Pain perception in neurodevelopmental animal models of schizophrenia

Miloslav Franek[1], Simon Vaculin[1], Anna Yamamotova[1], František Šťastný[2], Věra Bubeníková-Valešová[2], Richard Rokyta[1]

[1]Charles University in Prague, Third Faculty of Medicine, Department of Normal, Pathological and Clinical Physiology, Prague, Czech Republic

[2]Prague Psychiatric Center affiliated with the Charles University in Prague, Prague, Czech Republic

Corresponding author:

Miloslav Franek

Charles University in Prague, Third Medical Faculty, Department of Normal, Pathological and Clinical Physiology

Ke Karlovu 4

12000 Prague 2

Czech Republic

tel.: +420 224923905

fax: +420 224916896

E-mail address: franek@lf3.cuni.cz

Short title: Pain in animal models of schizophrenia

Summary

Animal models are important for the investigation of mechanisms and therapeutic approaches in various human diseases, including schizophrenia. Recently, two neurodevelopmental rat models of this psychosis caused by subunit selective *N*-methyl-D-aspartate receptor agonists - quinolinic acid (QUIN) and by *N*-acetyl-aspartyl-glutamate (NAAG) were developed. The aim of this study was to evaluate pain perception in these models. QUIN or NAAG was infused into lateral cerebral ventricles neonatally. In the adulthood, the pain perception was examined. The rats with neonatal brain lesions did not show any significant differences in acute mechanical nociception and in formalin test compared to controls. However, the neonatally lesioned rats exhibited significantly higher pain thresholds in thermal nociception. Increased levels of mechanical hyperalgesia, accompanying the sciatic nerve constriction (neuropathic pain), were also observed in lesioned rats. Although hyperalgesia was more pronounced in QUIN-treated animals, the number of c-Fos-immunoreactive neurons of the lumbar spinal cord was similar in experimental and control rats. We conclude that neonatal brain lesions attenuated the thermal perception in both nociceptive and neuropathic pain whereas mechanical pain was increased in neuropathic pain only. Thus, nociceptive and neuropathic pain belongs - in addition to behavioral changes – among the parameters which are affected in described animal models of schizophrenia.

Keywords: quinolinic acid, *N*-acetyl-aspartyl-glutamate, neuropathic pain, rat

Introduction

Schizophrenia is a neurodevelopmental disorder which afflicts about 1% of the human population worldwide. The etiology and pathogenesis of this psychosis is complex but involves the interplay of polygenic influences and environmental risk factors operating on brain maturation processes. Different theories as to the cause of schizophrenia and the heterogeneity of clinical symptoms have made it difficult to develop a valid animal model. A number of limited animal models have been developed to explore various theories of etiology, progression and treatment of this psychosis (Marcotte et al. 2001). Neonatal excitotoxic disconnection of the fronto-temporo-hippocampal complex may represent a heuristic model of schizophrenia-like behaviour (Bubenikova-Valesova et al. 2006; Lipska 2004; Stastny et al. 2005). Two of these models have been based on the neonatal intracerebroventricular (i.c.v.) infusion of substances, which activate *N*-methyl-D-aspartate receptor (NMDA-R), when overproduced by activated microglia: quinolinic acid (QUIN) (Heyes et al. 1996; Stastny et al. 2005) and *N*-acetyl-aspartyl-glutamate (NAAG) (Bubenikova-Valesova et al. 2006; Passani et al. 1998). Similarities in behavioral changes and changes in social interactions were observed between these models and a genetic model of reduced NMDA-R function (Duncan et al. 2004).

It is well known that QUIN and NAAG have also antinociceptive effects on the pain experience (Heyliger et al. 1998; Yamamoto et al. 2004). The effect might be related to a hypofunction of NMDA-R in rat lumbar spinal cord (Boyce et al. 1999). Also individuals with schizophrenia (and their relatives (Hooley and Delgado 2001)) are significantly less sensitive to direct painful stimuli (Dworkin 1994; Fishbain 1982; Kudoh et al. 2000; Rosenthal et al. 1990), but their subjective evaluation of the stimulus is similar to that of healthy control subjects (Blumensohn et al. 2002). It may reflect a dysfunction of glutamatergic system in these patients, which affects the transmission of the noxious stimuli.

To solve the problem we can use animal models even though there are difficulties to

evaluate the typical psychotic symptoms (hallucinations, lack of motivation, social withdrawal, attention deficits) in animals. However, it is important to know the nociceptive profile of these models can help to interpret the clinical results obtained in nociceptive tests owing to a validity of the neurodevelopmental animal models of schizophrenia. The changes of pain perception in animal models are not homogenous and depend on used model (Al Amin et al. 2004; Gao et al. 2004; Schneider and Przewlocki 2005). The purpose of our study was to describe nociceptive profiles of adult animals (which had been neonatally treated with icv QUIN or NAAG) with neonatal brain lesions induced by QUIN or NAAG.

