Research Paper

PAIN

OPEN

Antihyperalgesic effects of Meteorin in the rat chronic constriction injury model: a replication study

Jennifer Y. Xie[a], Chaoling Qu[b], Gordon Munro[c], Kenneth A. Petersen[c], Frank Porreca[b,*]

Abstract

Data from preclinical research have been suggested to suffer from a lack of inherent reproducibility across laboratories. The goal of our study was to replicate findings from a previous report that demonstrated positive effects of Meteorin, a novel neurotrophic factor, in a rat model of neuropathic pain induced by chronic constriction injury (CCI). Notably, 5 to 6 intermittent subcutaneous (s.c.) injections of Meteorin had been reported to produce reversal of mechanical allodynia/thermal hyperalgesia after injury, wherein maximum efficacy of Meteorin was reached slowly and outlasted the elimination of the compound from the blood by several weeks. Here, we evaluated the efficacy of Meteorin in reversing hindpaw mechanical hyperalgesia and cold allodynia in male, Sprague-Dawley rats with CCI. Nociceptive behavior was monitored before and after CCI, and after drug treatment until day 42 after injury. Systemic administration of recombinant mouse Meteorin (0.5 and 1.8 mg/kg, s.c.) at days 10, 12, 14, 17, and 19 after CCI produced a prolonged reversal of neuropathic hypersensitivity with efficacy comparable with that obtained with gabapentin (100 mg/kg, orally). Despite some protocol deviations (eg, nociceptive endpoint, animal vendor, testing laboratory, investigator, etc.) being incurred, these did not affect study outcome. By paying careful attention to key facets of study design, using bioactive material, and confirming drug exposure, the current data have replicated the salient findings of the previous study, promoting confidence in further advancement of this novel molecule as a potential therapy for neuropathic pain.

Keywords: Meteorin, Chronic constriction injury, Mechanical hyperalgesia, Cold allodynia, Neuropathic pain

1. Introduction

Preclinical scientific research has been increasingly criticized for a lack of reproducibility. Recent publications[3,7,14,22,25,32] have demonstrated an alarmingly low level of reproducibility in biomedical research fields. In a survey of 1576 scientists,[1] more than 70% could not reproduce published results and more than 50% could not reproduce findings from their own experiments, leading to discussion of a "reproducibility crisis." Data from psychology[22] and cancer biology[2] research showed only 40% and 11% of study results were reproducible, respectively.

Preclinical studies are often published based on a single laboratory's results and typically have implications for mechanistic understanding and progression of molecules towards human trials. Factors that can influence validity of conclusions include, but are not limited to, evaluation methods in a specific laboratory involving randomization, investigator blinding, inclusion of all data and statistical power. Other sources of variability include the strain, sex, source and handling procedures of animals, as well as the quality of the molecule (eg, pharmacokinetic profile and relevant tissue exposure) being evaluated. Inclusion of rigorous quality controls is necessary to help facilitate data replication. Moreover, even with highly standardized conditions, natural biological variability can be overlooked, resulting in false interpretation of data outcomes and/or failure of replication in other laboratory settings. Voelkl et al.[33] did a meta-analysis showing that results from multilaboratory (as few as 2-4 laboratories) studies produced ~30% to 40% increase of accurate prediction of the true effect size in animal models of stroke, myocardial infarction, and breast cancer. The National Institutes of Health has emphasized that key preclinical studies may first need to be validated independently especially when a potentially costly human clinical trial is proposed based on animal-model results.[5] Here, we attempted to replicate a recent single study, suggesting the efficacy of Meteorin in treating nerve injury–induced neuropathic pain.

Meteorin is a member of a newly described family of secreted proteins that is highly expressed in the mammalian brain throughout development and at lower levels in the postnatal brain.[12,21] Meteorin promotes gliogenesis and, similar to a number of other neurotrophic factors, enhances neurite outgrowth of nociceptive sensory neurons in dorsal root ganglia (DRG) isolated from rodents.[21,34] Based on these latter findings, Jorgensen et al.[13] proceeded to assess the efficacy of repeated systemic injections of Meteorin in 2 distinct rat models of peripheral nerve

Sponsorships or competing interests that may be relevant to content are disclosed at the end of this article.

[a] *Department of Basic Sciences, New York Institute of Technology College of Osteopathic Medicine, Arkansas State University, Jonesboro, AR, United States,* [b] *Department of Pharmacology, College of Medicine, University of Arizona Health Sciences Center, Tucson, AZ, United States,* [c] *Hoba Therapeutics ApS, Copenhagen, Denmark*

**Corresponding author. Address: Department of Pharmacology, University of Arizona HSC, Tucson, AZ 85724, United States. Tel.: (520) 626-7421, fax: (520) 626-4182. E-mail address: frankp@medadmin.arizona.edu (F. Porreca).*

PAIN 160 (2019) 1847–1855

http://dx.doi.org/10.1097/j.pain.0000000000001569

injury. Meteorin reversed mechanical and cold allodynia in rats with photochemical sciatic nerve injury, and mechanical allodynia and heat hyperalgesia in chronic constriction injury (CCI) rats. In both models, the maximum efficacy of Meteorin was reached slowly and outlasted the elimination of the compound from the blood (<24 hours after a single subcutaneous [s.c.] injection) by weeks,[13] suggesting potential "disease-modification." If true, this would differentiate Meteorin from currently available therapeutics.

We performed a replication study under strict experimental conditions of randomization and blinding aimed at confirming Meteorin efficacy in the rat CCI model. Our findings have reproduced key data sets in a different laboratory setting.

2. Methods

2.1. Animals

Pathogen-free, adult male Sprague-Dawley rats (150-200 g; Envigo, Indianapolis, IN) were housed in temperature (23 ± 3°C), light (20-40 lux, 12-hour light/12-hour dark cycle; lights on 07:00-19:00), and humidity (30%-70%) controlled rooms with standard rodent chow (Teklad 2018 Global Rodent Diet; Envigo) and tap water available ad libitum. Clear individually ventilated rat cages were used to house 3 rats per cage with the same cagemates before and after CCI surgery. Standard cage beddings (Corncob Bedding from Teklad; Envigo) were used. All animals were allowed to acclimate to the facilities for 3 to 5 days before being subject to behavioral tests. Diamond Twist nesting materials and PVC tubes were provided as environmental enrichment. All experiments were approved by the Institutional Animal Care and Use Committee of the University of Arizona. All procedures were conducted in accordance with the Guide for Care and Use of Laboratory Animals published by the National Institutes of Health and the ethical guidelines of the International Association for the Study of Pain. Animals were randomly assigned to treatment or control groups for the behavioral experiments.

