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Gene therapy for trigeminal pain in mice

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Abstract

The aim of this study was to test the efficacy of a single direct injection of viral vector encoding for encephalin to induce a widespread expression of the transgene and potential analgesic effect in trigeminal behavioral pain models in mice. After direct injection of HSV-1 based vectors encoding for human preproenkephalin (SHPE) or the lacZ reporter gene (SHZ.1, control virus) into the trigeminal ganglia in mice, we performed an orofacial formalin test and assessed the cumulative nociceptive behavior at different time points after injection of the viral vectors. We observed an analgesic effect on nociceptive behavior that lasted up to 8 weeks after a single injection of SHPE into the trigeminal ganglia. Control virus injected animals showed nociceptive behavior similar to naïve mice. The analgesic effect of SHPE injection was reversed/attenuated by subcutaneous naloxone injections, a μ-opioid receptor antagonist. SHPE injected mice also showed normalization in withdrawal latencies upon thermal noxious stimulation of inflamed ears after subdermal complete Freund's adjuvans injection indicating widespread expression of the transgene. Quantitative immunohistochemistry of trigeminal ganglia showed expression of human preproenkephalin after SHPE injection.

Direct injection of viral vectors proved to be useful for exploring the distinct pathophysiology of the trigeminal system and could also be an interesting addition to the pain therapists' armamentarium.

Keywords

gene therapy; trigeminal pain; animal model; behavioral test; enkephalin; viral vector

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Conflict of interest

The authors declare no conflict of interest.

Introduction

Trigeminal nerve-associated pain is very diverse and sometimes difficult to treat. Different types of chronic headaches including migraine and cluster headache, acute headaches such as postpunctural headache or meningitis-associated headache, intraoral pain such as dental pain, trigeminal neuralgia and many more pain pathologies depend upon sensory processing via trigeminal nerve branches. Potential treatments for trigeminal-associated pain is also diverse, ranging from simple nonsteroidal anti-inflammatory drugs such as ibuprofen, to opioids, triptans, oxygen, pentinoids (pregabalin and gabapentin), anti-epileptic drugs such as carbamazepine, ergotamines and others. Many of these drugs are usually not effective for any other type of pain, which emphasizes the distinct pathophysiology of the trigeminal system. Some trigeminal pain syndromes are treated by surgical interventions such as microvascular decompression (MVD) for trigeminal neuralgia[1]. Not surprisingly, unconventional treatments have been suggested for trigeminal pain such as intranasal oxytocin[2] or CO_2[3] application, CT-guided pulsed radiofrequency[4], stimulation of the sphenopalatine ganglia[5], and daily consumption of hot chilli peppers[6] or grape seeds[7].

In our study, we wanted to test the feasibility of a gene therapy based approach to treating trigeminal pain by delivering viral vectors by direct injection into the trigeminal ganglia of mice thereby changing the phenotype of transduced trigeminal neurons in order to alleviate craniofacial inflammatory pain after injection of formalin or complete Freund's adjuvant solution (CFA). Many trigeminal pain states, e. g. dental pain or temporomandibular joint pain, have a major inflammatory component[8]. We assessed inflammation induced nociceptive behavior at different time points after viral vector injection and transgene expression in trigeminal ganglia.

Results

Orofacial formalin test

Cumulative nociceptive behavior in naïve, SHZ.1-, and SHPE-injected mice, respectively, is shown in figure 1. Two-way analysis of variance revealed significant effects for time point ($F(2,3)=162.7$) and treatment ($F(2,3)=40.9$). *Post hoc* Bonferroni analysis showed significant differences for mice 2, 6, and 8 weeks after SHPE injection when compared to naïve or SHZ.1 injected mice. There was no significant difference between naïve and SHZ.1 injected mice at any time point tested.

Reversal of SHPE effects using naloxone

Figure 2 shows the effect of naloxone administered prior to injection of formalin on the nociceptive behavior in the orofacial formalin test 4 weeks after trigeminal injection of SHZ. 1 and SHPE viral vector, respectively. There was no significant effect naloxone injections in mice that received SHZ.1 vector. SHPE injected mice however showed a significant increase (t-test with Welch's correction) in nociceptive behavior after naloxone administration indicating an opioid-receptor mediated analgesic effect of SHPE.

Reversal of inflammatory hyperalgesia after trigeminal SHPE injection

Three days after subcutaneous injection of CFA into the rostral external part of the left ear all mice showed edema and erythema secondary to localized inflammation. Withdrawal latencies of CFA-injected mice were significantly shorter compared to naïve mice (Figure 3). Withdrawal latencies in mice that received SHZ.1 injections prior to CFA injections were not significantly different from CFA injected naïve mice. However, mice that received SHPE injections prior to CFA showed significantly longer withdrawal latencies to the noxious thermal stimulus compared to both naïve and SHZ.1 injected mice indicating an analgesic effect of trigeminal SHPE injections (one-way analysis of variance with *post hoc* Bonferroni comparisons, $F(3,12)=22.7$).

