal were calculated as the average number of cells per section for the six sections with highest number of cells counted.

The comparisons for all IEGPs, between the 2 and 6 h post-stimulation groups and the control (sham-operated) groups were done by means of two-way analysis of variance (ANOVA). During ANOVA, stimulation/non-stimulation, morphine/non-morphine and time point were used as independent variables while the number of cells was used as the dependent variable (Figs 2A–2E).

The bar graphs show mean values ±S.E.M. [N=18 sections, six sections with the highest numbers of positive cells in each spinal cord studied (N=3 per group)]. During ANOVA at each time point, stimulation and morphine pretreatment were used as independent variables while number of cells was used as the dependent variable. If a significant stimulation effect was found, that set of animals was compared with the sham group with *post hoc* Sidac's multiple comparison test. Since the morphine and saline sham operated (unstimulated) groups didn't show statistically different numbers of positive cells for both time points, as a control number of positive cells we used the average of six maximal numbers of positive cells from the 2 h morphine treated sham operated group, separately for each IEGP.

RESULTS

One week of morphine pretreatment increased the number of positive cells for all three IEGPs at both 2 h and 6 h post CS. The maximal IEGP's expression occurred 2 h after CS both with saline and with morphine pretreatment (Figs 1, 2A–2C).

There was statistically significant effects both of stimulation and of morphine treatment both 2 h and 6 h after CS (all effects: $p<0.0001$, two-way ANOVA, Sidac's *post hoc* test; on morphine and saline treated 2 and 6 h post-CS samples; 2 h group and 6 h group *vs.* controls; number of cells as dependent, treatment and stimulation as independent variables). For comparison within between groups we used the Sidac's *post hoc* test subsequent to two-way ANOVAnova. Statistical analysis showed highly significant differences ($p<0.0001$) between the saline and morphine treated groups and between the stimulated and non-stimulated groups except for the saline treated 6 h groups, where

there as no significant difference in Zif268 expression between stimulated and unstimulated animals.

Dorsal horn stimulation did not induce any significant change of the number of IEGP positive cells in the unstimulated side of the dorsal horn, which was approximately at the same level as sham operated (unstimulated) animals. The relative stimulus-induced increase of IEGP positive cells (Figs 3A, 3B) after morphine treatment compared to saline-treated rats [number of cells in the six sections with the highest number of positive cells from each morphine-treated animal (3 animals, 18 sections in each group) with means ± S.E.M] is shown in Figs 3A, 3B.

However, in stimulated animals 6 h post CS morphine caused a 70% increase of Arc, 42% increase of c-Fos and 80% increase of Zif268 positive cells compared to saline treatment. In the 2 h post CS group the morphine treatment led to a smaller relative increase, 34% of Arc, 33% of c-Fos and 64% of Zif268 positive cells compared to the saline treated group (Figs 3A, 3B). Morphine pretreatment gave had no effect on the number of IEGP positive cells without stimulation.

DISCUSSION

These results show that chronic morphine treatment by itself does not change the IEGP immunoreactivity in the dorsal horn SC after CS, but morphine treatment led to an increase in stimulus-induced expression, shown as the numbers of Arc, c-Fos and Zif268 positive cells at both (2 h, 6 h) time points.

To elicit IEGP expression we have used a CS that has been used before in our laboratory and has been shown to induce robust potentiation of stimulation-evoked single cell responses in the dorsal horn (Svendsen et al. 1997, Haugan et al. 2008b). The maximal number of positive cells was found 2 h post CS in both untreated and morphine treated rats, while the maximal relative increase in the number of positive cells in morphine-treated rats compared to the saline-treated group was 6 h post CS. Our results confirm previous showing peak expression of c-Fos occurred 2 h post CS. The morphine pre-treatment led to a 33–65% increase in IEGP positive cells at 2 h post CS and a 42–80% increase at 6 h post CS and S. The highest relative increase was found after 2 h post CS in Zif268 positive cells followed by Arc and c-Fos at the same time points. Research of Sukhatme and others (Sukhatme et al. 1988) observed the highest