2.2. Study design

The experiments were designed to follow the methods of Jorgensen et al. as closely as possible (see **Fig. 1** for study design). Chronic constriction injury was chosen as the experimental model to produce chronic neuropathic pain. Mechanical hyperalgesia and cold allodynia were selected as outcome measures. Mechanical and cold thresholds were assessed before CCI surgery and at days 10, 12, 14, 17, 19, 21, 26, 32, 39, and 41 after CCI. Meteorin (0.5 and 1.8 mg/kg, s.c.) or vehicle was administered at days 10, 12, 14, 17, and 19 after behavioral testing. At the end of the experiment and Meteorin unblinding, the vehicle-treated animals were reused to assess the efficacy of the reference compound, gabapentin (100 mg/kg, orally), against the same 2 output measures at day 42 after CCI. These rats were then crossed over to receive the corresponding treatment (switching between gabapentin and water treatment) at day 45 after a 3-day washout period. The grouping information for gabapentin and water was concealed from the experimenter until after completion of the entire study.

2.3. Blinding

All behavioral experiments were performed by an experimenter blinded to treatments. The blinding of the data collection was achieved by having 3 separate people prepare and code the solutions as follows: Experimenter 1 assigned the animals into different groups, experimenter 2 performed the drug treatments and evaluated the behavioral outcomes, and experimenter 3 unblinded the results and analyzed and plotted the data.

2.4. Chronic constriction injury

Chronic constriction injury surgeries were performed according to Bennett and Xie.[4] Briefly, rats were anesthetized with 5% isofluorane in air at 1 L/min and maintained with 2.5% isofluorane. The sciatic nerve of the left hindlimb was exposed at the midthigh level. Four loose ligatures using 4-0 chromic gut suture were placed around the exposed sciatic nerve with about 2 mm between ligatures. Sham-operated animals underwent the same procedure but without the ligatures to the sciatic nerve. No antibiotics or preoperative or postoperative analgesics were administered in accordance with approved IACUC procedures. Cold allodynia and mechanical hyperalgesia were assessed before and at days 10, 12, 14, 17, 19, 21, 26, 32, 39, and 42 after the surgery.

2.5. Assessment of cold allodynia and mechanical hyperalgesia

Cold allodynia was evaluated by placing each rat individually in an enclosed Plexiglas chamber (22 cm L × 11 cm W × 20 cm H) on a metal plate of the same dimension maintained at 4°C. The frequency and accumulated duration of guarding of the injured hindpaw were measured for 2 minutes. The rats were returned to their home cages afterwards. After 0.5 to 1 hour, the assessment of mechanical hyperalgesia was performed using a Randall–Selitto apparatus (Ugo-Basile algesimeter; Stoelting, Chicago, IL).[26] It consisted of measuring the withdrawal threshold of the hindpaw both ipsilateral and contralateral to the site of nerve injury in response to continuously increased pressure. A sharp withdrawal of the hindpaw or vocalization was taken as the endpoint. The paw withdrawal thresholds for each hindpaw were assessed 2 times consecutively, alternating between the 2 sides starting from the contralateral side. A cutoff at 500 g was used to avoid tissue injury. Mechanical thresholds were expressed as the average ipsilateral and contralateral paw withdrawal thresholds. Percent antihyperalgesia (%Antihyperalgesia) was calculated as: (after dose − before dose)/(baseline − before dose) × 100. One animal was removed from the experiment because of lack of mechanical hyperalgesia at day 10 after CCI before any treatment. All other animals were kept in the experiment, and no data with Meteorin treatment were excluded from analysis.

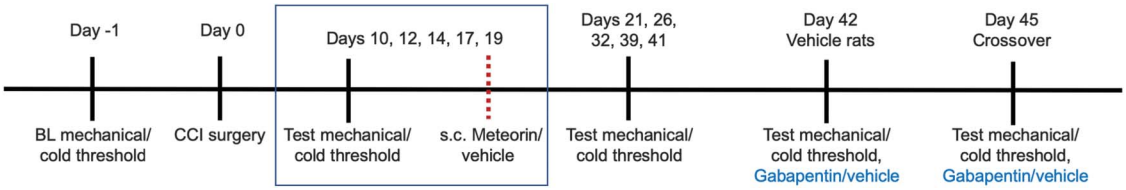

Figure 1. Schematic illustration of the study design. BL, naive baseline (day -1); CCI, chronic constriction injury; s.c., subcutaneous.

2.6. Measurement of Meteorin concentration in the plasma and brain

Separate cohorts of naive rats were treated with Meteorin (1.8 mg/kg, s.c.) acutely at day 1 or repeatedly at days 1, 3, 5, 8, and 10 (n = 3/group). Blood (~2 mL) was collected by cardiac puncture under isoflurane anesthesia at 4 hours after the last dose and immediately put into K3-EDTA–coated tubes. The samples were centrifuged at 3300*g* for 10 minutes at 4°C. Plasma (approximate 1.2 mL) was removed and then transferred to Micronic tubes and stored at −80°C. After blood collection, the animals were sacrificed through isoflurane overdose, and both brain hemispheres and the cerebellum (which was split into 2 halves) were harvested and the tissue weighed and stored at −80°C. Thereafter, one brain hemisphere and one half of the cerebellum were homogenized in ×10 radioimmunoprecipitation assay lysis buffer (Millipore, Catalogue no. 20-188), with added protease inhibitor cocktail (Sigma P8340; Sigma, St. Louis, MO) at 100 μL per 10 mL of lysis buffer. The mixture was left on a shaker for 45 minutes at 4°C and then centrifuged at 13,000*g* for 20 minutes at room temperature and the supernatant removed. The concentrations of Meteorin in plasma and brain samples were analyzed in duplicates according to the manufacturer instructions using a commercially available enzyme-linked immunospecific assay and reagent kit (Catalogue no.'s DY3475 and DY008) purchased from R&D Systems, Minneapolis, MN.

2.7. Drugs

Recombinant Meteorin (Catalogue no. 3475-MN) was purchased from R&D Systems Inc. Briefly, the sequence encoding mouse Meteorin (Q8C1Q4) was cloned and expressed in an NS0 mouse myeloma cell line. The recombinant mouse Meteorin was purified from the conditioned medium by ion exchange, hydrophobic interaction, and size exclusion chromatography. The buffer was exchanged into phosphate buffered saline (PBS) and the protein solution stored at −80°C. The purity of the preparation was 95% determined by densitometry scan. The bioactivity of Meteorin was confirmed by measuring its ability to enhance neurite outgrowth of E16 to E18 rat embryonic cortical neurons. Accordingly, Meteorin administered in the range of 60 to 300 ng/3 μL was shown to enhance neurite outgrowth (https://www.rndsystems.com/products/recombinant-mouse-meteorin-protein_3475-mn#product-datasheets). For pharmacology testing, Meteorin was dissolved in Dulbeccos PBS freshly each day at room temperature and injected s.c. at 5 mL/kg. The s.c. injections were made at the nape of the neck while the skin was gently pulled up. Gabapentin (Catalogue no. PHR1049; Sigma) was dissolved in deionized water and dosed orally at 2 mL/kg.

