Chemokine CXCL10 regulates pain behaviors via PI3K-AKT signaling pathway in mice

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ABSTRACT

The analgesic efficacy of morphine can be affected by a variety of factors. Our previous studies demonstrated that chemokine (CXC motif) ligand 10 (CXCL10) could induce algesia directly and attenuate the analgesic effect produced by a single dose of morphine. However, the underlying mechanism remains unclear. In the present study, we aimed to further investigate the mechanism of CXCL10-mediated inhibition on morphine analgesic effect. According to our findings, recombinant CXCL10 protein (rmCXCL10) could increase the phosphorylation of serine-threonine kinase AKT reduced by morphine in spinal cord. Blocking AKT activation by phosphoinositide 3-kinase (PI3K) inhibitor could effectively attenuate CXCL10-induced algesia, and reverse the decrease of paw withdrawal thresholds caused by the co-administration of morphine and rmCXCL10. Furthermore, rmCXCL10 could enhance the spinal expression of pro-inflammatory cytokines, including TNF-α, IL-6, and IL-1β, which could be blocked by PI3K inhibitor. In summary, these findings suggest that PI3K-AKT signaling pathway mediates the effect of CXCL10 on the regulation of morphine analgesia and the release of cytokines in spinal cord. Our study provides a new insight into the mechanism of chemokine-relative pain regulation.

1. Introduction

Despite a long time of research and progress, pain is still an important issue need to be resolved in the medical field. The worldwide prevalence of pain is estimated at 56% among the elderly and 41% in the general population (Jackson et al., 2015; Tsang et al., 2008). Although opioids, like morphine, are commonly used to alleviate acute and chronic pain, their analgesic effects remain highly unsatisfactory due to the complex endogenous molecular-cellular network (e.g. orexin receptor, carbon monoxide-releasing molecule), and the interference of exogenous agents (e.g. Harpagophytum procumbens extract, pioglitazone).

Chemokine is a family of small cytokines which was identified recently as novel regulators of morphine analgesia and tolerance (Campbell et al., 2013; Chen et al., 2007). As a member of CXC chemokine family, chemokine (CXC motif) ligand 10 (CXCL10) was previously reported by our studies showing an up-regulated expression with its receptor CXCR3 in cancer-induced bone pain and morphine tolerance (Bu et al., 2014; Wang et al., 2017), as well as its abilities to arouse pain and partially antagonize the analgesic effect of morphine (Ye et al., 2014). However, the intracellular processes that responsible for the effect of CXCL10 remain elusive.

Phosphoinositide 3 kinase-AKT (PI3K-AKT) is a signaling pathway that has been implicated in a broad range of human diseases. One important issue is the role played by PI3K-AKT in opioid-induced analgesic tolerance and hyperalgesia, which is initiated by the μ opioid receptor (MOR) agonist such as morphine (Xu et al., 2014). During the past few years, this signaling pathway has been clearly demonstrated

Abbreviations: CXCL10, chemokine (CXC motif) ligand 10; rmCXCL10, recombinant CXCL10 protein; PI3K, phosphoinositide 3-kinase; CXCR3, CXC chemokine receptor 3; PI3K-AKT, Phosphoinositide 3 kinase-AKT; MOR, μ-opioid receptor; AKT-ERK, AKT-extracellular regulated kinase; DMSO, dimethyl sulfoxide; i.t., intrathecal; PWTs, paw withdrawal thresholds; qRT-PCR, quantitative real-time polymerase chain reaction; GAPDH, Glyceraldehyde-3-phosphate dehydrogenase; DAPI, 4, 6-diamidino-2-phenylindole; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; PVD, polyvinylidene fluoride; HRP, horseradish peroxidase; SEM, standard error of means; ANOVA, analysis of variance; s.c., subcutaneous; INOS, inducible nitric oxide synthase; CNS, central nervous system; CX3CR1, CX3C chemokine receptor 1.