Methods

Animals

We have been using Wistar:Hann rats (SPF, BioTest, Konarovice Czech Republic) throughout. The litters were rearranged so that each contained eight males and two females. Mothers with their pups were housed in cages having free access to food and water. Animals were kept at 22 ± 2 °C (relative humidity was 40-70%) under 12-h light/12-h dark cycle. All experiments were approved by the Committee for Animal Care and Use of the 3rd Faculty of Medicine (Charles University, Prague) and conducted in accordance with the ethical guidelines of the International Association for the Study of Pain (Zimmermann 1983).

Neonatal brain lesions

Wistar male pups on postnatal day 12 (PND 12) were anesthetized by vaporized ethylether, fixed in a stereotaxic apparatus (Stoelting, Wood Dale, IL) using ear and tooth bars. Quinolinic acid (QUIN

group) or *N*-acetyl-aspartyl-glutamate (NAAG group) was slowly infused i.c.v. in a dose of 250 nmol/0.25 μL buffered isotonic saline/ventricle; one injection was given into each lateral cerebral ventricle by a 1 mL Hamilton syringe equipped with 26S needle with coordinates previously used (Bubenikova-Valesova et al. 2006; Lisy et al. 1994; Stastny et al. 2005). Sham-operated control rats (SHAM group) were i.c.v. injected with 0.25 mL of saline. Selection of sham-operated or lesioned animals was made randomly (from each litter 3 pups were treated with neuroactive substance, 3 pups were treated with saline and 2 male pups remained as naïve). The pups were weaned on the PND 28 (the day of birth was denoted as PND 0). According to the type of brain lesion three groups of animals were used in this study: QUIN, NAAG and corresponding SHAM groups (n = 4-8 animals per group in each pain test).

Neuropathic pain model

Persistent neuropathic pain in rats was evoked by chronic constriction of sciatic nerve (CCI) according to the model of Bennett and Xie (Bennett and Xie 1988). For this test, young adult animals with neonatal brain damage (or sham-operated animals) were used on PND 50-55. Rats were anesthetized with pentobarbital (40 mg /kg b.w., i.p.). Then the right sciatic nerve was exposed and, proximally to the trifurcation of the nerve, 5 mm of the sciatic nerve was freed from the adhering connective tissue. Around the loosened nerve four 4-0 chromic catgut sutures were loosely tied at intervals of approximately 1 mm. Finally, the incisioned muscles and skin were sutured in layers.

Acute nociceptive tests

Plantar test and von Frey test were performed 2-5 days before the CCI and then 2-3 weeks after this operation. Formalin test was performed one week before the CCI. Each animal was used in only one nociceptive test; they were not repeatedly exposed to pain.

Plantar test

Pain threshold for the thermal stimulation was determined using plantar test equipment (Ugo Basile, Comerio, Italy) as described previously (Prochazkova et al. 2009). Shortly, in this test the latency (in seconds) of hind limbs withdrawal to the noxious thermal stimulation was measured. Each animal was placed individually in a clear plastic box with a clear glass floor and was allowed to acclimatize for 10 min. Cut-off value was set to 22 s to prevent limb injury. The testing box was cleaned between each animal.

von Frey filaments

For the testing of mechanical allodynia and hyperalgesia von Frey calibrated nylon monofilaments (Touch-Test Sensory Evaluator, North Coast Medical Inc., Morgan Hill, USA) of different thicknesses were used as described elsewhere (Howard et al. 2005). With these filaments the plantar surface of hind limbs was exposed to varying degrees of pressure. The stimulus was applied five times at 2 s intervals for each successive filament until five clear withdrawal responses were elicited (100% response).

Formalin test

For chemical nociception, formalin test (injection of 50 ml of 2.5% formalin into the plantar surface of right hindpaw) was used. Scoring of nociceptive behavior began immediately after the formalin injection and continued for 1 hour. Nociceptive scores were determined for each five-minute time blocks using following behavioral categories: 0 – the injected paw is not favored; 1 – the injected paw has little or no weight placed on it; 2 – the injected paw is elevated and is not in contact with the surface; 3 – the injected paw is licked, bitten or shaken.

Visualization of c-Fos positive neurons

Two or three weeks after the CCI animals were deeply anesthetized with ketamine and perfused transcardially with saline followed by 4% formaldehyde. Lumbar laminectomy was performed and the L_{2-5} segments of the spinal cord were removed. Spinal cords were further fixed in 4% formaldehyde in 0.1 M phosphate-buffered saline (PBS; pH 7.4) for 2-3 days, and then soaked in 20% sucrose/PBS for cryoprotection. Lumbar spinal cords were cut into 50 mm transversal section with a freezing microtome and treated as free-floating sections in cold PBS. The immunohistochemical protocol was similar to that given elsewhere (Yamazaki et al. 2001). Briefly, after several rinses in PBS, sections were treated with 0.3% H_2O_2, rinsed in PBS and treated with normal blocking serum. Then the sections were incubated with a polyclonal c-Fos antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, USA; diluted 1:4000) overnight. Sections were then rinsed with PBS and incubated with biotinylated secondary antibody and subsequently with the ABC complex (Vectastain Elite Kit, Vector). Neurons were visualized by incubation with diaminobenzidine and intensified with nickel solution (DAB Kit, Vector).

