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TITLE: Nasal Oxytocin for the Treatment of Post-TBI Chronic Headache: Influence of Estrogen

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14. ABSTRACT: The funded project examines the impact of estrogen on the analgesic effect of nasally applied oxytocin in a rodent model of headache caused by traumatic brain injury. This report details efforts made during the first year plus of the funded project. The study began during Covid restrictions on laboratory work at Stanford, and so there were (and to some extent still are) delays secondary to the pandemic.However, we were able to achieve IACIC and ACURO protocol approval, recruit a qualified postdoctoral fellow, construct the TBI apparatus, begin demonstration of the allodynic effects of TBI and the analgesic effects of nasal oxytocin in pot-TBI rats. We were also able to begin electrophysiologic testing of trigeminal ganglia (TG) neurons from female rats – a prerequisite of testing of the impact of estrogen pretreatment on the effect of oxytocin as a inhibitor of these pain-sensing neurons. Finally, we used the time during which we could not do laboratory work to publish a paper describing a theory of the pathogenesis of menstrual migraine, which posited that the decrease in estrogen during menses drives decreases in TG oxytocin receptor activity.

15. SUBJECT TERMS
Traumatic brain injury, TBI, Post-traumatic headache, gender, hormones, sex

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1. INTRODUCTION:

The application of nasal application of oxytocin in rats demonstrating chronic head pain following induction of TBI decreases the animal's pain state and improves their performance in a test of depression behaviors. The aim of the experiments is to determine whether an induced increase in estrogen levels will enhance the inhibitory effect of OT on trigeminal neurons and pain behavioral sequelae to TBI in rats. To achieve this, we performed patch-clamp electrophysiological studies on the trigeminal ganglia (TG) of untreated female vs male rats as well as estrogen treated males to determine the effects of estrogen on oxytocin (OT) inhibition of TG excitability. We are also examining the effects of pretreatment of rats with estrogen on the analgesic efficacy of nasal OT in post-TBI male rats vs untreated male or female rats. Positive results of this project provide important information as to the optimal approach to the use of nasal oxytocin for alleviation of chronic migraine-like headache secondary to TBI for both male and female warfighters and veterans.

2. KEYWORDS:

Traumatic brain injury, TBI, Post-traumatic headache, gender, hormones, sex

3. ACCOMPLISHMENTS:

What were the major goals of the project?

Aim 1: Male rats will be given 4 daily injections of estradiol benzoate (EB) or vehicle, following which estradiol treated rats, as well as untreated male or female rats will be subjected to controlled traumatic brain injury and tested for the craniofacial analgesic effect of nasal oxytocin.
(significant delays resulted from Covid-19 pandemic)

- IACUC and ACURO protocol approval achieved **(04/2021)**
- Recruitment of postdoc **(9/2021)**
- Construction of TBI device **(10/2021)**
- Baseline TBI and facial mechanical pain sensitivity in male or female rats initiated **(02/2022)**
- Subcutaneous injection of estradiol for 4 days in males followed by facial sensitivity assessment **(10/2022)**
- Results statistically analyzed **(12/2022)**

Aim 2: Patch-clamp electrophysiological recordings of effects of oxytocin on trigeminal ganglia neurons from TBI injured rats with or without daily treatment with estradiol.

- Male rats euthanized after daily dosing with vehicle of estradiol for 4 days **(9/2022)**
- TG neurons from estradiol treated male, or untreated male or female rats extracted and assessed using current clamp electrophysiology (**10/2022)**
- Oxytocin (OT) added to the media and the excitability reassessed to determine potency of OT in decreasing excitability of male or female TG neurons **(5/2023)**
- Results will be statistically analyzed **(6/2023)**

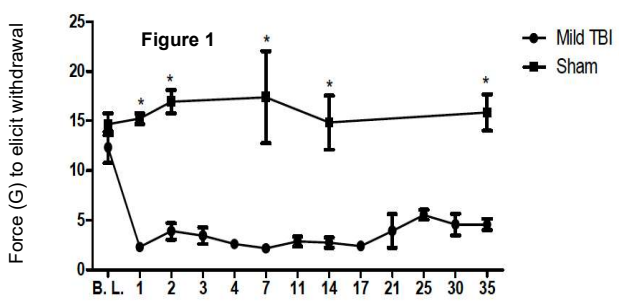
What was accomplished under these goals?