Semiquantitative immunohistochemistry

Figure 4 shows average brightness in trigeminal ganglia stained for human preproenkephalin after SHZ.1 and SHPE injections, respectively. Brightness in trigeminal neurons of mice that received SHPE was significantly lower compared to SHZ.1 injected mice (t test).

Discussion

This study sought to investigate the feasibility of a gene therapy based approach in two murine trigeminal pain models. Pohl et al.[9] showed almost two decades ago that expression of preproenkephalin A leads to measurable concentrations of met-enkephalin in dorsal root ganglia. Since this observation, efforts have been made to increase enkephalin expression in dorsal root ganglia by several groups in order to study analgesic effects and potentially treat pain. Many of these groups used viral-vector driven expression of an enkephalin transgene. We and others have used HSV-based viral vectors to introduce transgenes into primary afferents in previous studies. Usually, these viruses are applied directly onto the skin innervated by the terminals of nerve fibers that were targeted for transfection[10,11] exploiting the characteristics of HSV, *i.e.* the ability to infect and establish a latent infection in post-mitotic cells such as neurons.

In this study, we have chosen to directly inject the viral vectors into the trigeminal ganglia as opposed to applying it to the skin since we were interested in obtaining a widespread expression of the transgene with a single injection. Immunohistochemistry confirmed that trigeminal SHPE injection induced expression of the enkephalin transgene in trigeminal neurons. As a consequence, we observed a significant decrease in cumulative nociceptive behavior in the orofacial formalin test as well as a normalization of inflammation-induced decrease of withdrawal latencies to a noxious heat stimulus after injection of SHPE into the trigeminal ganglia. We have previously used infrared diode laser produced noxious heat stimuli in a rodent model[12]. For this study we injected CFA into the ear of mice inducing robust inflammation and a decrease in ear withdrawal latencies to noxious heat stimuli as compared to naïve animals. In this model, the analgesic effect of SHPE injections could be attenuated/reversed with naloxone, a μ-opioid receptor antagonist, demonstrating the opioid (and presumably enkephalin) dependency of this effect. The control vector did not reverse decreased ear withdrawal latencies to noxious heat stimulation after CFA-induced inflammation.

Goss et al.[13] administered SHPE by injecting it subcutaneously into hindpaws in rats. They also performed formalin tests and found a significant analgesic effect after SHPE injections for phase 2 of the test that lasted for about 4 weeks. After re-injecting the same viral vector the analgesic effect could be re-established. It is not clear why we observed a longer analgesic effect of at least 8 weeks, but differences between the trigeminal and peripheral nociceptive systems might be a possibility, which emphasizes the uniqueness of the trigeminal sensory system. Another reason could be that we have chosen to directly inject the viral vectors into the trigeminal ganglia as opposed to Goss et al. who administered the vectors subcutaneously into the receptive field of the DRG neurons. It could very well be that the amount of viral vectors reaching the dorsal root and trigeminal ganglia, respectively, depends on the administration route in a way that direct injection yields higher viral vector load than peripheral injection or simple inoculation. Lu et al.[14] injected a HSV-based vector encoding for human preproenkephalin into the knee joint of rats and later induced arthritis by injecting CFA. Interestingly, they did not only observe an analgesic effect in their model but also preserved synovial cytoarchitecture which was explained by the immunosuppressive and anti-inflammatory effects of met-enkephalin. Meunier et al.[15] have chosen to inoculate a similar viral vector into the vibrissa of rats. The depilation and scarification of the areas where the viral vectors were applied might present a potential confounding problem especially in a very sensitive like the vibrissa as it might induce altered sensitivity. Meunier et al. observed transgene-mediated expression of enkephalin as well as transport from the trigeminal neuron cell bodies to the peripheral terminals. They also observed an anti-allodynic effect in a trigeminal neuropathic pain model (chronic constriction of the infraorbital nerve). Comparable to our findings, this effect could be blocked by naloxone.

For this study, we have chosen to inject the virus directly into the trigeminal ganglia. Considering that neuroablative treatments such as radiofrequency ablation or glycerol rhizolysis using either neuronavigation or fluoroscopy via the foramen ovale are commonly used in patients with trigeminal neuralgia[16,17], the application by means of direct injection into the ganglia can be readily transferred to a clinical setting. Direct injection into the trigeminal ganglia might be even a more valuable approach since some tissues involved in trigeminal pain states such as the dura are not readily accessible. Therefore the widespread expression of the transgene observed in this study is an useful finding for future trials investigating potential benefits of gene therapy based treatments for e.g. migraine. However other application routes need to be evaluated as well. Vit et al.[18] have chosen a similar approach. They implanted a catheter into the skull of rats with the end in close proximity to the trigeminal ganglia. This catheter was used to inject adenoviral vectors encoding for glutamic acid decarboxylase (GAD) gene as well as several GABA antagonist. Six days after viral vector injection there was a significant reduction in nociceptive behavior in the second phase of the orofacial formalin test, but not in the first phase. This analgesic effect could be blocked by direct trigeminal administration of a $GABA_A$, but not by a $GABA_B$ antagonist. Unfortunately, this group did not perform experiments that determine duration of analgesic effect. Filipovic et al.[19] have also chosen direct injection into the trigeminal ganglia albeit using a different technique, i.e. through the infraorbital foramen and canal. Although this approach is more comparable to the techniques used in humans, it bears the