Statistical analysis

Statistical analysis was carried out by Origin 6 (Microcal Software, USA) and by Statistica 6.0 (StatSoft Inc., USA). Data are expressed as mean ± S.E.M. Statistical differences were evaluated using paired Student´s t-test for comparison of ipsilateral versus contralateral sides of the same experimental group. For comparison of the ipsilateral versus contralateral sides between groups one-way ANOVA test followed by post-hoc Fisher's test were used. Differences between means were considered statistically significant if $p < 0.05$. For the analysis of von Frey test the stimulus-response curves were constructed. The curves were fitted according to the equation $y=(y_{max} - y_{min})/$

$(1+(x/x_0)^n)+y_{min}$. The value x_0 corresponds to the force eliciting 50% of the maximum response and was defined as threshold value. The thresholds were used in subsequent comparisons within tested groups.

Results

Pain threshold for thermal stimulation

Thresholds of plantar test in animals without neuropathic pain were significantly different in both schizophrenia model groups (QUIN and NAAG) compared to control group (SHAM). As shown on Figure 1, the thermal pain threshold in SHAM group (6.7 ± 0.7 s) was significantly lower compared to groups neonatally treated with QUIN (8.3 ± 0.3 s) and/or NAAG (8.2 ± 0.3 s; $p<0.05$). It means that the sensitivity to the thermal stimulation was attenuated by neonatal brain damage. Latencies were measured on right and left hindlimbs, but no lateralization of pain threshold was observed in any of the tested groups.

Pain threshold for mechanical stimulation

There were no differences between QUIN-, NAAG- and SHAM-operated groups using von Frey filaments (data not shown). On whole scale of filaments (1 g – 180 g) we did not find any changes in the threshold for mechanical stimulation. It means that the neonatal brain lesion affected only the thermal hyperalgesia, but not the mechanical hyperalgesia (allodynia).

Formalin test

In early phase of the formalin test the acute chemical irritable pain was evaluated. Initially, we observed similar onset of the pain behavior in all three groups tested. This phase was shorter in

QUIN-treated group compared to NAAG-treated group or controls (Figure 2). The date obtained in the late phase of formalin test (inflammatory pain) showed the more accented pain perception in animals with neonatal brain lesions compared to controls (Figure 2). However, the differences did not reach statistical significance.

Neuropathic pain

Chronic constriction of the sciatic nerve was used as the model of peripheral neuropathic pain. The operation was performed on adult animals (PND 50-55). Two weeks after the operation the tests for mechanical and thermal allodynia/hyperalgesia were performed.

Thermal nociception under the neuropathic pain

As shown in Figure 3, the SHAM-operated group exhibited a significant decrease in the pain threshold on the ligated hind limb ($p < 0.05$) comparing threshold latencies of pain perception before (6.9 ± 1.0 s) and after the ligation (3.7 ± 1.2 s). This difference is typical for the peripheral neuropathic pain and was not observed either in QUIN-treated (before ligation 8.4 ± 0.3 s, after ligation 8.0 ± 1.3 s) and/or NAAG-treated animals (before ligation 8.2 ± 0.4 s, after ligation 6.8 ± 0.8 s).

Mechanical nociception in neuropathic pain

When von Frey filaments were used the changes in mechanical allodynia/hyperalgesia were observed in all three tested groups with CCI. In QUIN-treated group the threshold value (log of 50% of maximum response) was significantly lower in ligated hind limb (0.76 ± 0.22 log g) comparing to contralateral side (1.53 ± 0.16 log g; Figure 4A). Similar results were obtained in NAAG-treated (Figure 4B; 0.99 ± 0,24 log g and 1.65 ± 0.12 log g) on ipsilateral and contralateral

side, respectively) and in SHAM-operated animals (Figure 4C; 1.41 ± 0.11 log g and 1.94 ± log g on ipsilateral and contralateral side, respectively). In both neonatally lesioned groups the perception of mechanical stimulation was higher in comparison with SHAM-operated animals (on both ipsilateral and contralateral sides) but only in QUIN-treated group this effect reached statistical significance (Figure 4D; $p < 0.05$).

c-Fos protein expression in neuropathic pain

After nociceptive tests the rats with CCI were transcardially perfused and lumbar spinal segments were removed. Then c-Fos immunoreactive (IR) neurons in the spinal dorsal horn were visualized and their total number in this area was counted. The total number of c-Fos IR neurons was significantly higher on ligated side comparing to the contralateral side (Figure 5). This effect was observed in QUIN-treated (39.3 ± 3.5 and 23.0 ± 4.5 in ipsilateral and contralateral dorsal horn, respectively; $p < 0.05$), NAAG-treated (38.7 ± 3.6 and 27.3 ± 1.0 in ipsilateral and contralateral dorsal horn, respectively; $p < 0.05$) and SHAM-operated animals (30.3 ± 2.3 and 17.4 ± 2.4 in ipsilateral and contralateral dorsal horn, respectively; $p < 0.05$). The data suggest that there were no significant differences in the number of c-Fos IR neurons in animals with brain lesions in comparison with control group.