2.8. Data analysis

The statistical significance of differences between mean values was determined by either parametric or nonparametric analysis of variance followed by post hoc comparisons (the Dunnett or Tukey test) and/or the unpaired *t* test using GraphPad Prism 7. Differences were considered to be significant if $P < 0.05$. A specific power analysis was not used for estimation of group sizes in the current study. Rather, the group size was set to 8 based on empirical experience in our laboratory; similar group sizes (n = 8 for photochemical assay and n = 6 for CCI assay) were reported by Jorgensen et al. (2012). One rat was removed from the study because it failed to develop mechanical hyperalgesia at day 10 after CCI before administration of any treatment.

3. Results

3.1. Meteorin reversed mechanical hyperalgesia after chronic constriction injury

Two doses of recombinant mouse Meteorin (0.5 and 1.8 mg/kg, s.c.) or vehicle were given at days 10, 12, 14, 17, and 19 after CCI. Paw withdrawal threshold to mechanical pressure was assessed using the Randall–Selitto apparatus before the surgery and then on the same days of drug treatment after surgery before dosing. Behavior was subsequently monitored at days 21, 26, 32, 39, and 42 after CCI (**Fig. 2**).

Nerve injury produced a long-lasting reduction of the ipsilateral hindpaw mechanical threshold compared with the naive baseline ($P < 0.0001$) in CCI rats. The mean withdrawal threshold of the ipsilateral hindpaw was significantly reduced from a baseline of 194.75 ± 4.54 to 146.25 ± 4.30 g (day 10) after CCI and remained lower in the CCI vehicle-treated group at all the time points measured (**Figs. 2A and B**. *$P < 0.0001$ compared with baseline in the same group), while repeated s.c. administration of Meteorin reversed mechanical hyperalgesia in an apparent dose-related manner as the higher dose showed better efficacy than the lower dose. We did not construct a dose–response curve in these studies. The reduction of the mechanical hyperalgesia was significant at days 12, 14, 17, 19, 21, 26, 32, 39, and 42 compared with predose baseline (day 10 after CCI) and at days 14, 17, 19, 21, 26, and 32 compared with the vehicle control at the same time point after the highest dose (1.8 mg/kg, s.c.) of Meteorin tested ($P < 0.0001$). The peak effect (~100% antihyperalgesia) was reached at day 17 after 3 doses, and the antihyperalgesic effect was significant for more than 2 weeks after the termination of the treatment. The antihyperalgesic effect diminished at day 39 after CCI (20 days after the last dose of Meteorin). The lower dose (0.5 mg/kg, s.c.) of Meteorin produced ~60% reduction of mechanical hyperalgesia at days 14, 17, 21, 26, 32, and 39 after CCI compared with predose baseline ($P < 0.01$), and the antihyperalgesic effect was significantly different from vehicle treatment at days 14 and 17 ($P < 0.05$).

No significant change of mechanical threshold was observed at day 10 after CCI in the contralateral hindpaw (**Fig. 2C**). After the treatment, there was a small, but significant reduction of the paw withdrawal threshold at days 17 and 19 after CCI in the vehicle-treated group and at day 17 after low-dose Meteorin treatment compared with predose baseline at day 10, respectively ($P < 0.05$). However, no significant change was observed after high-dose Meteorin treatment ($P > 0.05$). No significant differences were detected between the treatment groups ($P > 0.05$), and no analgesic effects were observed because no treatment raised the paw withdrawal threshold above the presurgery baseline.

No observable side effects were noted during the entire study from any treatment groups. The animals' coat was smooth. Their locomotion and social behaviors appeared normal. No audible vocalization when handled. The body weight of the animals did not differ among groups during the course of the study (**Fig. 2D**).

3.2. Meteorin reversed cold allodynia after chronic constriction injury

The same study design was adopted to determine the guarding responses to noxious cold stimuli after CCI (**Fig. 3**). The frequency and accumulated duration of guarding were assessed using a cold metal plate set at 4°C before the surgery, and responses were assessed before the assessment of mechanical hyperalgesia on the same postsurgical days. By day 10 after CCI, cold allodynia developed in the ipsilateral paw of CCI rats manifested