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acting as the intracellular downstream phosphate kinases in MOR activation (Madishetti et al., 2014; Narita et al., 2002; Tian et al., 2015a). In addition, CXCL10 has been confirmed to be involved in the development of cancer-induced bone pain through AKT-extracellular regulated kinase (AKT-ERK) crosstalk pathway (Guan et al., 2015a; Guan et al., 2015b), and the interaction between CXCL10 and AKT signaling pathway was also observed in the process of glial activation (Williams et al., 2009). These aforementioned evidences lead to our speculation that morphine and CXCL10 may share the common signaling pathway PI3K-AKT for their actions in pain. Therefore, the aim of this study is to find out the potential role played by PI3K-AKT in the antagonistic effect of CXCL10 on morphine analgesia, and about their association with downstream pro-inflammatory cytokines induced by CXCL10, based on which a new therapeutic target for opioid-induced tolerance and hyperalgesia may be provided.

2. Materials and methods

2.1. Animals

All experimental procedures and protocols were subject to review and approval by Experimental Animal Care and Use Committee of Tongji Medical College, Huazhong University of Science & Technology. In addition, all the experiments were conducted in accordance with National Institutes of Health Guidelines (NIH publications No. 8023, revised 1978) for the Care and Use of Laboratory Animals and the Animal Research: Reporting of In Vivo Experiments (ARRIVE) 2009 Guidelines for Reporting Animal Research. In this study, male C57Bl/6 mice, weighing 18–22 g, were purchased from the Laboratory Animal Center of Tongji Medical College. Animals were housed under controlled conditions (22 ± 0.5 °C, relative humidity 40–60%, alternate light-dark cycles) with food and water ad libitum.

2.2. Drugs

Recombinant murine CXCL10 protein (rmCXCL10, PeproTech Inc. company, Rocky Hill, NJ, USA, Cat#250–16) was diluted by saline to 10 ng/μL. Morphine hydrochloride was diluted by saline to 1 mg/mL. LY294002 hydrochloride (Sigma, St. Louis, MO, USA, Cat#L9908), a specific inhibitor of PI3K, was diluted by 0.1% dimethyl sulphoxide (DMSO). The dosage for each drug used in this study was determined in the preliminary experiments.

2.3. Drug administration

Morphine was injected to mice subcutaneously. The intrathecal injection (i.t.) of LY294002 and rmCXCL10 was conducted using Hyden and Wilcox’s method. Briefly, mouse was gently restrained to maintain the back parallel to the ground. The needle of a 10 μL Hamilton microsyringe was punctured into subarachnoid cavity between L4 and L5 vertebrae of mice at an angle of 45 degrees. The correct puncture of needle was indicated by a slight flick of tail. Then a volume of 3 μL drug was applied through injection. The needle was removed 3 min after drug injection. The mice with hind limb paralysis or paresis after drug injection were excluded and euthanized with overdose of pentobarbital sodium. All drug-treatment groups were comprised in line with the random grouping criterion. Briefly, all the mice were ear-tagged with numerical identifiers, which corresponded to the random numbers obtained from Microsoft Excel 2013 (Microsoft Corporation, USA). Mice were split equally into different groups according to the random numbers.

2.4. Behavioral assessment

The mechanical nociceptive thresholds of mice were assessed by measuring the paw withdrawal thresholds (PWTs) via von Frey filaments as described previously (Ye et al., 2014). In simple terms, mice were tested individually within a deep rectangular stainless-steel tank and allowed 15 min of habituation prior to tests. To reduce stress, mice were handled under similar condition and tested using pain testing device for three times over a week before experiments. The region between foot pads in the plantar surface of right hind paw was stimulated by a series of von Frey filaments with logarithmically incrementing forces applied (0.008, 0.04, 0.07, 0.16, 0.4, 0.6, 1, 2, 4, 6, 8, 10 and 15 g). Abrupt paw withdrawal, licking or shaking was treated as positive response. Once a withdrawal response was established, the test would be repeated starting with the next descending von Frey filaments until no response occurred. A 10-s interval was scheduled between every two stimulations of filaments. The minimum amount of force which could trigger a response was recorded. Three trials were conducted on each mouse with a 10 min interval. The average value was treated as PWTs in grams (g).

All behavioral tests were carried out under blind conditions. The mice involved in the study were divided into different groups on a random basis, and the syringes containing different drugs were coded at random by a separate researcher. The drugs were injected in mice according to the random coding, and behavioral assessment was conducted by the researcher with no knowledge of grouping.