risk of damage to the second branch of the trigeminal nerve. These effects might play a minor role when a trigeminal pain state is already established, e.g. in trigeminal pain patients or in the study by Filipovic et al. where the rats had an infraorbital nerve constriction prior to trigeminal ganglia injection. For preclinical testing of potential benefits of therapies the direct injection into the trigeminal ganglia, our data indicates that the method suggested by Whitehead et al.[20] provides widespread expression of the transgene as indicated by the observed analgesic effects in the vibrissa and the ipsilateral ear with a single injection into the trigeminal ganglia. Peripheral application would have most likely required multiple injections since expression of the transgene delivered by HSV-based viral vectors is limited to transfected primary afferents[21].

Interestingly, we have observed an effect of age on pain-associated behavior in the orofacial formalin test in Swiss-Webster mice. There was a steady decline in pain-associated behavior with increasing age. This effect was also observed in naïve rats and can thus not be explained by viral vector related effects. In addition, although we have not performed a conclusive study, we have not seen any signs of neural degeneration in trigeminal ganglia microscopic examination. We were unable to find any studies that have looked into age-dependent effects on baseline nociceptive behavior in the paw formalin test in Swiss-Webster mice. In a previous study where we investigated the orofacial formalin test in naïve Balb/C mice we did not observe a similar age-dependent decrease in pain associated behavior[22]. It has been shown that different mouse strains show different sensitivity in the formalin test[23], to our knowledge however, there are no studies that systematically investigated age-dependent changes for differerent mouse strains. This discrepancy emphasizes the need for appropriate controls in studies and the use of the correct behavioral test and appropriate mouse strain.

While secondary to the pain models used in this study we were not able to test for baseline changes in pain perception, previous studies have shown that the analgesic effects of viral vectors encoding for preproenkephalin become effective under noxious stimulation leaving baseline pain perception unaffected[24]. This is an important point since acute pain serves the purpose of tissue protection and should thus not be attenuated by the treatment.

A similarly constructed virus has been used in several rodent[10–15,25–27] and a non-humane primate[28] pain model. A feasibility study in humans using a similar viral vector has recently been published[29]. Fink et al. found a dose-response curve with analgesic effects lasting at least 28 days with the highest dose investigated after a single administration of the preproenekphalin encoding HSV-based vector in patients that were on high doses of chronic opioids. The findings of this study emphasize the potential rapid adaptation of this novel approach to treat trigeminal pain in humans.

In summary, gene therapy using an enkephalin encoding herpes viral vector approach was successfully used for treating trigeminal pain conditions in mice in different behavioral pain models. Gene therapy using this viral vector or other similarly constructed ones might be a useful addition to the armamentarium of pain therapists in the future.

Methods

Animals

Adult male Swiss Webster mice (22–25 g; 4–5 weeks; Charles River Laboratories, Wilmington, MA) were housed in a 12-h light: 12-h dark environment and provided food and water ad libitum. Effort was made to minimize discomfort and to reduce the number of animals used. All animal procedures were approved by Stanford University (Stanford, CA) Institutional Animal Care and Use Committees.

Viral Vectors

Initial stocks of replication-defective, ICP4-herpes viruses encoding either lacZ (SHZ.1; formerly named DZ) [30] or human preproenkephalin (SHPE) [25] under control of human cytomegalovirus immediate-early promoter/enhancer and the complementing 7B cell line were generously provided by J. C. Glorioso and M. A. Bender at the University of Pittsburgh.

Trigeminal Injections

Mice were injected in groups of 10 based on a technique described by Whitehead et al.[20]. Each mouse from a group was weighed. The mouse whose weight most closely approximated the mean weight was sacrificed to determine the stereotactic coordinates for the 9 remaining mice. After an intraperitoneal injection of pentobarbital (100mg/kg) sufficient to kill this mouse, the stereotactic coordinates (x, y and z) for bregma were determined. The cranium and underlying brain tissue were removed and the stereotactic injector was placed in the middle of the left trigeminal ganglia. The stereotactic coordinates were recorded and compared to the coordinates for bregma.