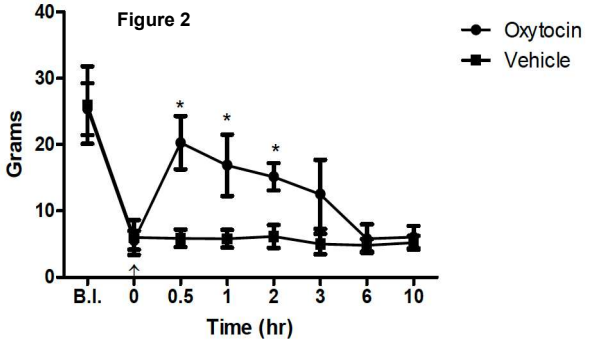
Unfortunately, the Covid-19 pandemic slowed our progress in this project considerably. This was manifested both in terms of bringing on a postdoc (Dr. Bharadwaj), achieving IACUC approval, equipment development, and education of the postdoc as to the model.

That being say, we have had significant progress.

1. We brought on and trained a postdoc (Vimala Bharadwaj) from Dr. Porreca's laboratory at University of Arizona, where she performed work on post-TBI head pain in rodents.
2. We achieved IACUC and ACURO approval of our protocol.
3. We built and initiated the TBI device to be used – which was an improvement over our previously developed model.
4. We published a paper, supported by this grant, on the importance of the addition of magnesium to the analgesic potency of intranasal oxytocin in rodent headpain models. This enhancement of efficacy is due to a Mg++ binding site which allows for much higher affinity binding of oxytocin to its receptor (https://doi.org/10.3390/pharmaceutics13071088).
5. We tested the behavioral effects of the TBI device in normal, control, male rats.
6. We then demonstrated the analgesic effects of intranasal oxytocin in rats that had demonstrated craniofacial hypersensitivity following TBI (Figures 1 and 2).
7. We demonstrated, in vitro in current clamp electrophysiological experiments, that oxytocin was significantly more potent in inhibiting trigeminal ganglia neurons from male vs female rats (Figure 3).
8. We then demonstrated that, pretreatment of male rats with estrogen for 4 days prior to trigeminal ganglia removal caused the in vitro potency of oxytocin in testing these ganglia to match that of ganglia neurons from female rats (Figure 3).

Figures 1 and 2 below show that, following TBI (but not sham injury), male rats demonstrated a profound and persistent increase in cranial mechanical allodynia (Figure 1), and that intranasal delivery of 10 ug of oxytocin significantly ($p < 0.05$) reversed this allodynia for 2-3 hours (Figure 2).





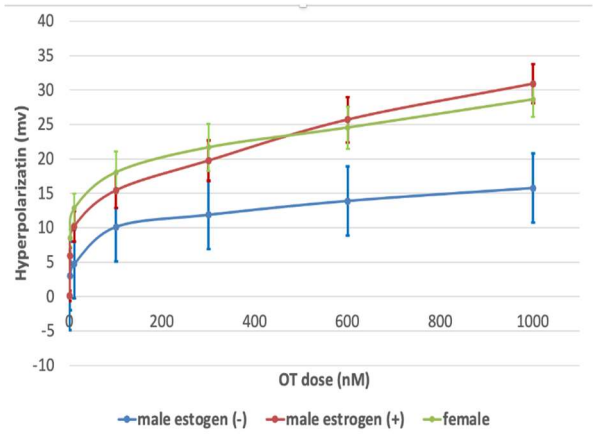


Figure 3. Current clamp recordings of rat TG demonstrate that oxytocin concentration-dependently inhibited the excitability of trigeminal ganglia neurons from both male and female post-TBI rats as assessed by increases in resting membrane potential (hyperpolarization). The potency of oxytocin was significantly greater in neurons from female rats. Pretreatment of males for 4 days with estrogen induced comparable potency for neurons from male vs female rats.

What opportunities for training and professional development has the project provided?

This project provided training opportunities for Dr. Vimala Bharadwaj, a new postdoc in the lab. Dr. Bharadwaj gained greater proficiency in protocol preparation, manuscript preparation, device construction, behavioral testing, and electrophysiology. All of these things will serve her well in her goal to become an academic scientist.

How were the results disseminated to communities of interest?