2.5. Quantitative real-time polymerase chain reaction (qRT-PCR)

Following deep anesthesia with 1% pentobarbital sodium (50 mg/kg, intraperitoneal injection), L3-L5 spinal cord segments of the mice were removed. Total RNA extraction and reverse transcription procedure were conducted using RNAiso Plus (Takara, Shiga, Japan, Cat#9108) according to the manufacturer’s instructions. The specific primers for mouse cytokines and endogenous control mouse Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were obtained from PrimerDepot and shown as follows (Table 1). Step One Real-Time PCR System (Applied Biosystems, Carlsbad, CA, USA) was applied to conduct the qRT-PCR. The relative quantification of mRNA was performed using 2−ΔΔCt method. The data were indicated as fold changes normalized to the control group.

2.6. Immunofluorescent staining

After being deeply anesthetized with 1% pentobarbital sodium, mice were perfused with saline, and then with 4% ice-cold paraformaldehyde. The L3-L5 spinal cord segments were removed and post-fixed for 24 h at 4 °C, prior to being dehydrated in a 30% sucrose solution. After being deeply anesthesia with 1% pentobarbital sodium, mice were perfused with saline, and then with 4% ice-cold paraformaldehyde. The L3-L5 spinal cord segments were removed and post-fixed for 24 h at 4 °C, prior to being dehydrated in a 30% sucrose solution. After being treated with 0.3% Triton X-100 and blocked with 10% donkey serum for 40 min at room temperature, 30 μm-thick sections were incubated with primary antibodies overnight at 4 °C and secondary antibodies for 3 h at room temperature and subsequently stained with 4, 6-diamidino-2-phenylindole (DAPI) for 10 min. Details of antibodies used are included as supplementary table (Suppl.). The fluorescent images were captured using Upright Fluorescence Microscope (DM2500, Leica, German). A total of five spinal sections were selected at random for each mouse and immunoreactivities were measured in a blinded fashion. The stained sections were analyzed using Image Pro Plus 4 software (Media Cybernetics, Maryland, MD, USA).

2.7. Western blots

L3-L5 spinal cord segments of mice were quickly removed for dissection. The proteins of spinal cord tissue were extracted using immunoprecipitation assay lysis buffer according to the manufacturer’s instructions (Beyotime, Wuhan, China). The protein concentration of supernatants was determined through bicinchoninic acid assay. After being denatured by boiling, 30 μg of protein derived from each sample was loaded on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel. Electrophoresis was conducted at 60 V
constant voltage for stacking gel and 100 V for separating gel. Subsequently, the proteins were electro-transferred (200 mA, 90–120 min) to a polyvinylidene fluoride (PVDF) membrane (Millipore, Bellerica, MA, USA). After being blocked with 5% bovine serum albumin for 2 h at room temperature, the membranes were incubated with specific primary antibodies overnight at 4°C followed by horseradish peroxidase (HRP)-conjugated secondary antibodies for 2 h. Antibodies used are listed in Suppl. Finally, proteins were detected by enhanced chemiluminescence detection system (Beyotime, Wuhan, China) and visualized by means of X-ray film exposure. The ImageJ analysis system (NIH, Bethesda, MD) was employed to conduct quantification for the specific bands. The levels of AKT and p-AKT were denoted as density relative to the density of GAPDH. All of the western blots were performed for a minimum of three times.

2.8. Statistical analysis

The animal sample size for behavioral experiment was determined by means of power analysis with the assistance of SSize2021 software (National University of Singapore, Singapore) (version 2). With the anticipated population proportion \( P_1 = 0.95, P_2 = 0.05 \), significance level 0.05 and power of test 0.09, the sample size was estimated as four per group. The data was indicated as the mean ± standard error of means (SEM). Behavioral test was analyzed by two-way repeated measure analysis of variance (ANOVA) (treatment group \( \times \) time) to detect overall differences among treatment groups, followed by Bonferroni’s test to detect the changes of PWTs after drug administration over time. The results of qRT-PCR and western blots were analyzed by one-way ANOVA. Individual comparisons were performed with unpaired t-test. With the statistical significance set to \( P < 0.05 \), all statistical analyses were carried out using GraphPad Prism 5.0 software (GraphPad Software Inc.).