The remaining mice were anesthetized with 2–3 vol% sevoflurane in oxygen. The cranium was shaved with clippers and desinfected with 10% betadine solution. After placing the mouse in the stereotactic device, a midline incision was made to expose the skull. Stereotactic coordinates of bregma were found and the microinjector was directed above the left trigeminal ganglia using the previously determined coordinates. A small burr hole trepanation (~3mm^2) allowed to lower the microinjector towards the trigeminal ganglia. Four μl of the viral vector solution (2×10^6 plaque-forming units, pfu) were injected over 30 sec. The wound was closed with clips and the mouse was allowed to recover to complete consciousness on a heating pad. Wound clips were removed between day 10 to 12 post surgery.

Behavioral Test

Orofacial Formalin Test—Two, 6, and 8 weeks after trigeminal injections mice were tested for analgesic effects using the orofacial formalin test. Mice were habituated to the testing scenario on 3 consecutive days prior to the experiment for at least 20 minutes by the same experimenter. Test cages were custom-built from clear plastic boxes (17 × 10.5 × 7 cm). Holes were drilled into the sides to allow for ventilation, and the outer side of the top of the boxes was taped with black paper to maximize contrast and facilitate analysis of video recordings.

After habituation, 8 animals were tested per session. Mice were placed in an induction chamber and anesthetized using 2 vol% of sevoflurane in oxygen. After reaching an adequate level of anesthesia (*i.e.*, animals lying still not responding to tail tweak), animals were taken out of the plexiglass cylinder one at a time and 10 μl of 2.5% formalin was injected subcutaneously into the left vibrissa. Immediately after, mice were placed in the testing cage and videotaped for 1 hour using 4 cameras (2 animals per camera). Data were stored for offline analysis at the end of the experiment. Animals could be observed during the experiment on a monitor. The animals' cumulative nociceptive behavior, *i.e.* scratching the injection site with the hindpaw or face-washing like behavior, was measured cumulatively with a stopwatch offline by an experimenter, who was blinded to the treatment. The observation period of 60 minutes was split up into 20 bins of 3 minutes each. Each animal was assessed individually. Data were entered into a Microsoft EXCEL table for further analysis.

Naloxone injection—To test for potential reversal of SHPE induced analgesic effects and thus opioid dependency, we injected mice with the SHZ.1 and SHPE viral vector, respectively (n=18 per group). Six weeks after viral injections, animals were anesthetized using 2 vol% of sevoflurane in oxygen. They were then injected with formalin as described above but also received a subcutaneous injection of naloxone 0.3 mg/kg[31]. The dose of naloxone was chosen based on the findings of Vaccarino et al.[31]. This group has shown a reversal of opioid (morphine) induced analgesia in the paw formalin (5%) test in Balb/C mice for naloxone doses of 0.1 and 0.3mg/kg, respectively. When the dose of naloxone was increased to 10mg/kg however, morphine-induced analgesia was significantly potentiated by naloxone.

CFA injection—Twelve mice (4 naïve, 4 SHZ.1 injected, 4 SHPE injected, 2 weeks after viral vector injection) were lightly anesthetized with 2 vol% sevoflurane in oxygen and injected subdermally in the rostral external part of the left ear with 5μl complete Freund's adjuvant solution (CFA; suspension of heat-killed Mycobacterium tuberculosis in mineral oil (Sigma, St. Louis, MO) or vehicle (mineral oil). This method produces a robust inflammatory response and behavioral hyperalgesia[11].

Laser Test—Two days after CFA injection, mice were lightly anesthetized with urethane (600 mg/kg ip) and placed with minimal restraint on a heating pad to maintain their body temperature at 37°C. It has been shown in previous studies[32,33] that light urethane anesthesia (500–600 mg/kg) does not alter withdrawal latencies to noxious thermal stimuli. The laser beam was directed via the fiberoptic cable to the rostral external part of the left ear. Characteristic responses to laser irradiation, i. e. thermal noxious stimulation, was a retraction of the stimulated ear for 1–3 s. Laser stimulation with this setting was terminated rapidly after response of the stimulated ear or after a maximal response (cut-off) latency of 30 s to prevent tissue damage[12].

The stimulation site was changed after each long pulse allowing at least 2 min in between 2 stimuli. The testing sessions were videotaped for off-line analysis of responses. The off-line analysis was performed by an investigator blinded to the treatment groups, who determined

the latency of the response to the long pulse (with an accuracy of 0.1 s). Data were entered into a Microsoft EXCEL table for further analysis.

Immunohistochemistry

After the end of the behavioral tests, animals were deeply anesthetized with tribromoethanol (500 mg/kg) and perfused by cardiac puncture with PBS followed by 4% paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.5). Trigeminal ganglia were removed and incubated further in fixative for 1–2 h at 4°C.