Publication of paper, supported by this grant, on an improvement in the delivery formulation of intranasal oxytocin (Bharadwaj VN, Meyerowitz J, Zou B, Klukinov M, Yan N, Sharma K, Clark DJ, Xie X, Yeomans DC. Impact of Magnesium on Oxytocin Receptor Function. Pharmaceutics. 2022 May 21;14(5):1105. doi: 10.3390/pharmaceutics14051105. PMID: 35631690; PMCID: PMC9144867).This publication reports behavioral and patch clamp electrophysiological evidence that the addition of magnesium ions to the oxytocin significantly enhances the potency of the treatment.

Publication of paper, supported by this grant, on a theory of menstrual migraine (Bharadwaj VN, Porreca F, Cowan RP, Kori S, Silberstein SD, Yeomans DC. A new hypothesis linking oxytocin to menstrual migraine. Headache. 2021 Jul;61(7):1051-1059. doi: 10.1111/head.14152. Epub 2021 Jun 14. PMID: 34125955). This paper described the importance of estrogen, as well as other endogenous biochemicals in the mediation of menstrually-associated migraine – which is common in post-TBI female headache patients.

Publication of paper, supported by this grant, on the nervous system distribution of oxytocin after nasal application (Bharadwaj VN, Tzabazis AZ, Klukinov M, Manering NA, Yeomans DC. Intranasal Administration for Pain: Oxytocin and Other Polypeptides. Pharmaceutics. 2021 Jul 16;13(7):1088. doi: 10.3390/pharmaceutics13071088. PMID: 34371778; PMCID: PMC8309171).

Presentation of data at the Annual American Headache Society Conference.

Describe briefly what you plan to do during the next reporting period to accomplish the goals and objectives.

We plan to begin behavioral testing of post-TBI male and female rats following daily injections of estradiol as well as investigations into the impact of this estrogen treatment on the analgesic effect of intranasal oxytocin.

4. IMPACT:

What was the impact on the development of the principal discipline(s) of the

project?

The overall impact of the project is to determine the importance of estrogen (endogenous or exogenous) on the craniofacial analgesic effect of intranasally applied oxytocin. If it turns out that estrogen plays a key role in this analgesia, then this may dictate that the potency of oxytocin is likely to depend on the sex of the patient and, for females, the time within the menstrual cycle.

The immediate impact of the results so far is that we have established that inhibitory effect of oxytocin on trigeminal (likely pain-sensing) neurons is sex dependent and that this difference is dependent on estrogen level. As estrogen has been shown to induce the expression of oxytocin receptors within the nervous system, it is likely that estrogen will play a key role in the application of oxytocin to post-TBI headache.

What was the impact on other disciplines?

The above-described papers have the potential to affect how physicians treat menstrual migraine, the dependence of oxytocin receptor binding on magnesium, and he demonstration of the neural deposition of nasally applied polypeptides.

What was the impact on technology transfer?

Nothing to Report

What was the impact on society beyond science and technology?

Again, the only result of note so far is the paper we were able to publish as the result of the funding as well as the restrictions placed on us by Covid. The results of our studies have the potential to impact patients with persistent headache, including menstrual migraine. If the results of the laboratory work pan out, this could lead to the clinical implementation of oxytocin treatment for post-TBI headache. This work will also be useful to society in that nasally applied oxytocin has been suggested to be useful for a variety of medical and psychiatric issues.

5. CHANGES/PROBLEMS:

Nothing to Report

Actual or anticipated problems or delays and actions or plans to resolve them

The cause of the delays, so far, have been related to the Covid Pandemic.

Changes that had a significant impact on expenditures

Because of the delays in laboratory work caused by the pandemic, the costs of the project are projected to be beyond the original budget as the personnel costs were continuing despite our inability to perform experiments.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Significant changes in use or care of human subjects

Nothing to Report

Significant changes in use or care of vertebrate animals

Nothing to Report

Significant changes in use of biohazards and/or select agents

Nothing to Report

6. PRODUCTS:

☐ **Publications, conference papers, and presentations**

Journal publications.

Published:
Bharadwaj VN, Meyerowitz J, Zou B, Klukinov M, Yan N, Sharma K, Clark DJ, Xie X, Yeomans DC. Impact of Magnesium on Oxytocin Receptor Function. Pharmaceutics. 2022 May 21;14(5):1105. doi: 10.3390/pharmaceutics14051105. PMID: 35631690; PMCID: PMC9144867.