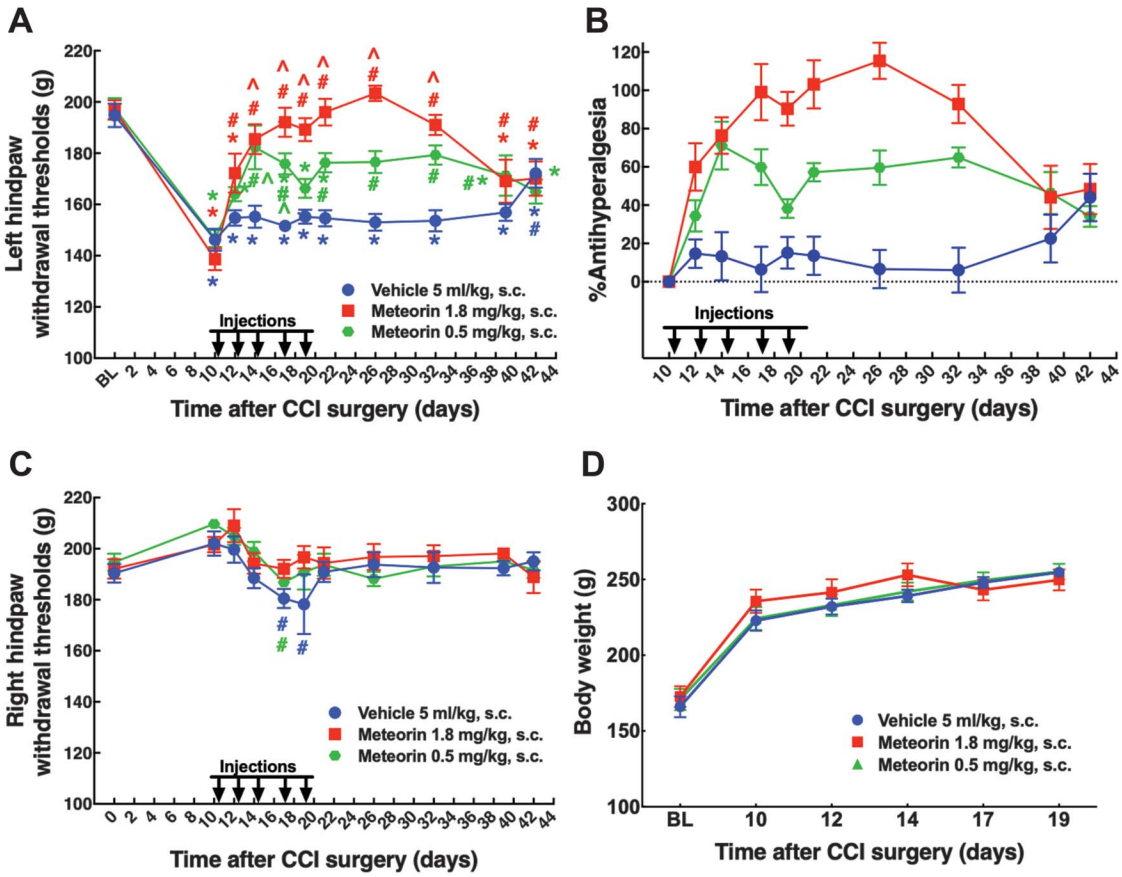

Figure 2. The effect of Meteorin on mechanical hyperalgesia induced by CCI in male SD rats. Hindpaw withdrawal thresholds over time were assessed before and after CCI surgery at days 10, 12, 14, 17, 19, 21, 26, 32, 39, and 42 using a Randall–Selitto apparatus. Meteorin (0.5, 1.8 mg/kg, s.c.) or vehicle (Dulbecco's PBS, 5 mL/kg, s.c.) was given at days 10, 12, 14, 17, and 19 after CCI immediately after hindpaw mechanical responses had been assessed. (A) Left (ipsilateral to the nerve injury) hindpaw withdrawal thresholds. (B) %Antihyperalgesia of the left hindpaw. (C) Withdrawal threshold of the right hindpaw. (D) Chronological changes of body weights. Chronic constriction injury induced robust mechanical hyperalgesia compared with presurgery (naive) baseline (*$P < 0.05$). Meteorin reversed mechanical hyperalgesia compared with predose baseline (#$P < 0.05$) or vehicle control (^$P < 0.05$) at the same time point. A small, yet significant, change in the contralateral paw withdrawal threshold was observed in the vehicle-treated group at days 17 and 19 after CCI. This change was not observed in the high-dose (1.8 mg/kg) Meteorin-treated group. The body weights were comparable among all treatment groups. The repeated-measure two-way ANOVA post hoc Tukey multiple comparison test was used for statistical analysis. N = 7 to 8/group. ANOVA, analysis of variance; BL, naive baseline (day -1); CCI, chronic constriction injury; s.c., subcutaneously; SD, Sprague-Dawley.

as increases of total number of guarding frequency and accumulated guarding duration in 2 minutes to noxious cold stimuli on the 4°C cold plate (**Fig. 3**). The cold response diminished over time in the vehicle-treated group, suggesting resolution of cold allodynia or adaptation to the testing apparatus. Meteorin at the dose of 1.8 mg/kg shortened the accumulated guarding duration at days 12, 14, 17, 19, 21, 26, and 42 (**Figs. 3A and B**) and decreased the guarding frequency at day 19 (**Figs. 3C and D**) ($P < 0.05$ compared with day 10 after CCI before the drug treatment). No significant effects were observed after 0.5 mg/kg Meteorin treatment.

3.3. Gabapentin reversed mechanical hyperalgesia and cold allodynia of the ipsilateral paw

Gabapentin is a first-line therapy for neuropathic pain and was used here as a standard reference comparator. Therefore, oral gabapentin (100 mg/kg) was used as a positive control at the end of the studies. Rats previously treated with vehicle for Meteorin were tested at days 42 to 45 after CCI using a crossover design, so that rats that received gabapentin first were given vehicle after 3 days and vice versa allowing for assessment of the antihyperalgesic effects of gabapentin. Two hours after treatment, gabapentin significantly raised the withdrawal threshold of the ipsilateral (left) hindpaw in CCI rats compared with predose (day 10) baseline or vehicle control ($P < 0.0001$, **Fig. 4A**). % Antihyperalgesia of the ipsilateral hindpaw showed 100% reversal of the mechanical hyperalgesia by gabapentin (**Fig. 4B**). No change was observed to the contralateral (right) hindpaw by gabapentin ($P > 0.05$, data not shown). Similarly, gabapentin significantly decreased cold allodynia induced by CCI. At 2 hours after dose, the guarding duration (**Fig. 4C**) and frequency (**Fig. 4D**) were significantly lower than that of vehicle control ($P < 0.05$).

3.4. Levels of Meteorin in rat plasma and brain after acute and repeated systemic administration

Separate cohorts of naive rats were dosed with acute (at day 1) or repeated Meteorin (at days 1, 3, 5, 8, and 10), and their blood and brain samples were harvested for the assessment of plasma and brain exposure (**Fig. 5**). Accordingly, 4 hours after acute injection at day 1, the plasma concentration of Meteorin was 416.7 ± 24.5 ng/mL, and 4 hours after the last injection after repeated administration at day 10, the plasma concentration was 713 ± 46.8 ng/mL. By contrast, in the same rats, only very low levels of mouse Meteorin were detected in the brain parenchyma after