For immunohistochemistry, the trigeminal ganglia were placed in PBS containing 30% sucrose for 24 h and thereafter blocked. Cryostat sections (40 μm) were washed three times in PBS containing 4% normal goat serum and 0.3% Triton X-100, then were incubated for 12 h with gentle agitation at 4°C with a 1:500 dilution of mouse mAb PE-24. Antibody PE-24 binds to amino acids 175–185 of the human preproenkephalin sequence[34] and does not cross-react with either rat or bovine preproenkephalin as bacterially expressed products[26]. As a control, other sections were processed in parallel without primary antibody. Sections were incubated further in a 1:50 solution of Texas Red-conjugated goat anti-mouse (The Jackson Laboratory) in PBS for 1 h in the dark (22°C) with constant agitation. The sections then were washed three times with PBS and were incubated with a met-enkephalin antibody (Peninsula Laboratories; 1:250 solution prepared as for PE-24). After 12 h, the sections were washed three times in PBS and were transferred to a 1:50 solution of sheep anti-rabbit FITC (The Jackson Laboratory) for 1 h in the dark. The sections were washed three times in PBS, were mounted on gelatin-coated slides, air dried, cover-slipped with Fluoromount, and examined using a Leica DMXA microscope (Leica Microsystems GmbH) at a magnification of 40×. Briefly, images of each slice were captured with a Sony F-707 digital camera (Sony, New York, NY) and imported into Adobe Photoshop (Adobe Systems Incorporated, San Jose, CA). The trigeminal ganglia of both naive and SHPE–infected mice were analyzed for optical density of standardized areas of interest normalized against background. Each region of interest was measured for area and mean optical brightness using Image J version 1.26 (National Institutes of Health, Bethesda, MD). Eighteen slides (9 SHZ.1 injected and 9 SHPE injected, respectively) were coded so that the evaluator was blinded to condition.

Statistics

Cumulative nociceptive behavior during phase 2 of the formalin test (10^{th} to 60^{th} minute) was tested for statistically significant differences using a two-way analysis of variance with *post hoc* Bonferroni analysis.

Naloxone effects after SHZ.1 and SHPE injections, respectively, were tested for statistically significant differences using an unpaired two-tailed t test. For unequal variances we used the Welch's correction.

For withdrawal latencies upon noxious thermal laser stimulation after CFA injection, we used a one-way analysis of variance with *post hoc* Bonferroni analysis to test for significant differences.

Immunohistochemical data was tested for significant differences between the mean brightness values for the SHPE injected versus SHZ.1 injected trigeminal neurons using an one-way analysis of variance with *post hoc* Bonferroni analysis after background correction.

For all statistical test the significance level was set to $p<0.05$.

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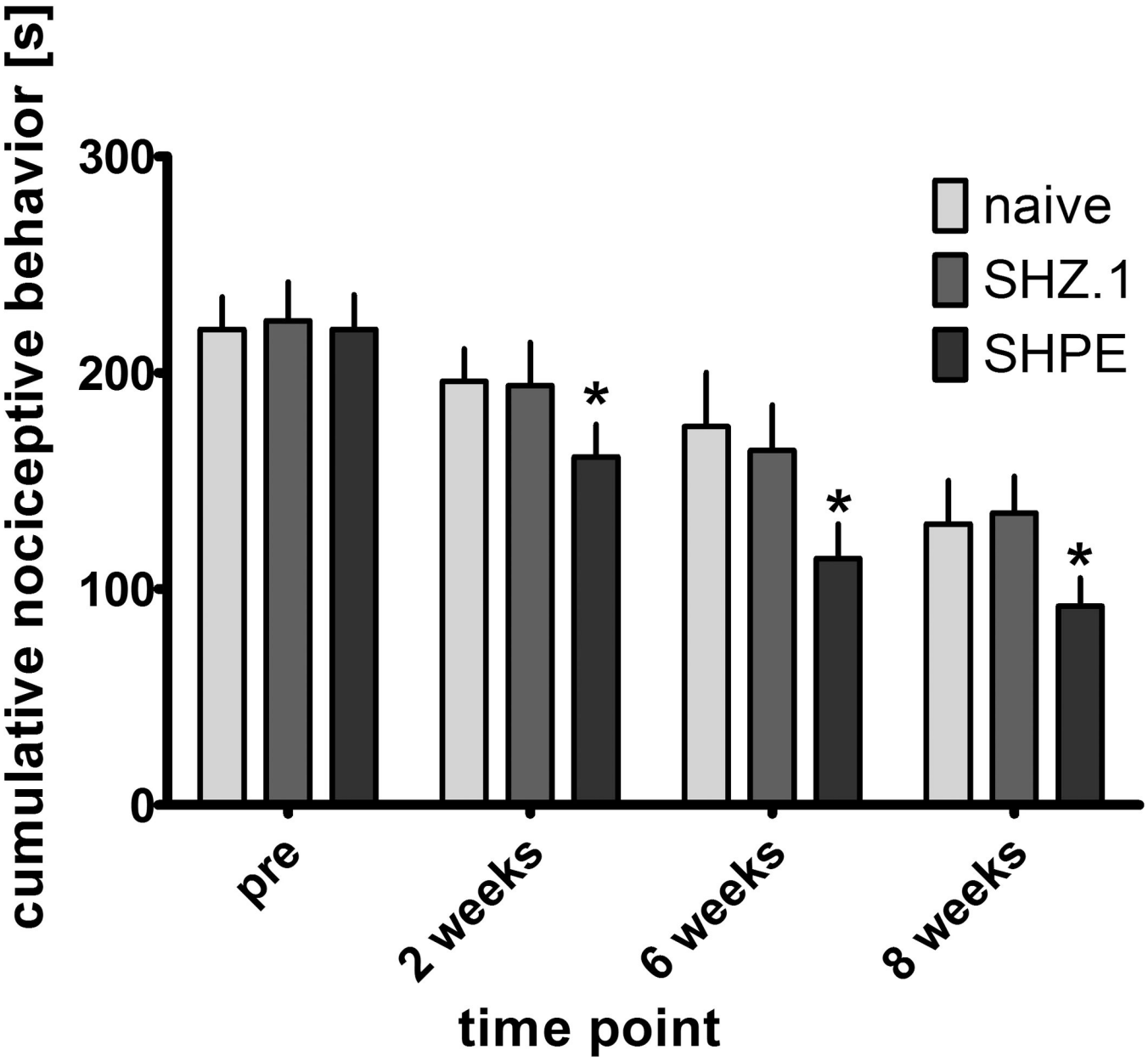