Bharadwaj VN, Porreca F, Cowan RP, Kori S, Silberstein SD, Yeomans DC. A new hypothesis linking oxytocin to menstrual migraine. Headache. 2021 Jul;61(7):1051-1059. doi: 10.1111/head.14152. Epub 2021 Jun 14. PMID: 34125955.

Bharadwaj VN, Tzabazis AZ, Klukinov M, Manering NA, Yeomans DC. Intranasal Administration for Pain: Oxytocin and Other Polypeptides. Pharmaceutics. 2021 Jul 16;13(7):1088. doi: 10.3390/pharmaceutics13071088. PMID: 34371778; PMCID: PMC8309171.

Books or other non-periodical, one-time publications.

Nothing to Report

Other publications, conference papers and presentations.

Nothing to Report

☐ **Website(s) or other Internet site(s)**

Nothing to Report

☐ **Technologies or techniques**

Nothing to Report

☐ **Inventions, patent applications, and/or licenses**

Nothing to Report

☐ **Other Products**

Nothing to Report

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

David C. Yeomans, PhD
Principle Investigator
ORCID #0000-0002-9389-8539
Person Months: 2
Contribution: Dr. Yeomans has overseen all work performed in this project to date.
Funding support: The work described in this project has no other funding support.

Michael Klukinov, MD
Senior Research Scientist
ORCID # 0000-0002-5229-6777
Person Months: 3
Contribution; Dr. Klukinov has overseen construction of tBI apparatus and initial behavioral experiments. He has also contribute to the publication discussed above
Funding Support: The work described in this project has no other funding support.

Vimala Bharadwaj, PhD
Postdoctoral Fellow
ORCID # 0000-0002-6243-0861
Person Months: 3
Dr. Bharadwaj has contributed to the preparation of the publication described above, the behavioral testing, and the elecrophysiological testing
Funding Support: The work described in this project has no other funding support.

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Active

1. 2 R01 HL 00000 – 13 Neuronal and behavioral responses to spinal cord injury. Yeomans, Co-I
2.
3. UG3NS115637. Clinical Translation of Ultrasonic Ketamine Uncaging for Non-Opioid Therapy of Chronic Pain, Yeomans, Co-I
4. R61NS122298. Discovery and validation of novel biomarker signature of peripheral painful neuropathy. Yeomans, Co-I

What other organizations were involved as partners?

Nothing to Report

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS:

QUAD CHARTS:

9. APPENDICES:

Appendix 1: ACURO Approval Letter

Appendix 2: Published Paper (Bharadwaj et al., A new hypothesis linking oxytocin to menstrual migraine)

Appendix 3: Published Paper (Bharadwaj et al., Imapact of Meagnesium on Oxytocin Receptor Function.)

Appendix 4: Published Paper (Bharadwaj et al., Intranasal Administration for Pain.)

DEPARTMENT OF THE ARMY

HEADQUARTERS, U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
810 SCHREIDER STREET
FORT DETRICK, MD 21702-5000
April 12, 2021

Director, Office of Research Protections
Animal Care and Use Review Office (ACURO)

Subject: Approval of Proposal Number PR202508, Award Number W81XWH-21-1-0186 entitled, "Nasal Oxytocin for the Treatment of Post-TBI Chronic Headache: Influence of Estrogen"

David Yeomans
The Leland Stanford Junior University
Stanford, CA, US

Dear David Yeomans:

Reference: (a) DOD Instruction 3216.01, "Use of Animals in DOD Conducted and Supported Research and Training"
(b) US Army Regulation 40-33, "The Care and Use of Laboratory Animals in DOD Programs"

In accordance with the above references, ACURO protocol PR202508.e001 entitled, "Estrogen Oxytocin treatment after mTBI," IACUC protocol number 33945, Protocol Principal Investigator David Yeomans, is approved by ACURO as of 04/09/2021 for the use of rats and will remain so until modification, expiration or cancellation. This protocol was approved by the The Leland Stanford Junior University IACUC on 02/26/2021; IACUC approval expires 02/23/2024.