Discussion

In the present study we compared pain perception of two recently described neurodevelopmental models of schizophrenia based on the neurotoxicity of intracranially increased levels of endogenous NMDA-R agonists, QUIN and/or NAAG, in neonatal rat pups on PND 12. The weak agonists (Shave et al. 2001; Stastny et al. 2005), with selective affinity to heteromers containing NR2B (QUIN) or NR2D subunits (NAAG), evoked nerve cell damage in periventricular rat brain region

with a maximum in the hippocampus (Bubenikova-Valesova et al. 2006; Kudoh et al. 2000).

In the first model quinolinic acid, an endogenous metabolite of tryptophan with a partial selectivity for NR2B subunit containing NMDA-R (de Carvalho et al. 1996), was used. Although QUIN itself produced prolonged decrease in pain sensitivity in tail flick test (Heyliger et al. 1998), the pain-related changes observed in this study can be related to the decreased expression of NR2B protein in hippocampi of rats with the neonatal brain lesion (Skuba et al. 2004). The loss of the subunit protein seemed to be comparable to the action of subunit selective NMDA-R antagonists in alleviating chronic pain (Boyce et al. 1999; Chizh et al. 2001). In contrast to our results, adult rats with neonatal ventral hippocampal lesion exhibited decreased latencies for the thermal and mechanical nociception (Al Amin et al. 2004). Possible explanation of the difference can be in the contribution of the anterior cingulate cortex and amygdala in pain perception in adult rats (Gao et al. 2004). We assume that these brain regions can be damaged by QUIN infused intraventricularly, but not by ibotenic acid infused into the ventral hippocampus (Lipska et al. 1992).

In the case of the second model of schizophrenia, NAAG, an agonist at group II metabotropic glutamate receptors (mGluRs) (Neale et al. 2005), was infused to neonatal rat brains in the equimolar dose as in the case of QUIN. NAAG is one of the most abundant neurotransmiter in rat brain and is up to date the only known endogenous ligand of mGluR3. Its precise physiological function remains unknown. Previous reports have demonstrated that NAAG interacts not only at postsynaptic mGluRs, preferring subtype 3 to subtype 2 (Wroblewska et al. 1997), but at higher concentrations, also at NMDA-R heteromers (Losi et al. 2004; Shave et al. 2001), containing NR2D subunit. We previously showed that the interaction of NAAG with NMDA-Rs at high micromolar-low millimolar concentrations is responsible for neurotoxicity of this dipeptide in rat hippocampus (Bubenikova-Valesova et al. 2006; Pliss et al. 2000). Also NAAG-induced damage of the blood-brain barrier seems to be involved in this process (Pliss et al. 2002). Moreover, elevated levels of extracellular NAAG, evoked by the inhibition of glutamate-carboxypeptidase II, produced an analgesic effect in animal pain models (Neale et al. 2005; Yamamoto et al. 2004).

The pain properties of these models were examined using followed pain tests: plantar test (acute thermal nociception), test with von Frey filaments (acute mechanical nociception) and formalin test (acute chemical irritable nociception and inflammatory nociception). For the chronic neuropathic pain the CCI model was used and following pain tests were performed in this model: plantar test (thermal hyperalgesia and allodynia), test with von Frey filaments (mechanical allodynia) and visualization of c-Fos positive neurons (spontaneous chronic pain). c-Fos protein is a marker of neuronal activity and its expression is evoked by various types of noxious stimulation (Coggeshall 2005).

We found the decreased pain perception in animals with both types of neurotoxic lesion only in thermal nociception test (plantar test). In other performed tests (mechanical nociception – von Frey filaments, chemical nociception – formalin test) the sensitivity to painful stimulation remained unchanged. These findings indicate that the neonatal brain lesion caused by QUIN or NAAG has only a limited influence on nociceptive pain perception in adulthood.