3. Results

3.1. The co-localization of CXCR3 and MOR in spinal cord

In order to examine the cellular localization of CXCR3 and MOR in spinal cord, immunofluorescent staining was conducted in mice without any treatment as the blank control. Both CXCR3 and MOR can be detected in the dorsal horn of the spinal cord and MOR exhibited a ring-like structure. As shown by the merged images, CXCR3 was surrounded by MOR, indicating the co-localization of CXCR3 and MOR (Fig. 1). These results provided spatial evidences for the subsequent experiments to explore the intracellular interaction between CXCR3 and MOR after being activated by their agonists, respectively.

![Fig. 1. Expressions of CXCR3 and MOR in spinal cord. Co-localization of CXCR3 and MOR was detected by immunofluorescence staining in spinal dorsal horn in blank control mice. CXCR3 was surrounded by MOR as indicated by arrows (h), a and e. Immunostaining of CXCR3 (green). b and f. Immunostaining of MOR (red). c and g. Immunostaining of nuclei stained by DAPI (blue). d and h. CXCR3 merged with MOR and nuclei. Scale bar: 100 μm (a, b, c and d); scale bar: 50 μm (e, f, g and h). n = 5 mice in each group. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)](image-url)
3.2. CXCL10 and morphine could influence the phosphorylation of AKT in opposite ways

In our previous study, CXCL10 could directly induce algesia and attenuate the analgesic effect of a single dose of morphine. (Ye et al., 2014) To further investigate the potential signaling pathways associated with the effect of CXCL10, mice were treated with saline (3 μL, i.t.), rmCXCL10 (30 ng, i.t.), morphine (10 mg/kg, s.c.) or rmCXCL10 combined with morphine, respectively. The protein expressions of AKT and p-AKT in spinal cord were examined 2 h after drug administration. There was no significant difference in the expression of AKT among four groups (Fig. 2A and B). Morphine could reduce the expression of p-AKT, whereas rmCXCL10 facilitated the phosphorylation of AKT compared with saline-treated mice (P < 0.05). As shown in Fig. 2A and C, the suppressed expression of p-AKT by morphine could be completely reversed by rmCXCL10 (P < 0.05), suggesting the possibility that morphine and rmCXCL10 have opposite effects on AKT-related pathways.

3.3. The cellular localization of p-AKT in spinal cord

To further explore the role of phosphorylated AKT in CXCL10-induced activation of intracellular signaling pathway, mice received either saline (3 μL, i.t.) or rmCXCL10 (30 ng, i.t.). The cellular localization of p-AKT in spinal cord was then assessed by immunofluorescence staining 2 h later. As shown in Fig. 3, p-AKT was extensively distributed in spinal dorsal horn and its expression was significantly upregulated in rmCXCL10-treated mice compared to that in saline-treated mice (P < 0.05).

3.4. Blocking PI3K signaling pathway could inhibit CXCL10-induced algesia and attenuation of morphine analgesia

To investigate the role of AKT-related signaling pathway in CXCL10-induced activation of intracellular signaling pathway, PI3K, which is an important upstream activator of AKT, was blocked by its inhibitor LY294002. Firstly, mice were treated with saline (3 μL) or LY294002 (5 μg), respectively, followed by rmCXCL10 (30 ng) 30 min later. The expression of p-AKT in spinal cord was examined 2 h after drug administration by Western-blot analysis. As shown in Fig. 4A, the pretreatment with LY294002 could inhibit the phosphorylation of AKT induced by rmCXCL10 (P < 0.05). Secondly, to determine whether the inhibition of PI3K activation could affect CXCL10-induced algesia, mice were pretreated with LY294002 (5 μg) or DMSO (0.1%, 3 μL), followed by saline (3 μL) or rmCXCL10 (30 ng) 30 min later, respectively. Data were collected during behavioral tests conducted at baseline and then at 30, 60, 90, and 120 min after drug administration. As shown in Fig. 4B, mice treated with DMSO and rmCXCL10 were characterized by a rapid decrease in PWTs from 30 to 120 min after drug administration, whereas pretreatment with LY294002 inhibited the effect of rmCXCL10 on mechanical nociceptive threshold and maintained the PWTs at the baseline level (P < 0.05). Thirdly, as our previous study showed that PWTs were lower in mice receiving rmCXCL10 and morphine than those in morphine-treated mice (Ye et al., 2014), in this study, mice were treated with LY294002 (5 μg) or 0.1% DMSO (3 μL), respectively, followed by rmCXCL10 (30 ng, i.t.) and morphine (10 mg/kg) 30 min later. According to the results, pretreatment with LY294002 resulted in an increase of PWTs in mice receiving rmCXCL10 and morphine, and the analgesic effect of morphine could be restored 30 min after drug administration (P < 0.05) (Fig. 4C). These results demonstrate that CXCL10 could attenuate the analgesic effect of morphine by activating PI3K-AKT signaling pathway.