Required Actions:

A. Submit to ACURO for review and approval prior to implementing:

- IACUC-approved de novo reviews of the protocol
- IACUC-approved significant changes to this protocol (see guidance document)

B. Notify ACURO within 5 business days of any of the following:

- Any noncompliance, suspensions or adverse events (see guidance document)
- Receipt of notification that the institution is under investigation by USDA
- AAALAC, International accreditation status change

For further assistance, please contact ACURO at (301) 619-6694, FAX (301) 619-4165, or via e-mail: usarmy.detrick.medcom-usamrmc.other.acuro@mail.mil.

NOTE: Do not construe this correspondence as approval for any contract funding. Only the Contracting Officer or Grant Officer can authorize expenditure of funds. It is recommended that you contact the appropriate Contract Specialist or Contracting Officer regarding the expenditure of funds for your project.

Sincerely,

Dawn C. Fitzhugh, VMD, MPH, DACLAM
Colonel, US Army
Director, Animal Care and Use
Review Office

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Cheryle Aird
Dr. Stephen Felt
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REVIEW ARTICLE

A new hypothesis linking oxytocin to menstrual migraine

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Abstract

Objective: To highlight the emerging understanding of oxytocin (OT) and oxytocin receptors (OTRs) in modulating menstrual-related migraine (MRM).

Background: MRM is highly debilitating and less responsive to therapy, and attacks are of longer duration than nonmenstrually related migraine. A clear understanding of the mechanisms underlying MRM is lacking.

Methods: We present a narrative literature review on the developing understanding of the role of OT and the OTR in MRM. Literature on MRM on PubMed/MEDLINE database including clinical trials and basic science publications was reviewed using specific keywords.

Results: OT is a cyclically released hypothalamic hormone/neurotransmitter that binds to the OTR resulting in inhibition of trigeminal neuronal excitability that can promote migraine pain including that of MRM. Estrogen regulates OT release as well as expression of the OTR. Coincident with menstruation, levels of both estrogen and OT decrease. Additionally, other serum biochemical factors, including magnesium and cholesterol, which positively modulate the affinity of OT for OTRs, both decrease during menstruation. Thus, during menstruation, multiple menstrually associated factors may lead to decreased circulating OT levels, decreased OT affinity for OTR, and decreased expression of the trigeminal OTR. Consistent with the view of migraine as a threshold disorder, these events may collectively result in decreased inhibition promoting lower thresholds for activation of meningeal trigeminal nociceptors and increasing the likelihood of an MRM attack.

Conclusion: Trigeminal OTR may thus be a novel target for the development of MRM therapeutics.

KEYWORDS
estrogen, magnesium, menstrual migraine, oxytocin, oxytocin receptor, therapeutics

INTRODUCTION

Migraine disproportionately affects women.[1] There is little sex difference in migraine prevalence before puberty; yet after puberty, women are two to three times more likely to experience from migraine compared with men.[2] In many women, migraine attacks are associated with the menstrual cycle, a condition termed menstrual-related migraine (MRM). Approximately 42%–70% of female patients

Abbreviations: CGRP, calcitonin gene-related peptide; CNS, central nervous system; IL-6, interleukin-6; Mg^{2+}, magnesium ion; MRM, menstrual-related migraine; mRNA, messenger ribonucleic acid; Na^{+}, sodium ion; OT, oxytocin; OTR, oxytocin receptor; PVN, paraventricular nuclei; TG, trigeminal ganglion; TNC, trigeminal nucleus caudalis.

report a worsening of migraine headaches during perimenstrual time periods.[3–7] MRM is more severe and incapacitating with higher frequency compared with nonmenstrual migraine.[8,9] Moreover, MRM attacks have a longer duration and are more difficult to treat than nonmenstrual attacks.[2,5,9] Therefore, it is critical to understand the unique underlying pathogenesis peculiar to MRM and to develop therapies to manage this debilitating type of migraine.

The pathogenic relationship between menstruation and migraine has been the subject of much research but remains unclear. The estrogen withdrawal theory postulates that a drop in estrogen is the cause of migraine attacks in vulnerable women.[10] A decrease in estrogen is often strongly linked with factors that also likely contribute to MRM. For example, menstruation has been associated with a decrease in circulating magnesium that temporally coincides with an MRM attack,[11] and ionized magnesium blood levels were found to be significantly lower in MRM attacks compared with non-MRM attacks in MRM patients.[12] Magnesium infusion is abortive in migraine, and magnesium supplementation can be prophylactic for MRM,[13–15] but the mechanism by which these effects occur is not known.