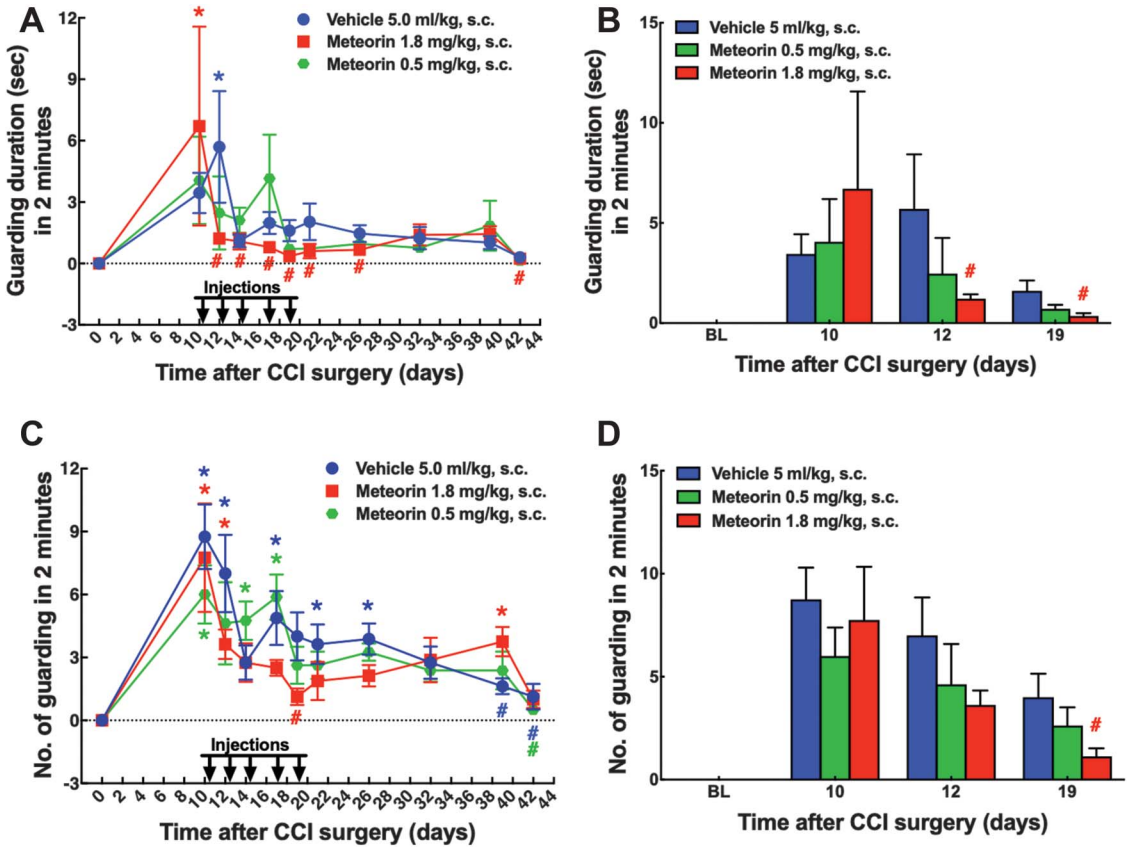

Figure 3. The effect of systemic Meteorin on cold allodynia induced by CCI in male SD rats. The response of the ipsilateral (left) hindpaw in 2 minutes to a noxious cold stimulus on a 4°C cold plate was assessed before and after CCI surgery at days 10, 12, 14, 17, 19, 21, 26, 32, 39, and 42. Meteorin (0.5, 1.8 mg/kg, s.c.) or vehicle (Dulbecco's PBS, 5 mL/kg, s.c.) was given at days 10, 12, 14, 17, and 19 after CCI. (A) Accumulated duration of guarding. (B) Bar graph illustrating the change of guarding duration at early and peak efficacy time points. (C) Guarding frequency. (D) Bar graph illustrating the change of guarding frequency at early and peak efficacy time points. Chronic constriction injury induced significant cold allodynia as assessed using both measures (*$P < 0.05$ compared with presurgery [naive] baseline). This effect diminished over time with repeated measurement. High-dose Meteorin (1.8 mg/kg) significantly reduced the guarding duration at days 12, 14, 17, 19, 21, 26, and 42 and guarding frequency at day 19 after CCI compared with predose baseline (#$P < 0.05$). The data were analyzed using the repeated-measure two-way ANOVA with the Tukey multiple comparison test (A) and the Friedman test with the Dunn multiple comparison test (B). N = 8/group. ANOVA, analysis of variance; CCI, chronic constriction injury; s.c., subcutaneously; SD, Sprague-Dawley.

acute (0.97 ± 0.04 ng/mL/g brain tissue) and repeated (1.06 ± 0.16 ng/mL/g brain tissue) Meteorin administration.

4. Discussion

The data from this study have replicated key features and conclusions supporting Meteorin efficacy in neuropathic pain as originally reported by Jorgensen et al.[13] These include reversal of mechanical and cold hypersensitivity, time to onset of analgesic efficacy, and prolonged duration of action upon cessation of Meteorin treatment. Importantly, a strict blinding regimen was used to eliminate potential experimenter bias that could interfere with interpretation of the results, and the experiments were performed using well-characterized biological material.

We adopted the overall study design used in the original report of Jorgensen et al. (**Fig. 1** and **Table 1**). Accordingly, male Sprague-Dawley rats were subjected to CCI and, upon developing appropriate neuropathic hypersensitivity, were then administered recombinant mouse Meteorin at the same time point (day 10) after injury. We ensured that Meteorin was sourced from the same vendor, wherein different protein batches were provided as a unit of mass per vial (25 μg) with a specific activity range estimated using the same neurite extension bioassay. Thereafter, identical handling (eg, avoidance of freeze-thawing, etc.), formulation, and dosing of Meteorin were followed, a process that was facilitated by one of the original authors of the Jorgensen article in the current study (G.M.). Thus, we are confident that the quality and functional bioactivity of Meteorin were as similar as possible in the 2 independent studies.

Nevertheless, a number of protocol deviations did occur as highlighted in **Table 1**. We obtained our rats from Envigo as this is the provider we standardly use for experimental research. Sprague-Dawley rats are an outbred strain and have been reported to develop different levels of neuropathic hypersensitivity and sensitivity to analgesics after CCI depending on the vendor from which they are sourced.[16] However, we do not believe this to have been an issue per se, since if the effect size of Meteorin could not have overcome the potential difference among Sprague-Dawley rats from different vendors, the translational value of this study would have been highly diminished. Another key difference is that we measured mechanical and thermal hindpaw thresholds using distinct reflex sensory testing methods. Numerous reports have described the presence of mechanical allodynia after CCI injury, albeit a wide range of effect magnitude has been reported.[6,8,15,19] However, previous studies in our laboratory have not demonstrated a consistently reproducible tactile allodynia in the CCI model. Moreover, data from many previously reported studies may reflect the presence of