Figure 1.

Cumulative nociceptive behavior in phase 2 of the orofacial formalin test at different time points after trigeminal injections (mean ± SD). *: indicates significantly different effect compared to naïve animals ($p<0.001$, two-way analysis of variance with *post hoc* Bonferroni analysis). There was no statistically significant difference between naïve and SHZ.1 injected mice at any time point.

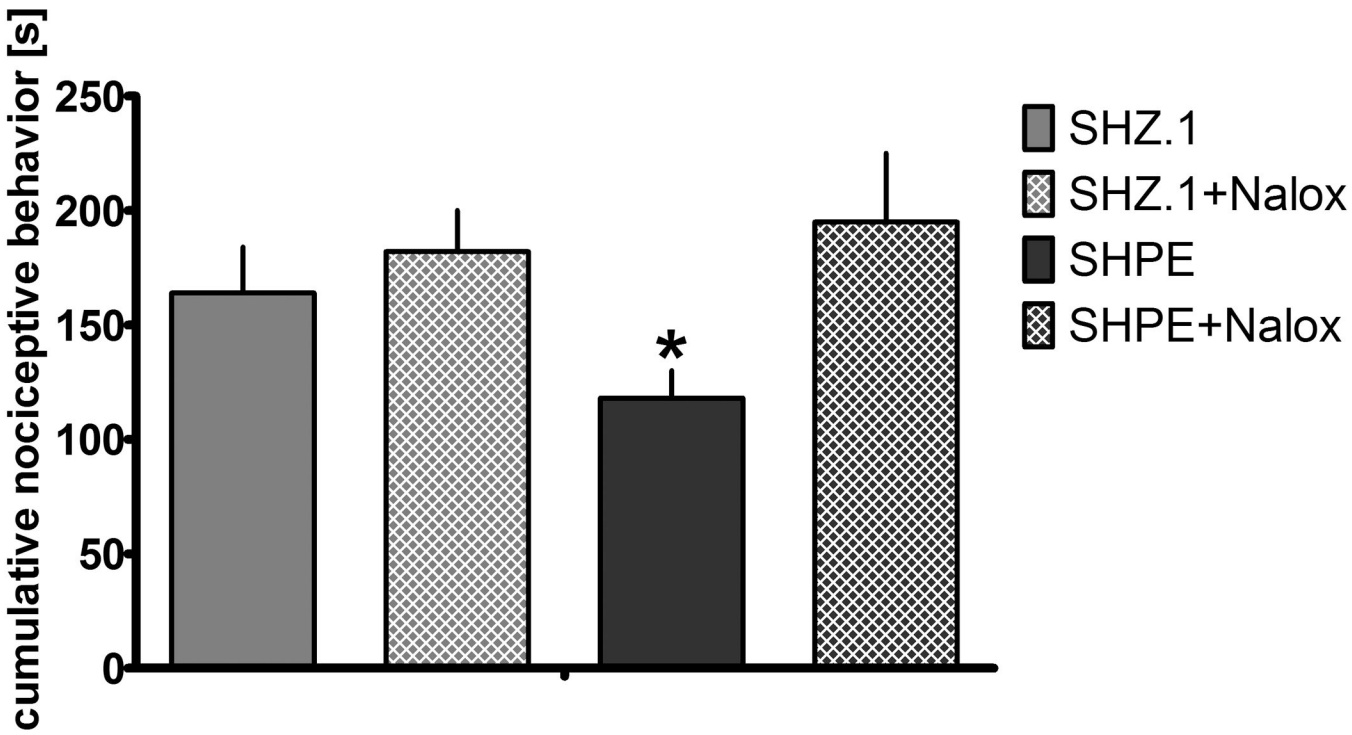

Figure 2.