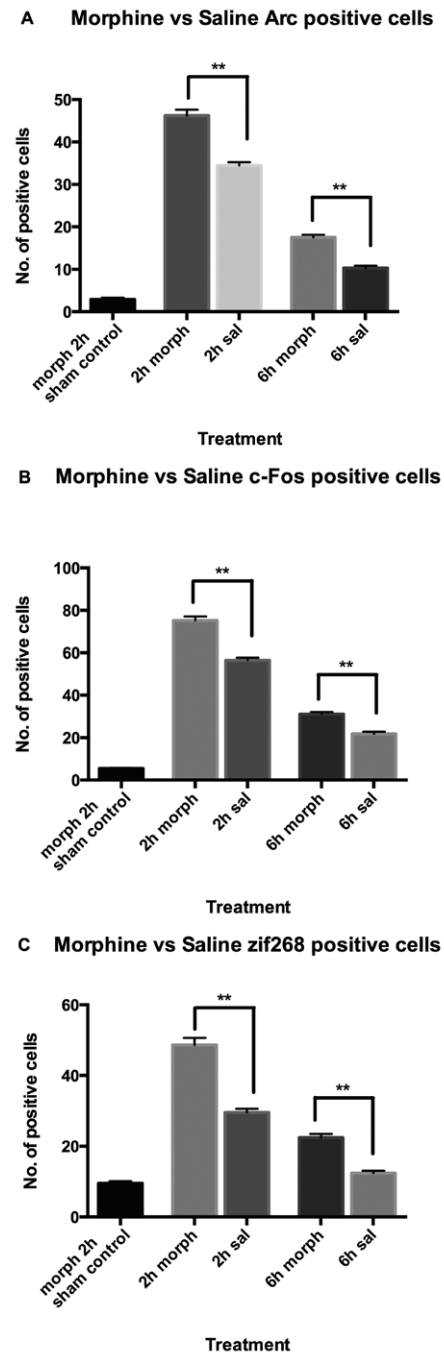

Fig. 2. Effects of saline/ morphine treatment on the number of Arc, Zif268 and c-Fos positive cells 2 and 6 h after CS. For each time point, for each IEGP the mean of number of positive cells of the 6 sections with highest numbers of cells (N=3 animals) was calculated, and the graphs show the means ±S.E.M. of these values. The morph 2 h sham control bar represents the number of positive cells in morphine treated animals 2 h post-sham operation (N=3), calculated separately for Arc, Zif268 and c-Fos, ** $p < 0.01$, two-way ANOVA with Tukey's *post hoc* tests.

levels of Zif268 mRNA elevation at, 60 min after stimulation. The time difference between highest mRNA expression and maximal protein expression could befits with the time needed for actual protein synthesis. According to our findings, the maximal number of Zif268 IEGP positive cells was found 2 h post CS with a decreasing trend towards the 6 h point. Our results correspond to previous studies. (Haugan et al. 2008b, Hossaini et al. 2010) in which the number of Arc positive cells at the maximal point of expression was lower than the number of Zif268. The SC level with maximal number of positive cells corresponded to the results of our previous study (Bojovic et al. 2015) and was found at the border between SC segments L3 and L4.

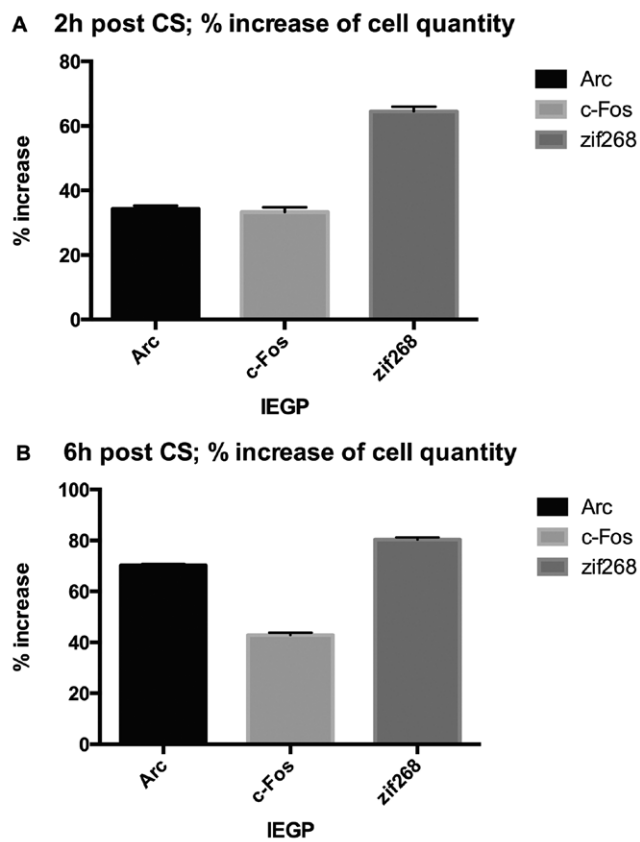

Fig. 3. Percentage of Arc, c-Fos and Zif268 positive cell increase due to morphine effects 2 and 6 h after CS. Percent increase of IEGP positive cells after morphine treatment compared to saline-treated rats (number of cells in the six sections with the highest number of positive cells from each morphine-treated animal (3 animals, 18 sections in each group). Means ±S.E.M. Every value from morphine treated rats was compared with the value of same IEGP from saline treated animals.