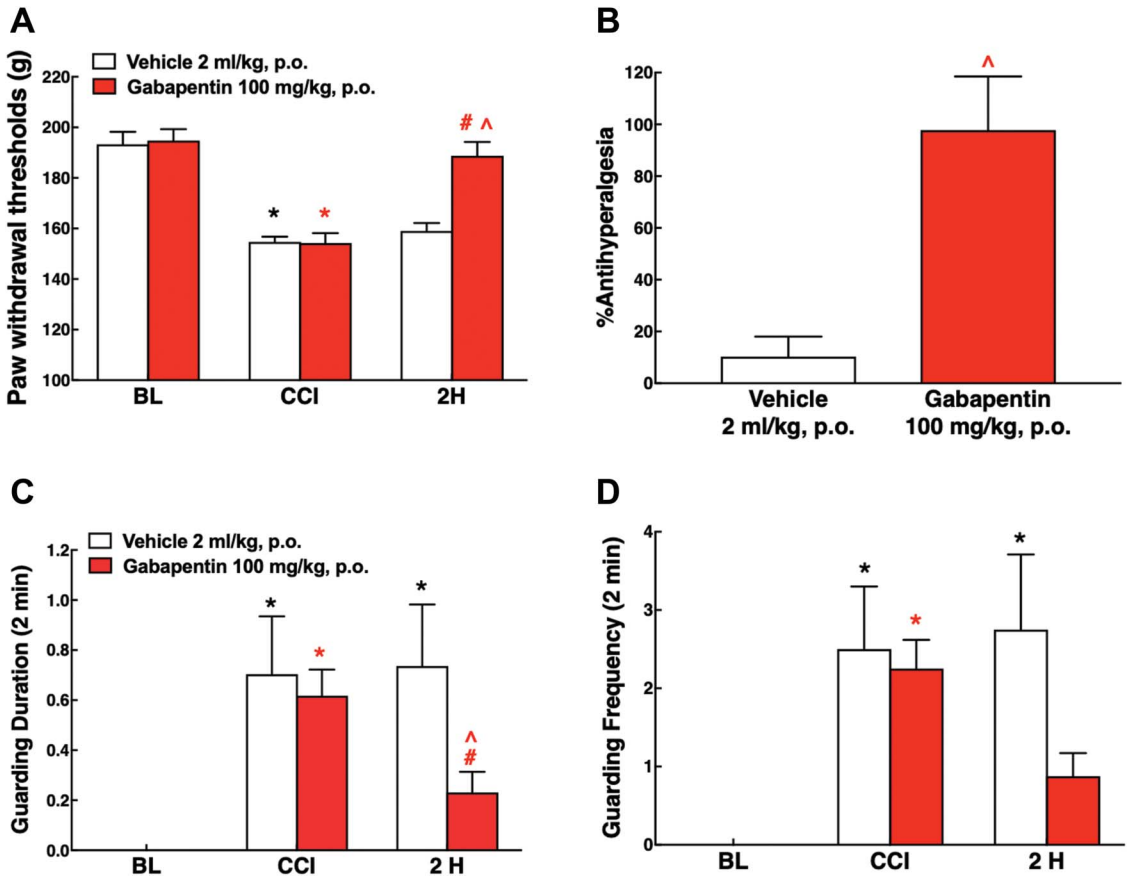

Figure 4. Antihyperalgesic effects of gabapentin on mechanical hyperalgesia in CCI rats. At day 45 after CCI injury, the ipsilateral paw withdrawal threshold (A and B) to a noxious mechanical stimulus, and guarding duration (C) and frequency (D) to a noxious cold stimulus were measured before and 2 hours after gabapentin (100 mg/kg, p.o.) or vehicle (DiH_2O, 2 mL/kg, p.o.) administration. Chronic constriction injury induced significant mechanical hyperalgesia (A and B) and cold allodynia (C and D) for both measures (*$P < 0.05$ compared with presurgery [naive] baseline). Gabapentin significantly reversed CCI-induced mechanical hyperalgesia in the ipsilateral hindpaw compared with predose baseline (#$P < 0.05$) or vehicle control (^$P < 0.05$). The data were analyzed using the repeated-measure two-way ANOVA post hoc Tukey multiple comparison test (A, C, and D) or unpaired *t* test (B). N = 8/group. ANOVA, analysis of variance; BL, naive baseline (day -1); CCI, chronic constriction injury (D45 after surgery); p.o., orally.

mechanical hyperalgesia rather than allodynia per se, given that the upper range of von Frey filaments used evoked a reflex nociceptive response at baseline before injury which by definition excludes allodynia.[17,20,27–31,36] Again, pragmatic reasons informed our decision to assess the effect of Meteorin on cold allodynia in CCI rats compared with the thermal hyperalgesia reported by Jorgensen et al. (2012);[13] note that Meteorin was shown to attenuate cold hypersensitivity in a rat model of photochemical-induced ischaemic neuropathic pain by the same authors. Although multiple studies report the presence of heat hyperalgesia in the CCI model,[35–37] we have previously observed that the injured hindpaw displays a curved plantar surface (ie, "cupping") that does not easily allow for focused application of the radiant heat stimulus to the injured paw.

Despite the differences in the sensory output measures evaluated, our results remain highly consistent with those reported by Jorgensen et al. Thus, we observed that Meteorin produces an inhibition of neuropathic hypersensitivity in CCI rats that reached a peak effect of almost 100% reversal to baseline after the third injection of Meteorin. In addition, we also observed similar efficacy against mechanical and cold endpoints with gabapentin at study end, the inclusion of which helps align the sensitivity of our assay testing conditions with other laboratories worldwide. However, although Meteorin efficacy was originally described as being relatively slow in onset, only becoming clearly apparent after 2 injections, statistical analysis of the current data with an all pairwise test (which allowed for comparison with the corresponding predose baseline threshold value) revealed that the high dose of Meteorin alleviated mechanical hypersensitivity already after the first injection. We do not believe that this finding can be attributed to different exposure levels of Meteorin between the 2 studies because pharmacokinetic analysis of blood at 4 hours after either acute or 5 repeated s.c. injections of Meteorin confirmed that plasma levels in the current study aligned with those reported previously.[13] While Meteorin seemed to accumulate in plasma after repeated injections, plasma concentrations in the ng/mL range are cleared within 24 hours[13] and so are highly unlikely to account for the profound efficacy obtained with Meteorin that persisted >2 weeks after termination of the treatment in each study. Considered together, our data not only provide confirmation of Meteorin efficacy in this preclinical model, but also increase our confidence in the efficacy of Meteorin in neuropathic pain because its effects are not limited to specific sensory modalities or observable under specific laboratory conditions.

It is noteworthy that brain levels of Meteorin were about 500-fold lower than in plasma, a finding consistent with the size and structure of Meteorin. Moreover, the measured brain concentrations were in the order of 20-fold lower than that required to mediate neurite extension in cultured DRG explants (EC_{50} = 20 ng/mL).[21] Taken together, this suggests that systemically

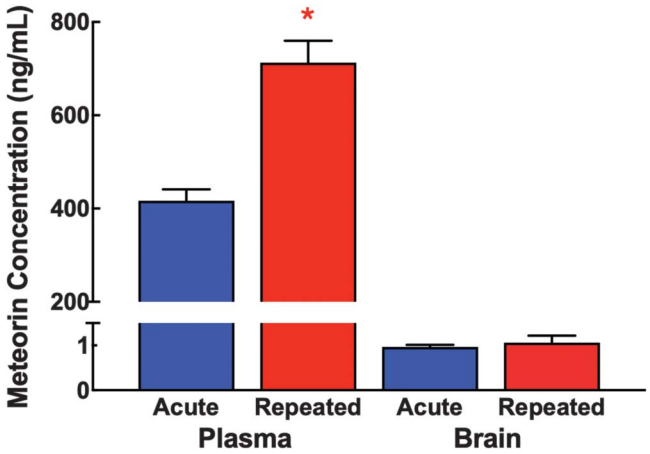

Figure 5. Meteorin concentrations in the rat plasma and brain. Plasma and brain samples were obtained from naive rats at 4 hours after the acute injection or 4 hours after the last injection of Meteorin (1.8 mg/kg, s.c.) after 5 repeated administration at days 1, 3, 5, 8, and 10. Data are presented as mean ± SEM. The concentrations of Meteorin were determined by enzyme-linked immunospecific assay. *$P < 0.05$ compared with acute administration in plasma. The unpaired *t* test was used (N = 3/group).

administered Meteorin most likely has a peripheral site of action, albeit it does not readily inform on potential underlying mechanism(s) of action. Although the receptor for Meteorin remains to be identified, potential insights can be gleamed from other studies showing that it promotes endothelial maturation,[23] angiogenesis,[23] and glial cell differentiation.[21,34] Peripheral nerve injury can induce morphological and plasticity changes in glial cells within DRG which in turn have been linked to spinal cord microglial activation and subsequent central sensitization.[9,10,18] Meteorin on the other hand induces axonal extension of peripheral sensory neurons in DRG[11] and trigeminal cell cultures through a process that also seems to involve activation of neighbouring satellite glia possibly promoting secondary effects that are either directly neuritogenic, act in concert with Meteorin, or increase the responsiveness of injured neurones to other trophic factors.[21] It is reasonable to speculate that these processes might be relevant in explaining the slow onset and long-lasting effects of Meteorin to alleviate tactile and cold hypersensitivity after CCI but such conclusions require future investigation.