The CCI model appears to be one of the most frequently used models for the study of mechanism and treatment of neuropathic pain. Our findings demonstrating the presence of thermal and mechanical hyperalgesia/allodynia during 2 weeks after CCI are consistent with the definition of this model (Bennett and Xie 1988). We observed both types of pain in the control group, which corresponds with our previous study (Franek et al. 2004). In the animals with neurodevelopmental models of schizophrenia the sensitivity to thermal and mechanical stimulation was different. While the pain threshold for thermal stimulation was not affected by CCI, in the pain threshold for mechanical stimulation we observed significant decrease in both SHAM and QUIN groups. Moreover, in the QUIN group the mechanical pain threshold was significantly lower then in control group. It means that the rats with described brain lesions are not sensitive to thermal hyperalgesia after CCI, but are sensitive to mechanical hyperalgesia/allodynia, even more than the control animals are. The difference in the development of thermal and mechanical pain perception in a model of neuropathic pain was described previously (Hofmann et al. 2003). Similar differences of

thermal and mechanical pain after CCI were described in analgesic actions of some drugs (baclofen, amitryptiline)(De Vry et al. 2004). These facts indicate different mechanisms of thermal and mechanical hyperalgesia/allodynia in neuropathic pain. The symptoms of mechanical hyperalgesia/allodynia are considered to be more reliable than thermal hyperalgesia. This hypothesis is supported by more consistent pathophysiological mechanisms underlying the mechanical allodynia in different types of neuropathic pain (Bridges et al. 2001).

There are conflicting views about the correlation between numbers of c-Fos IR neurons in the spinal cord and severity of hyperalgesia/allodynia (Munglani et al. 1999; Yamazaki et al. 2001). In our study, the CCI significantly increases the number of ipsilateral c-Fos IR neurons compared to the number of contralateral ones in all groups. However, the increase of the neurons paralleled the development of both thermal and mechanical hyperalgesia/allodynia in sham operated rats only. In QUIN and NAAG rats thermal hyperalgesia did not develop at all, so the increase of the IR neurons rather followed the development of mechanical hyperalgesia/allodynia. We could summarize that if there is a correlation then it is between the number of c-Fos IR neurons and mechanical, but not thermal, hyperalgesia/allodynia after CCI. We hypothesize, that ipsilateral increase of c-Fos IR neurons might follow ongoing mechanical stimulation of the injured hindlimb during normal walking after CCI. Moreover, it supports the hypothesis about the significance of mechanical hyperalgesia/allodynia mentioned in previous paragraph.

We may conclude that the perception of thermal stimulation is attenuated in both neuropathic and nociceptive pain in described animal models based on the neonatal i.c.v infusion of QUIN and/or NAAG. Mechanical pain is exacerbated in neuropathic rats in both models while the perception of acute chemical and inflammatory pain (formalin test) remains unchanged and comparable to controls. Thus, nociceptive and neuropathic pain belongs - in addition to behavioral changes and changes in social interactions - to the parameters which are affected in the neurodevelopmental animal models of schizophrenia. These findings highlight the importance of neonatal period in the development of pain pathways.

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Legend to the figures

Figure 1. Plantar test withdrawal latencies in rats without neuropathic pain. Both groups with neonatal brain lesion (QUIN and NAAG) show lower sensitivity to thermal stimulation compared to control (SHAM). Latencies were measured on both hindlimbs.

The height of each bar represents the mean time ± S.E.M. of results obtained from 5-8 animals. * $p < 0.05$ compared to SHAM group.

Figure 2. Nociceptive responses induced by injection of 50 ml 2.5% formalin in hind paw. Differences between QUIN (n=8), NAAG (n=5) and SHAM (n=7) groups in any phase of formalin test were not significant.

Figure 3. Plantar test withdrawal latencies in rats 2-3 weeks after CCI. Significant difference before (dark bars) and after ligation (light bars) only in SHAM group was observed.

The height of each bar represents the mean time ± S.E.M. of results obtained from 5-7 animals. * $p < 0.05$.

Figure 4. Mechanical hyperalgesia/allodynia in rats 2-3 weeks after CCI. Stimulus-response curves of ligated and contralateral hind limb for QUIN (A), NAAG (B) and SHAM (C) group. In all groups the threshold values (the force required to elicit 50% of the maximum response) on the ligated side (light bars) were significantly higher compared to unaffected side (dark bars) (D). In QUIN group both ligated and contralateral thresholds were lower compared to SHAM-operated animals.

Values represent the mean ± S.E.M. of results obtained from 5-7 animals. * $p < 0.05$, § $p < 0.05$ compared to SHAM group.

Figure 5. Number of c-Fos-IR neurons in the spinal dorsal horn (segments L3-L5) in animals after CCI. In all groups the number of neurons on the ligated side (light bars) was significantly higher compared to unaffected side (dark bars), but there were no differences between QUIN, NAAG and SHAM groups.

The height of each bar represents the mean ± S.E.M. of results obtained from 5-7 animals. * $p < 0.05$.