3.5. CXCL10 could increase the expression of cytokines via PI3K-AKT signaling pathway

It has been reported that chemokines like CXCL4, CXCL16 could regulate the production of pro-inflammatory cytokines (Gouwy et al., 2016; Liang et al., 2018). Here we assumed that CXCL10 would display similar characteristics in controlling expressions of downstream pro-inflammatory cytokines. In order to verify this conjecture, mice were subjected to intrathecal injection of saline (3 μL, i.t.), morphine (10 mg/kg, s.c.), rmCXCL10 (30 ng, i.t.), or morphine combined with rmCXCL10, respectively. Pro-inflammatory cytokines including TNF-α, IL-10, inducible nitric oxide synthase (iNOS), IL-1β, and IL-6 in spinal cord were detected 2 h after drug administration. As indicated by the results, a single dose of morphine has no impact on the expressions of IL-10, iNOS, IL-1β, or IL-6 (P > 0.05), despite its effectiveness in reducing the mRNA level of TNF-α (P < 0.05). By contrast, we observed that mice treated with rmCXCL10 showed increased expressions of TNF-α, IL-10,
Neuropeptides, IL-1β, and IL-6, which were partially inhibited by co-administration of morphine (P < 0.05). Nevertheless, the mRNA levels of above-mentioned cytokines in mice receiving morphine and rmCXCL10 remained significantly higher than in the saline-treated mice (P < 0.05) (Fig. 5A). These results suggest that rmCXCL10 and morphine could produce opposite effects on the expression of pro-inflammatory cytokines.

A necessary step was conducted subsequently to determine whether PI3K-AKT signaling pathway is contributed to the CXCL10-induced production of pro-inflammatory cytokines. Mice were treated with LY294002 (5 μg, i.t.) or 0.1% DMSO (3 μL, i.t.), followed by saline (3 μL, i.t.) or rmCXCL10 (30 ng, i.t.) 30 min later, respectively. The expression of aforementioned cytokines in spinal cord was detected 2 h after drug administration. As revealed by the results, LY294002 had no impact on the expression of TNF-α, IL-10, iNOS, IL-1β, and IL-6. Moreover, rmCXCL10 could lead to an increase in the expressions of all the above-mentioned cytokines, which were partially inhibited by co-administration of LY294002 (P < 0.05) (Fig. 5B). These results demonstrate that CXCL10 could be effective in increasing the expression of pro-inflammatory cytokines by activating PI3K-AKT signaling pathway.

### 4. Discussion

The antinociceptive effect of morphine is modulated by multifactorial factors and some chemokines are well known to be significantly associated with the attenuation of morphine-induced analgesia by binding to their receptors (Lin et al., 2017). As a member of the chemokine family, the CXCL10 was also found in our previous study to be temporarily increased in response to a single dose of morphine and to be able to exert a counteraction against morphine. These results suggest that CXCL10 and morphine may act through an unknown mechanism to oppositely regulate pain in spinal cord (Ye et al., 2014). In the present work, a further investigation was conducted into the potential molecular mechanism associated with the correlation between CXCL10 and morphine, and the major results of the present study were shown in the schematic diagram (Fig. 6).