We also measured mechanical thresholds for the uninjured hindpaw of CCI rats in our experiment. Typically, this measure is not reported in experimental studies involving neuropathic pain models. We noted that the mechanical pressure threshold for the uninjured hindpaw showed a small, yet significant, reduction at days 17 and 19 after CCI that was reversed by Meteorin. Whether this small decrease of mechanical threshold is an experimental artifact or a consequence of central sensitization, possibly reflecting mirror pain, remains to be further elucidated. Significant changes of contralateral hindpaw mechanical thresholds have been reported previously in a sciatic nerve CCI induced by a cuff injury in rats.[24] In our study, the magnitude of the paw pressure threshold reduction in the contralateral hindpaw was small compared with that of the ipsilateral hindpaw. Whether this statistically significant reduction is biologically relevant remains to be explored. Nonetheless, it highlights the value of assessing thresholds for both the ipsilateral and contralateral hindpaw in such models.

In conclusion, our study has replicated key findings of a single previous preclinical study supporting the potential efficacy of Meteorin in the treatment of neuropathic pain. The consistency of results, despite inherent protocol deviations, suggests that the biological effects are robust, with further studies required to

Table 1

Comparison of key features of rat CCI study design and methods with Jorgensen et al.

	Current study	Jorgensen et al., 2012
Housing conditions	Temperature, light, food, cage size, and enrichment provided	No information provided
Body weight	Male SD rats starting weight at 175-200 g sourced from Envigo	Male SD rats starting weight at 250-280 g sourced from Charles River Laboratories*
Rat model	CCI with 4 loose ligatures (about 2 mm apart) using 4-0 chromic gut sutures	CCI with 4 loose ligatures (about 1 mm apart) using 4-0 chromic gut sutures
Output measures	Cold allodynia using 4°C cold plate (innocuous cold stimuli); mechanical hyperalgesia (noxious pressure stimuli) using the Randall–Selitto test	Thermal hyperalgesia using Hargreaves apparatus (noxious heat stimuli); mechanical allodynia using von Frey filaments (innocuous tactile stimuli); and weight-bearing using incapacitance meter
Source of Meteorin	R&D systems	R&D systems
Dosing regimen of Meteorin	0.5 and 1.8 mg/kg, s.c.; 5 injections at days 10, 12, 14, 17, and 19 after CCI	0.1, 0.5, and 1.8 mg/kg, s.c.; 5 injections at days 10, 12, 14, 17, and 19 after CCI
Testing time points	Behaviors were monitored at baseline and days 10, 12, 14, 17, 19, 21, 26, 32, and 39 after CCI before each Meteorin administration.	Behaviors were monitored at baseline and days 10, 12, 14, 17, 19, 21, 26, 32, and 39 after CCI before each Meteorin administration.
Blinding methods	The behavioral tests were evaluated by an experimenter blinded to the treatment groups and subsequent data analysis.	The behavioral tests were evaluated by an experimenter blinded to the treatment groups.
Pharmacokinetic study	Terminal plasma and brain samples were collected from trunk blood at 4 hours after the acute or repeated administration of Meteorin (1.8 mg/kg, s.c.) in naive rats.	Serial plasma samples were collected from awake naive rats via a jugular vein catheter at 0, 0.5, 2, 7, 24, 31, 48, and 72 hours after an acute dose of Meteorin (0.5 or 2 mg/kg, s.c.) treatment.

* Vendor used for CCI rats is assumed based on the vendor used for providing rats for Meteorin pharmacokinetic analysis.
CCI, chronic constriction injury; s.c., subcutaneously; SD, Sprague-Dawley.

uncover the underlying mechanism(s) for the observed effects. The replication of Meteorin efficacy across independent laboratories also provides support for future development of this molecule as a potential therapy.

Conflict of interest statement

The authors have no conflict of interest to declare.

Acknowledgements

Funding for laboratory costs of performing these studies was provided by Hoba Therapeutics. G. Munro and K.A. Petersen are employees at Hoba Therapeutics.

Article history:
Received 17 December 2018
Received in revised form 21 February 2019
Accepted 6 March 2019
Available online 21 March 2019