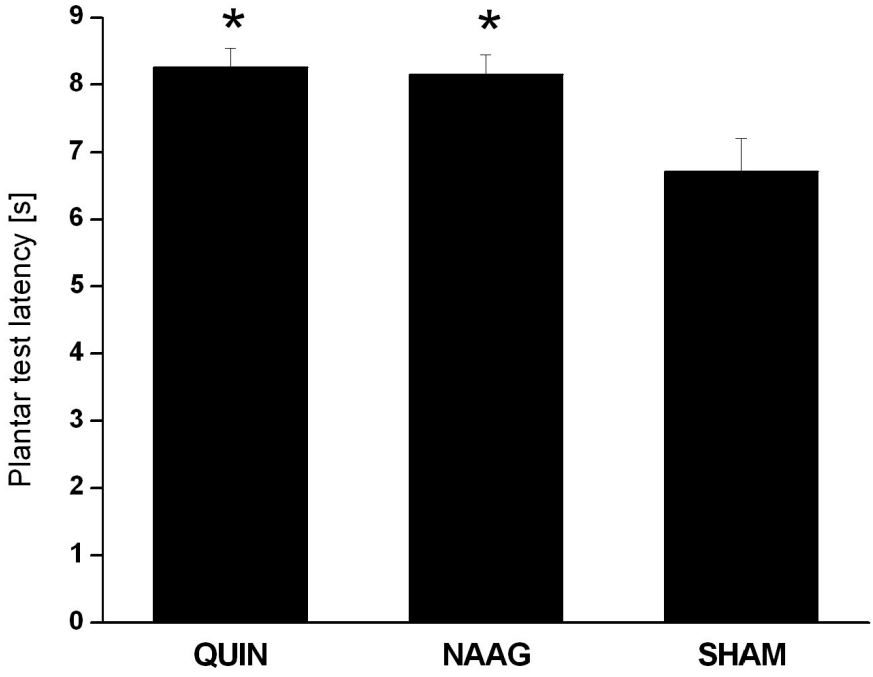

Fig 1

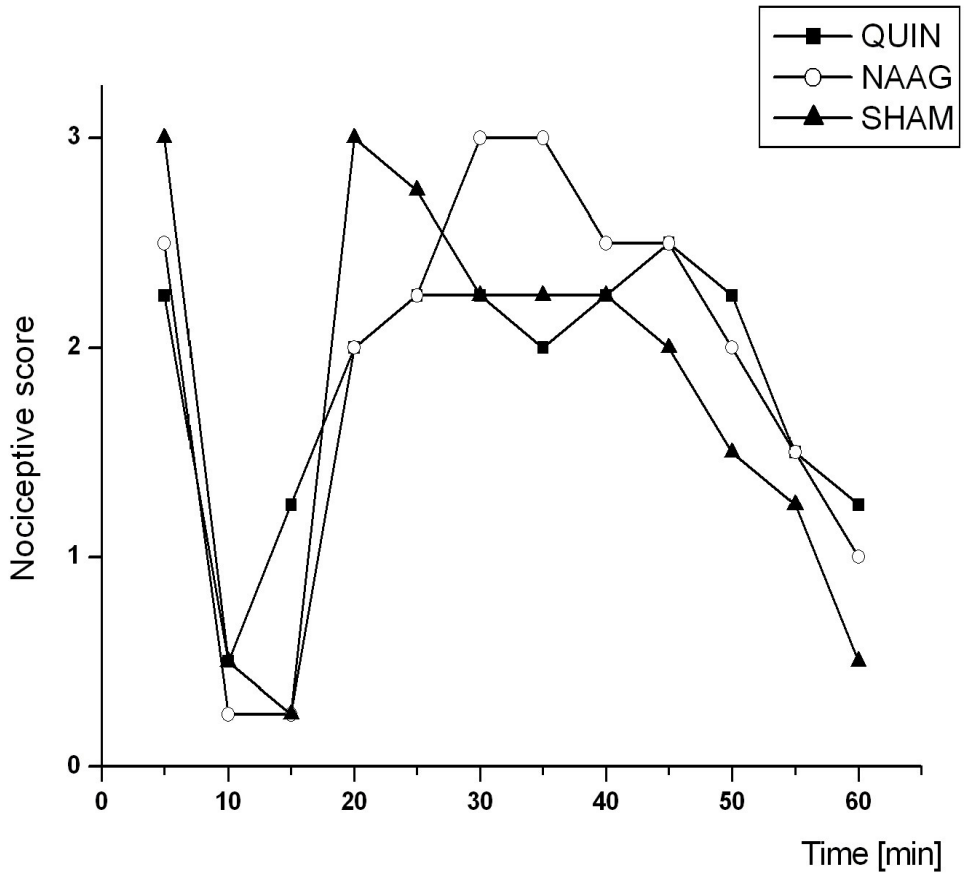
QUIN
NAAG
SHAM
Nociceptive score
0
1
2
3
0
10
20
30
40
50
60
Time [min]