Effect of naloxone on SHPE mediated analgesic effect (mean ± SD). The analgesic effect of trigeminal SHPE injections was reversible with subcutaneous naloxone injection. *: indicates significantly different effect compared to SHPE+Nalox ($p<0.0001$, unpaired t test with Welch's correction).

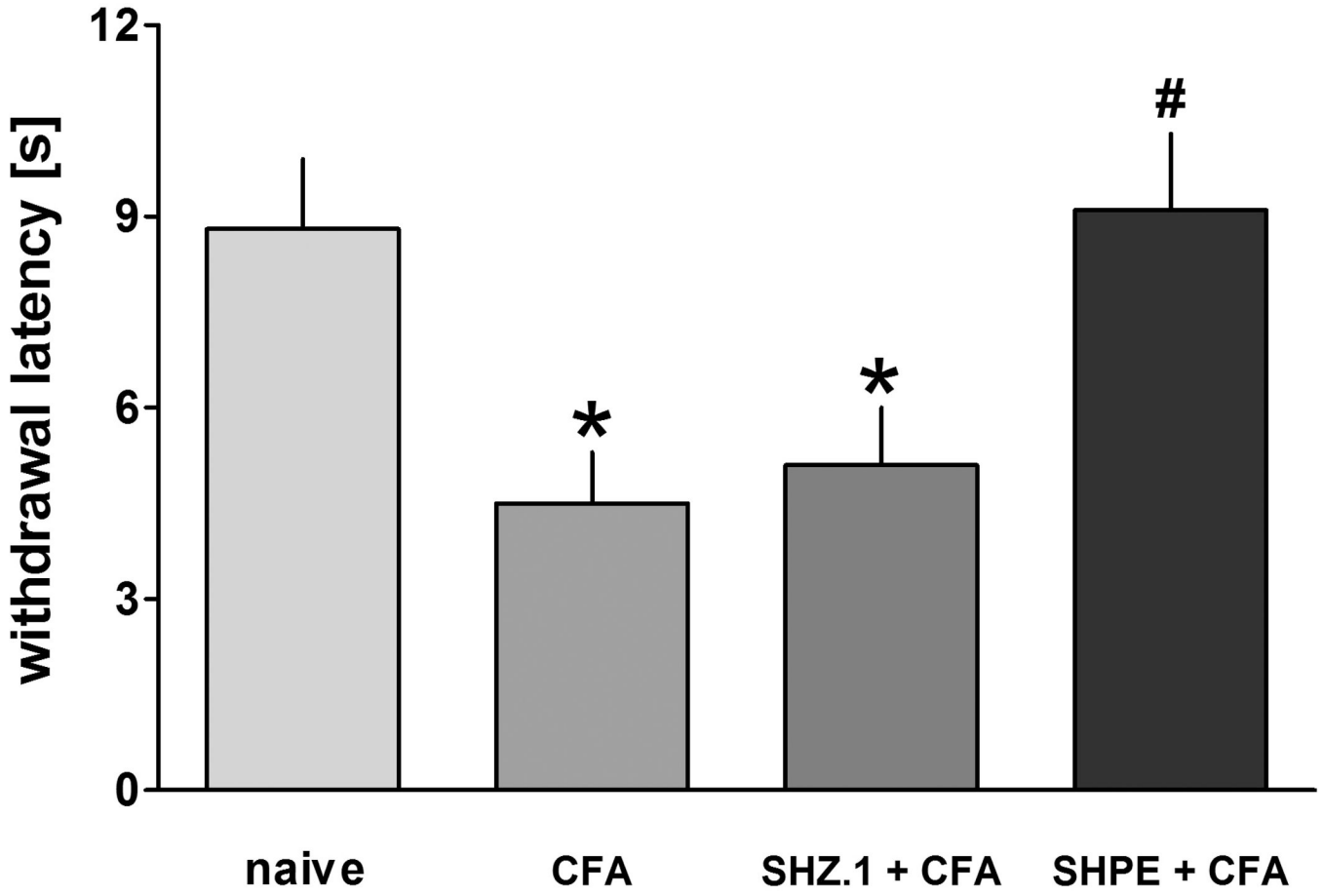

Figure 3.