Fos-like immunoreactivity has been found to be elevated after CS in chronic opioid treated animals compared to the controls (Robbins et al. 2012). Although c-Fos protein and Fos-like immunoreactivity is widely used as a marker for neuronal activity in the spinal cord, the exact function of the individual studied IEGPs in opioid enhancement of pain is not known.

By reviewing the results of this study we suggest that there is a relationship between IEGP expression and the mechanisms of opioid-induced changes in nociception.

Although Arc is induced by CS stimulation that triggers LTP in the spinal cord, it is not yet known whether Arc expression is causally involved in potentiation of CS-evoked firing or central sensitization. In the brain, Arc is indispensable for major forms of protein synthesis-dependent synaptic plasticity including LTP, LTD, and homeostatic scaling (Bramham et al. 2010). Arc expression after CS could be involved in any of these plasticity processes. Recent work suggests that Arc may function in both LTP and LTD in the same cell, but in different subcellular compartments and with different time courses insert refs with your system (Okuno et al. 2012, Korb et al. 2013, Panja et al. 2014). Biochemicaly, Arc is flexible modulator protein capable of reversible self-oligomerization and interaction with a number of binding partners (Myrum et al. 2015). Arc function in dentate gyrus LTP is linked to cytoskeletal regulation in dendritic spines (Fukazawa et al. 2003, Matsuzaki et al. 2004, Messaoudi et al. 2007). Arc function in LTD is linked to endocytosis of AMPA glutamate receptors (Shepherd et al. 2006 Neuron), while scaling involves transport of Arc to the nucleus and decreased transcription of the GluA1 subunit of the AMPA receptor (Korb et al. 2013). It has been Zif268, zinc–finger transcription factor, has been shown to have an important role in LTP maintenance (Jones et al. 2001) and to be induced in neurons as a response to CS (Knapska and Kaczmarek 2004). C-Fos is classified as a proto-oncogene and used as a marker of nociceptive neuronal activation (Munglani and Hunt 1995). It is as well used as a neural marker of pain and detected in response to stimulation (Harris 1998). In accordance with our results, long-lasting morphine treatment did not alter the baseline c-Fos expression compared to controls (Rohde et al. 1996) (in our case saline treated animals) but led to a marked increase of c-Fos expression after nociceptive sensitization, supporting results from earlier studies (Rohde et al. 1997,

Robbins et al. 2012). This could be interpreted as that the neurons were in a state of latent hyper-excitability (Fry et al. 1980). The objective of Rohde et al was to determine if a formaline subcutaneous injection as a noxious stimulus can show a difference in expression of c-Fos proteins between morphine pellet pre-treated rats and control rats. The authors recorded a significantly increased Fos-like immunoreactivity at L4/L5 segments compared to the control rats. However the results of Rohde et al found showed an increased in Fos-like immunoreactivity in both ipsilateral and contralateral sides of the spinal cord, whereas. This finding differs from our study where we were able to find only ipsilateral increases in Fos immunoreactivity were observed in the present study (Rohde et al. 1997). The obje. ctive of Robbins et al (Robbins et al. 2012) sought to was to determine the differences of Fos immunoreactivity throughout the medullary dorsal horn between conditionally stimulated chronic morphine-treated animals and non-stimulated chronic morphine treated controls. The authors used thermal corneal stimulation as the conditional stimulation and similarly to our results found a significant increase in the number of Fos-positive neurons in the superficial laminae.

CONCLUSION

These results indicate that chronic systemic treatment with morphine leads to strong amplification of the stimulus-induced Arc, c-Fos and Zif268 expression in the dorsal horn spinal cord compared to opioid naive animals. CS was has previously been shown to increase IEGP expression in the dorsal horn spinal cord (Bojovic et al. 2015). Studies of Haugan and others (Haugan et al. 2008a) showed that chronic morphine pre-treatment facilitates the induction of LTP after CS compared to non-morphine treated animals. Our current study revealed that morphine pre-treatment enhances IEPG expression in the dorsal horn spinal cord after CS. These findings support the strengthen our hypothesis that the researched studied IEGP expression contributes to 's might be involved in mechanisms of central facilitation.

Taken together, Takingen the previous in consideration, we can strongly argue for that CS in morphine pre-treated subjects might lead to a higher degree of central facilitation. The implication for clinical care is that general long-term morphine treatment may promote hyperalgesia though enhancement of nociception-induced synaptic plasticity. In terms of the pathogenesis of chronic pain, it will be important to establish causal roles for This fact suggests that general long-term morphine treatment should be thoroughly considered since it may lead to enhancementa higher level of nociception-induced synaptic plasticity. However currently there is no direct evidence present that Arc, c-Fos and or Zif268 in are directly involved in the process of central facilitation in such a way that it may be the foundation for treatment to prevent the chronification of pain and the mechanisms underlying this enhancement of IEGP expression needs to be further explored.

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