References

[1] Baker M. 1,500 scientists lift the lid on reproducibility. Nature 2016;533:452–4.
[2] Begley CG, Ellis LM. Drug development: raise standards for preclinical cancer research. Nature 2012;483:531–3.
[3] Benjamin D, Mandel DR, Kimmelman J. Can cancer researchers accurately judge whether preclinical reports will reproduce? PLoS Biol 2017;15:e2002212.
[4] Bennett GJ, Xie YK. A peripheral mononeuropathy in rat that produces disorders of pain sensation like those seen in man. PAIN 1988;33:87–107.
[5] Collins FS, Tabak LA. Policy: NIH plans to enhance reproducibility. Nature 2014;505:612–13.
[6] Ding Y, Yao P, Hong T, Li H, Zhu Y, Han Z, Zhou G. The analgesic effect of early hyperbaric oxygen treatment in chronic constriction injury rats and its influence on nNOS and iNOS expression and inflammatory factor production. Mol Pain 2018;14:1744806918765837.
[7] Fiala C, Diamandis EP. How to reduce scientific irreproducibility: the 5-year reflection. Clin Chem Lab Med 2017;55:1845–8.
[8] Ghoreishi-Haack N, Priebe JM, Aguado JD, Colechio EM, Burgdorf JS, Bowers MS, Cearley CN, Khan MA, Moskal JR. NYX-2925 is a novel N-Methyl-d-Aspartate receptor modulator that induces rapid and long-lasting analgesia in rat models of neuropathic pain. J Pharmacol Exp Ther 2018;366:485–97.
[9] Goncalves NP, Vaegter CB, Pallesen LT. Peripheral glial cells in the development of diabetic neuropathy. Front Neurol 2018;9:268.
[10] Hanani M, Huang TY, Cherkas PS, Ledda M, Pannese E. Glial cell plasticity in sensory ganglia induced by nerve damage. Neuroscience 2002;114:279–83.
[11] Jorgensen JR, Fransson A, Fjord-Larsen L, Thompson LH, Houchins JP, Andrade N, Torp M, Kalkkinen N, Andersson E, Lindvall O, Ulfendahl M, Brunak S, Johansen TE, Wahlberg LU. Cometin is a novel neurotrophic factor that promotes neurite outgrowth and neuroblast migration in vitro and supports survival of spiral ganglion neurons in vivo. Exp Neurol 2012;233:172–81.
[12] Jorgensen JR, Thompson L, Fjord-Larsen L, Krabbe C, Torp M, Kalkkinen N, Hansen C, Wahlberg L. Characterization of Meteorin—an evolutionary conserved neurotrophic factor. J Mol Neurosci 2009;39:104–16.
[13] Jorgensen JR, Xu XJ, Arnold HM, Munro G, Hao JX, Pepinsky B, Huang C, Gong BJ, Wiesenfeld-Hallin Z, Wahlberg LU, Johansen TE. Meteorin reverses hypersensitivity in rat models of neuropathic pain. Exp Neurol 2012;237:260–6.
[14] Kandela I, Aird F; Reproducibility Project: Cancer B. Replication Study: discovery and preclinical validation of drug indications using compendia of public gene expression data. Elife 2017;6:e17044.
[15] Kashkin VA, Shekunova EV, Titov MI, Eliseev II, Gureev MA, Porozov YB, Makarova MN, Makarov VG. A new tridecapeptide with an octaarginine vector has analgesic therapeutic potential and prevents morphine-induced tolerance. Peptides 2018;99:61–9.
[16] Kristensen PJ, Heegaard AM, Hestehave S, Jeggo RD, Bjerrum OJ, Munro G. Vendor-derived differences in injury-induced pain phenotype and pharmacology of Sprague-Dawley rats: does it matter? Eur J Pain 2017;21:692–704.
[17] Li SS, Tu WZ, Jia CQ, Jiang X, Qian XR, Yang GH, Hu QM, Chen WC, Lu B, Jiang SH. KCC2-GABAA pathway correlates with the analgesic effect of electro-acupuncture in CCI rats. Mol Med Rep 2018;17:6961–8.
[18] Lim H, Lee H, Noh K, Lee SJ. IKK/NF-kappaB-dependent satellite glia activation induces spinal cord microglia activation and neuropathic pain after nerve injury. PAIN 2017;158:1666–77.
[19] Lin JP, Chen CQ, Huang LE, Li NN, Yang Y, Zhu SM, Yao YX. Dexmedetomidine attenuates neuropathic pain by inhibiting P2X7R expression and ERK phosphorylation in rats. Exp Neurobiol 2018;27:267–76.
[20] Liu H, Zhou J, Gu L, Zuo Y. The change of HCN1/HCN2 mRNA expression in peripheral nerve after chronic constriction injury induced neuropathy followed by pulsed electromagnetic field therapy. Oncotarget 2017;8:1110–16.
[21] Nishino J, Yamashita K, Hashiguchi H, Fujii H, Shimazaki T, Hamada H. Meteorin: a secreted protein that regulates glial cell differentiation and promotes axonal extension. Embo J 2004;23:1998–2008.
[22] Open Science C. Psychology: estimating the reproducibility of psychological science. Science 2015;349:aac4716.
[23] Park JA, Lee HS, Ko KJ, Park SY, Kim JH, Choe G, Kweon HS, Song HS, Ahn JC, Yu YS, Kim KW. Meteorin regulates angiogenesis at the gliovascular interface. Glia 2008;56:247–58.
[24] Pitcher GM, Ritchie J, Henry JL. Nerve constriction in the rat: model of neuropathic, surgical and central pain. PAIN 1999;83:37–46.
[25] Poldrack RA, Baker CI, Durnez J, Gorgolewski KJ, Matthews PM, Munafo MR, Nichols TE, Poline JB, Vul E, Yarkoni T. Scanning the horizon: towards transparent and reproducible neuroimaging research. Nat Rev Neurosci 2017;18:115–26.
[26] Randall LO, Selitto JJ. A method for measurement of analgesic activity on inflamed tissue. Arch Int Pharmacodyn Ther 1957;111:409–19.
[27] Ren H, Jin H, Jia Z, Ji N, Luo F. Pulsed radiofrequency applied to the sciatic nerve improves neuropathic pain by down-regulating the expression of calcitonin gene-related peptide in the dorsal root ganglion. Int J Med Sci 2018;15:153–60.
[28] Riffel APK, Santos MCQ, de Souza JA, Scheid T, Horst A, Kolberg C, Bello-Klein A, Partata WA. Treatment with ascorbic acid and alpha-tocopherol modulates oxidative-stress markers in the spinal cord of rats with neuropathic pain. Braz J Med Biol Res 2018;51:e7097.
[29] Rutten K, Gould SA, Bryden L, Doods H, Christoph T, Pekcec A. Standard analgesics reverse burrowing deficits in a rat CCI model of neuropathic pain, but not in models of type 1 and type 2 diabetes-induced neuropathic pain. Behav Brain Res 2018;350:129–38.
[30] Scheid T, Moraes MS, Henriques TP, Riffel APK, Bello-Klein A, Poser GLV, Ethur EM, Partata WA. Effects of methanol fraction from leaves of Schinus terebinthifolius raddi on nociception and spinal-cord oxidative biomarkers in rats with neuropathic pain. Evid Based Complement Alternat Med 2018;2018:5783412.
[31] Sumizono M, Sakakima H, Otsuka S, Terashi T, Nakanishi K, Ueda K, Takada S, Kikuchi K. The effect of exercise frequency on neuropathic pain and pain-related cellular reactions in the spinal cord and midbrain in a rat sciatic nerve injury model. J Pain Res 2018;11:281–91.
[32] Tierney W, Schweinsberg M, Jordan J, Kennedy DM, Qureshi I, Sommer SA, Thornley N, Madan N, Vianello M, Awtrey E, Zhu LL, Diermeier D, Heinze JE, Srinivasan M, Tannenbaum D, Bivolaru E, Dana J, Davis-Stober CP, du Plessis C, Gronau QF, Hafenbrack AC, Liao EY, Ly A, Marsman M, Murase T, Schaerer M, Tworek CM, Wagenmakers EJ, Wong L, Anderson T, Bauman CW, Bedwell WL, Brescoll V, Canavan A, Chandler JJ, Cheries E, Cheryan S, Cheung F, Cimpian A, Clark MA, Cordon D, Cushman F, Ditto PH, Amell A, Frick SE, Gamez-Djokic M, Grady RH, Graham J, Gu J, Hahn A, Hanson BE, Hartwich NJ, Hein K, Inbar Y, Jiang L, Kellogg T, Legate N, Luoma TP, Maibeucher H, Meindl P, Miles J, Mislin A, Molden DC, Motyl M, Newman G, Ngo HH, Packham H, Ramsay PS, Ray JL, Sackett AM, Sellier AL, Sokolova T, Sowden W, Storage D, Sun X, Van Bavel JJ, Washburn AN, Wei C, Wetter E, Wilson CT, Darroux SC, Uhlmann EL. Data from a pre-publication independent replication initiative examining ten moral judgement effects. Sci Data 2016;3:160082.
[33] Voelkl B, Vogt L, Sena ES, Wurbel H. Reproducibility of preclinical animal research improves with heterogeneity of study samples. PLoS Biol 2018;16:e2003693.

[34] Wright JL, Ermine CM, Jorgensen JR, Parish CL, Thompson LH. Over-Expression of Meteorin drives gliogenesis following striatal injury. Front Cell Neurosci 2016;10:177.
[35] Zhang YL, Liu YG, Chen DJ, Yang BL, Liu TT, Li JJ, Wang XQ, Li HR, Liu ZX. Microencapsulated Schwann cell transplantation inhibits P2X2/3 receptors overexpression in a sciatic nerve injury rat model with neuropathic pain. Neurosci Lett 2018;676:51–7.
[36] Zhao H, Liu S, Wang C, Wang Q, Liu W, Gong M. Contralateral monoarthritis exacerbated chronic constriction injury-induced pain hypersensitivity through upregulating inducible nitric oxide synthase. J Pain Res 2018;11:1433–43.
[37] Zhao Y, Yang Z. Effect of Wnt signaling pathway on pathogenesis and intervention of neuropathic pain. Exp Ther Med 2018;16: 3082–8.