Effect of CFA injection on ear withdrawal latency to noxious thermal laser stimulation (mean ± SD). CFA injection lead to significantly reduced withdrawal latencies in naïve and SHZ.1 injected mice ($p<0.0001$, one-way analysis of variance with *post hoc* Bonferroni analysis, indicated by *). Compared to naïve and SHZ.1 injected mice, withdrawal latencies in SHPE injected animals were significantly longer (one-way analysis of variance with *post hoc* Bonferroni analysis, indicated by $^{\#}$).

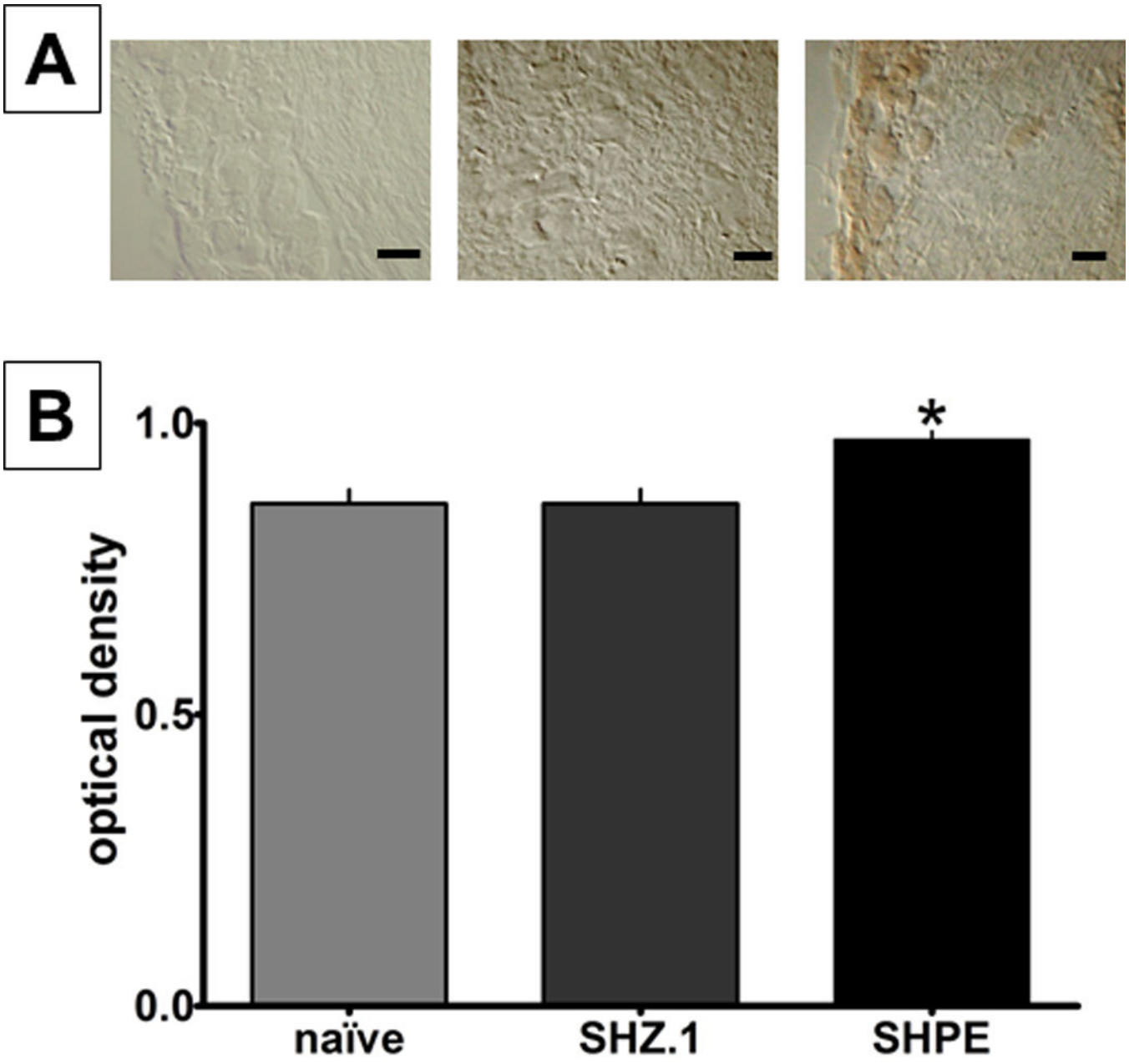

Figure 4.

A) Immunohistochemical images of trigeminal ganglia stained for met-enkephalin, left: naïve, middle: after SHZ.1 injection, right: after SHPE injection. Scale bar: 20μm.

B) Optical density in in naïve trigeminal ganglia neurons and after SHZ.1 and SHPE injections, respectively, and immunohistochemical staining for met-enkephalin (mean ± SD). After injection of SHPE, a significant increase in optical density was observed compared to naïve and SHZ.1 injected mice ($p<0.0001$, one-way ANOVA with *post hoc* Bonferroni analysis, indicated by *).