Fig 2

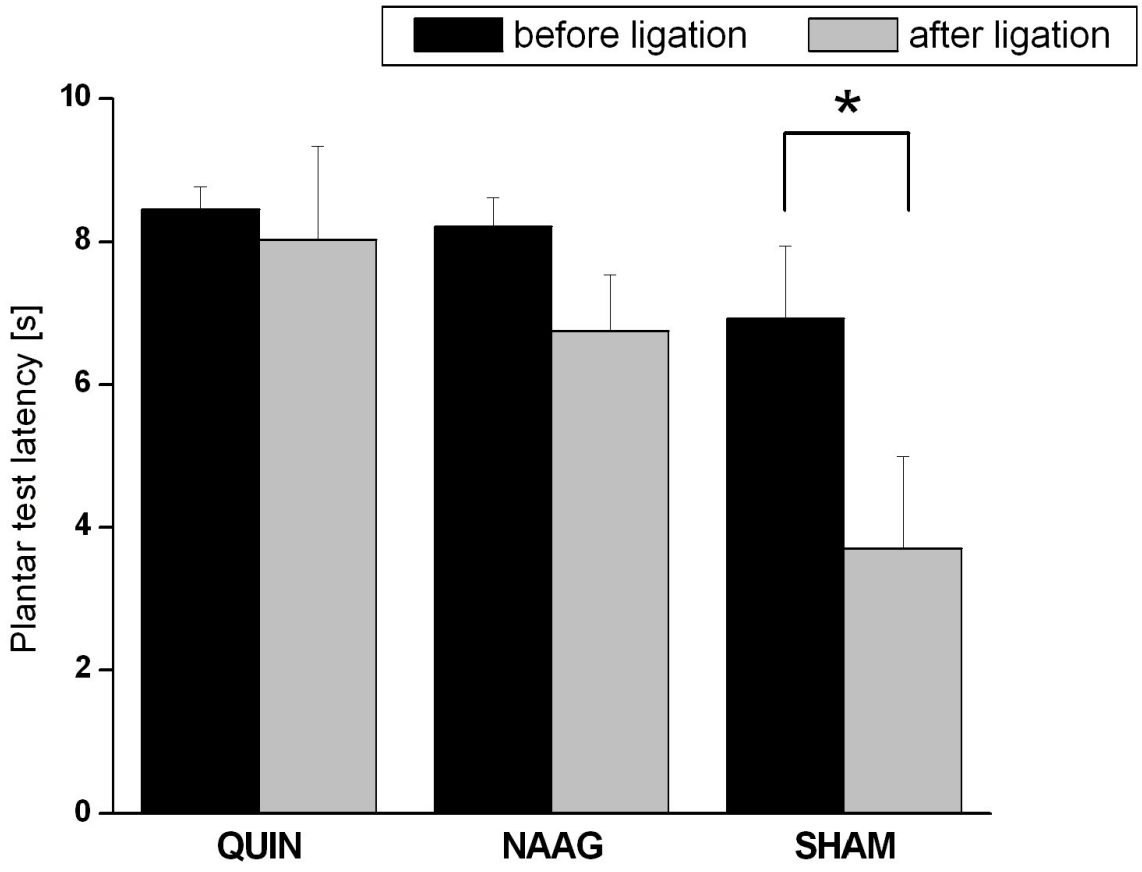
before ligation
after ligation
*
Plantar test latency [s]
10
8
6
4
2
0
QUIN
NAAG
SHAM

Fig 3

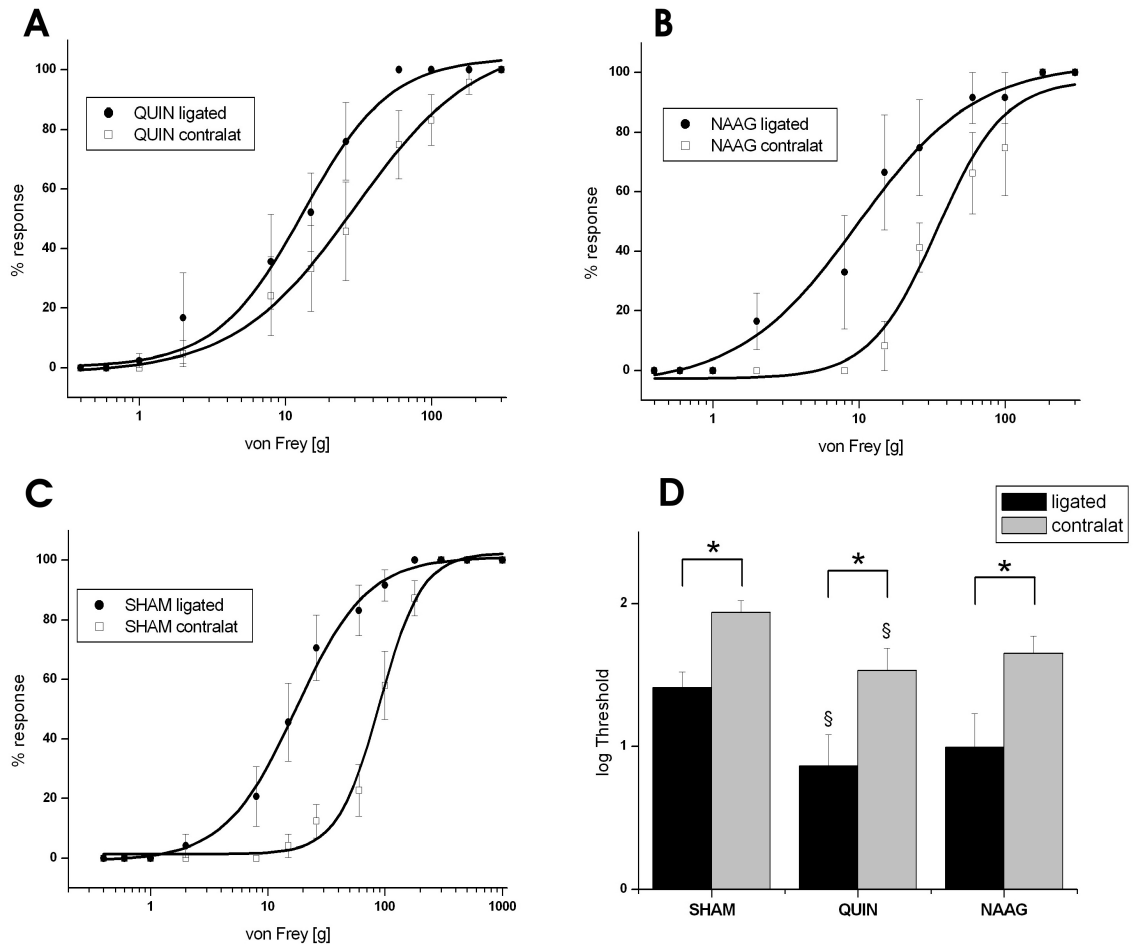
A
QUIN ligated
QUIN contralat
% response
von Frey [g]
B
NAAG ligated
NAAG contralat
% response
von Frey [g]
C
SHAM ligated
SHAM contralat
% response
von Frey [g]
D
ligated
contralat
log Threshold
SHAM
QUIN
NAAG
*
§