The chemokine receptor pair CXCL10/CXCR3 is widely expressed throughout the central nervous system (CNS) and produces different responses in CNS pathology (Klein et al., 2005; Loetscher et al., 2001). At the level of spinal cord, our findings presented the closely co-located CXCR3 and MOR as a spatial evidence for the interaction between these two receptors. This ligand-induced receptor activation was triggered by...
the inhibitory effect of morphine on AKT activation at the same time. Induce the transformation of AKT to phosphorylated AKT, and attenuate However, blocking PI3K-AKT signaling pathway could have inhibitory downstream effect. According to our results, AKT was also co-localized of AKT, abolished the decreased expression of p-AKT induced by morphine and further induced algesia through PI3K-AKT signaling pathway. Heterologous desensitization was originally thought to be the most common mechanism underlies the interactions between chemokine receptors and opioid receptors. Some scholars argued that the this process can usually be achieved by giving rise to the heterodimer or oligomeric complex of the two receptors (Patel et al., 2006; Sengupta et al., 2009; Burbassi et al., 2010; Suzuki et al., 2002). Our results seemed to contradict with these findings and suggested that the co-localization of CXCR3 and MOR in spinal cord provides a functional basis for the interactions between these two receptors. The similar results have also been obtained by Heinisch et al., who demonstrated the co-localization of MOR-CXCR4, MOR-CX3C chemokine receptor 1 (CX3CR1) on individual neurons in multiple sites of the nervous system (Heinisch et al., 2011). These evidences exemplifies the heterologous desensitization of MOR by chemokine receptors typically occurs not only following a process termed “heterodimerization”, but also at the level of receptor signaling (Steele et al., 2002). And the co-localization of CXCR3 with MOR opens up a possibility to achieve cross-desensitization by altering PI3K-AKT signaling. Therefore, rather than the formation of heterodimers, the different effects on intracellular signaling pathway are speculated to account for the mechanism of interaction between CXCR3 and MOR.

Pro-inflammatory cytokines, which have been produced during neurogenic inflammation, are clearly involved both in pain generation and morphine tolerance (Hutchinson et al., 2008; Matsuda et al., 2019). The increased synthesis of these cytokines can be promoted by activated chemokine-receptor pair following morphine exposure (Roeckel et al., 2016; Zhang et al., 2017). According to the study reported previously, CXCL10/CXCR3 signaling plays a negative feedback role in morphine analgesia (Ye et al., 2014). Based on these facts, we inferred that proinflammatory cytokines were under the opposite pattern of regulation by both CXCL10 and morphine and acted as algogenic molecules. This hypothesis was then experimentally demonstrated by us. In our study, the production of IL-10, IL-1β, iNOS and IL-6 were incapable of being induced by one single dose of morphine, but could be up-regulated in response to CXCL10. It is well known that CXCR3 is the main receptor for CXCL10. In line with this fact, recent studies showed evidences on the highly CXCR3-dependent pro-inflammatory cytokine release induced by CXCL10 in arthritis and ischemia reperfusion induced pain models (Lee et al., 2017; Yu et al., 2018). We are therefore reasonably confident that the elevated proinflammatory cytokine release reported in current paper are similarly initiated by the binding of CXCL10 to its receptor CXCR3.

The activation of PI3K-AKT is well documented to induce morphine and release of pro-inflammatory cytokines (Muro et al., 2018; Yang et al., 2015), and bear association with the effect of morphine analgesia (Tian et al., 2015b). According to our results, morphine is partially responsible for the reduced levels of pro-inflammatory factors as induced by CXCL10, which confirms that morphine is not involved in the activation of pro-inflammatory cytokine, but act as an inhibitor of pro-inflammatory cytokine activation. When co-administered with CXCL10 and PI3K inhibitor, the level of inflammatory factors showed decline, suggesting that PI3K-AKT is the exact signaling pathway for CXCL10 to initiate intracellular pro-inflammatory signals, thus causing the mediation of algesia and having effect on morphine analgesia. This means that blocking PI3K-AKT signaling pathway will suppress the CXCL10-induced pro-inflammatory factors, thereby allowing an enhanced morphine potency.

5. Conclusion

In the present study, it was demonstrated that CXCL10 could induce algesia and reverse morphine analgesia through PI3K-AKT signaling pathway in physiological condition. Moreover, activated PI3K-AKT pathway has a positive effect on CXCL10-induced inflammatory mediation. Our study is hoped to provide a novel target for maintaining the analgesic effect of morphine by blocking CXCL10-CXCR3-PI3K-AKT signaling pathway.
Declaration of Competing Interest
None declare.

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Appendix A. Supplementary data
Supplementary data to this article can be found online at https://doi.org/10.1016/j.npep.2022.102243.

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