Gene therapy for trigeminal pain in mice
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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 herpes simplex virus type 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 naive 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 subdural complete Freund’s adjuvant 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.
injections before CFA showed significantly longer withdrawal latencies to the noxious thermal stimulus compared with both naive 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 with 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. 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, exploiting the characteristics of HSV, that is, the ability to infect and establish a latent infection in postmitotic 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, as we were interested in obtaining a widespread expression of the transgene with a single injection. Immunohistochemistry

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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. 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 with naive animals. In this model, the analgescic 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. 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 a different route for vector injection: first, into the trigeminal ganglia as opposed to Goss et al. who administered the vectors subcutaneously into the receptive field of the dorsal root ganglia neurons. It could very well be that the amount of viral vectors reaching the dorsal root and trigeminal ganglia 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. 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. 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 very likely used similar or presumably enkephalinergic, 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, as 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 example, migraine. However, other application routes need to be evaluated as well. Vit et al. 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 gene as well as several gamma-aminobutyric acid antagonist (GABA). 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 GABAA, but not by a GABAB antagonist. Unfortunately, this group did not perform experiments that determine duration of analgesic effect. Filipovic et al. have also chosen direct injection into the trigeminal ganglia albeit using a different technique, that is, 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 have a minor role when a trigeminal pain state is already established, for example, in trigeminal pain patients or in the study by Filipovic et al. where the rats had an infraorbital nerve constriction before trigeminal ganglia injection. For preclinical testing of potential benefits of therapies, the direct injection into the trigeminal ganglia, our data indicate that the method suggested by Whitehead et al. 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, as expression of the transgene delivered by HSV-based viral vectors is limited to transfected primary afferents. In summary, 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 naive 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 naive Balb/C mice, we did not observe a similar age-dependent decrease in pain-associated behavior. It has been shown that different mouse strains show different sensitivity in the formalin test. To our knowledge, however, there are no studies that systematically investigated age-dependent changes for different mouse strains. This discrepancy emphasizes the need for appropriate controls in studies and the use of the correct behavioral test and appropriate mouse strain.

Although 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. This is an important point as 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 rodent16–19,23–27 and a non-humane primate28 pain model. A feasibility study in humans using a similar viral vector has recently been published. 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 preproenkephalin 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.
Vaccarino et al. 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 0.3 mg kg\(^{-1}\), respectively. When the dose of naloxone was increased to 10 mg kg\(^{-1}\), however, morphine-induced analgesia was significantly potentiated by naloxone.

CFA injection. Twelve mice (four naïve, four SHZ.1 injected, four 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 5 μl CFA (suspension of heat-killed Mycobacterium tuberculosis in mineral oil (Sigma, St. Louis, MO, USA) or vehicle (mineral oil). This method produces a robust inflammatory response and behavioral hyperalgesia.

Laser test. Two days after CFA injection, mice were lightly anesthetized with urethane (600 mg kg\(^{-1}\) i.p.) and placed with minimal restraint on a heating pad to maintain their body temperature at 37 °C. It has been shown in previous studies\(^\text{22,33}\) that light urethane anesthesia (500-600 mg kg\(^{-1}\)) 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, that is, 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 (cutoff) latency of 30 s to prevent tissue damage.\(^\text{12}\)

The stimulation site was changed after each long pulse allowing at least 2 min in between two 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\(^{-1}\)) 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, and 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\(^\text{34}\) and does not cross-react with either rat or bovine preproenkephalin as bacterially expressed products.\(^\text{26}\) 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, West Grove, PA, USA) 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, San Carlos, CA, USA; 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, Wetzlar, Germany) at a magnification of × 40. Briefly, images of each slice were captured with a Sony F-707 digital camera (Sony, New York, NY, USA) and imported into Adobe Photoshop (Adobe Systems Incorporated, San Jose, CA, USA). The trigeminal ganglia of both naïve 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, USA). Eighteen slices (nine from each SHPE injected and nine SHPE injected, respectively) were coded so that the evaluator was blinded to condition.

Statistics

Cumulative nociceptive behavior during phase 2 of the formalin test (10th–60th min) was tested for statistically significant differences using a two-way analysis of variance with post hoc Bonferroni analysis.
Naloxone effects after SHZ1 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 were tested for significant differences between the mean brightness values for the SHPE-injected versus SHZ1-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 \( P < 0.05 \).

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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