Fig 4

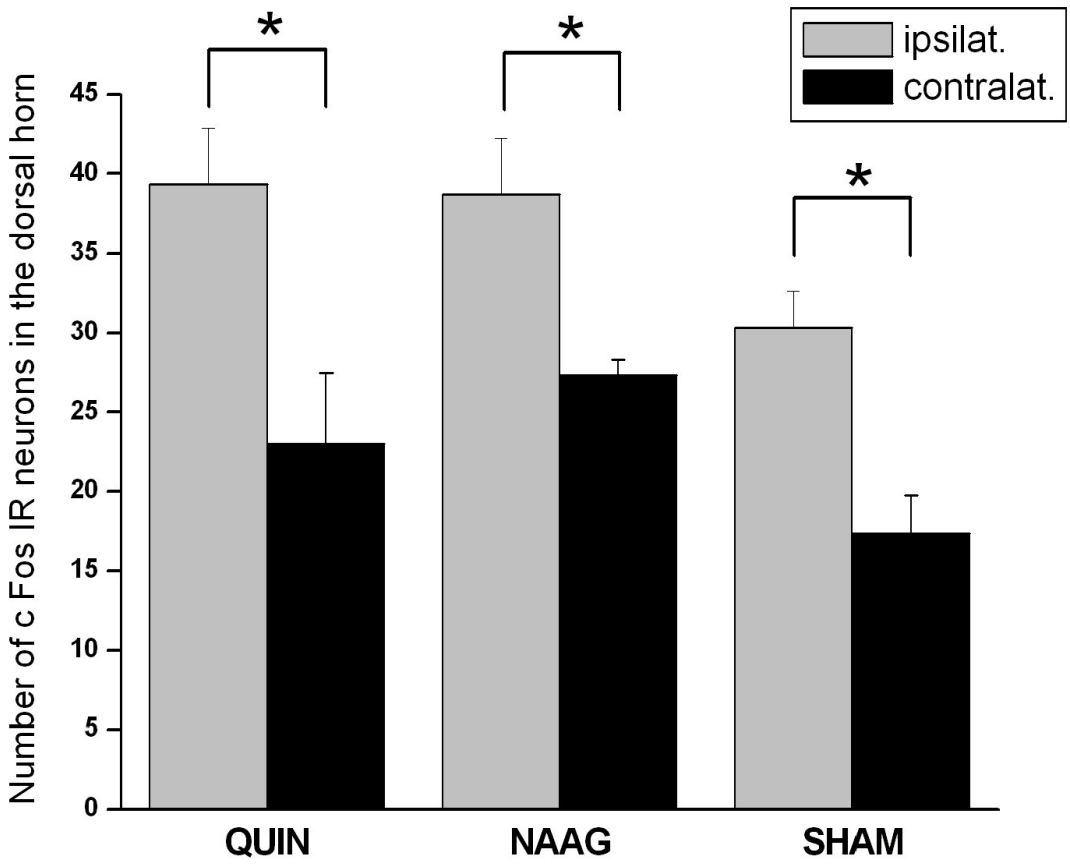
ipsilat.
contralat.
Number of c Fos IR neurons in the dorsal horn
0
5
10
15
20
25
30
35
40
45
*
*
*
QUIN
NAAG
SHAM

Fig 5