Uncertainties such as these suggest the need for a unitary theory that integrates knowledge of physiological mechanisms underlying MRM more broadly. We propose that the actions of oxytocin (OT) on trigeminal oxytocin receptors (OTRs) may help to unify numerous apparently disparate observations. Clinical[16–18] and preclinical studies strongly support an antinociceptive role of OT in the trigeminal pain system[16,19,20] as well as in the spinal cord.[21,22] OT has been reported to directly inhibit neuronal excitability of cells in the trigeminal ganglion (TG) and to elicit a robust inhibition of nociceptive responses in the trigeminal nucleus caudalis (TNC).[16,20] Therefore, we hypothesize that decreased tonic OT/OTR activity in the perimenstrual period may lower thresholds for activation of trigeminal nociceptive afferents and thus increase the probability of a pain attack. In this review, we suggest that, coincident with menstruation, there are changes in multiple factors that can affect trigeminal OTR activity and thus neuronal excitability, increasing the likelihood of and perhaps dictating a migraine attack.

We first discuss the estrogen withdrawal and magnesium deficiency theories with their respective limitations and propose a theory related to OT for MRM pathogenesis. We also consider the interaction of OT and the OTR, key clinical observations of OT treatment in migraine, and how the mechanism of action of OT in pain modulation may play an important role in promoting MRM. Finally, the therapeutic implications of the proposed OT theory are discussed.

METHOD

We reviewed the literature on MRM on PubMed/MEDLINE database including clinical trials and basic science publications. Inclusion criteria were based on key search items, "pathophysiology of menstrual migraine," "pathogenesis of menstrual migraine," "therapeutics and menstrual migraine," "estrogen withdrawal," "magnesium and migraine," "oxytocin," "oxytocin receptors," "oxytocin and intranasal delivery," "oxytocin and migraine," "blood serum levels and menstrual cycle," and "blood serum levels and migraine." The authors reviewed the citations from review papers involving MRM and OTRs. The final reference list was generated based on relevance to the topics covered in this review.

ESTROGEN WITHDRAWAL THEORY

The estrogen withdrawal theory for MRM, first described over 45 years ago, proposed that the decline in estrogen levels during the late luteal phase could trigger a migraine attack.[10] In this report, women who had MRM and were given estradiol treatment had a delay in their migraine attacks until the level of estradiol dropped once more to pretreatment levels.[10] Studies from the same group suggested that a period of estrogen "priming" with several days of high estrogen level exposure is a necessary precursor for MRM that resulted from estrogen "withdrawal."[23,24] Other clinical studies[25–28] have supported the conclusion that decreased levels of estrogen increase susceptibility for MRM attacks.[12]

The reason fluctuations in estrogen can promote MRM remains uncertain. Estrogen withdrawal has been hypothesized to lead to multiple pronociceptive sequelae including the sensitization of the trigeminal system, modulation of neurotransmitter systems, increased synthesis of neuropeptides, and altered reactivity of microglia.[13,29] Nonetheless, periods of high estrogen appear to increase neuronal excitability and decrease the threshold for spreading depression[30] and sometimes are associated with increased incidence of migraine with aura.[23] Furthermore, estrogen has been used as a therapy to prevent migraine attacks.[24] These paradoxical observations suggest that the effect of estrogen on migraine is complex and multifaceted.[30] One possibility is that the rapid decrease, rather than absolute estrogen levels, is the critical factor contributing to MRM. Additionally, the fluctuation of other factors over the menstrual cycle may also play important roles in MRM pathogenesis. Herein, we suggest that a key hormonal factor in the pathogenesis of MRM attack is OT/OTR activity, which is modulated by estrogen level but is also dependent on other factors that vary over the menstrual cycle.

MAGNESIUM DEFICIENCY

Magnesium (Mg^{2+}) is an essential metallic cation that plays an important role in numerous cellular functions including the maintenance of neuronal transmembrane electric potentials. Mg^{2+} serum concentration has been identified to be an independent risk factor in migraine, and patients have their lowest serum levels of Mg^{2+} during migraine attacks.[31] In fact, the odds of acute migraine headache attack is increased 35-fold when serum levels of Mg^{2+} drop below normal levels.[31] Critically, menstruation has been associated with a decrease

in circulating Mg^{2+}[11] with the timing of this decrease coinciding with MRM attack. In MRM patients, intracellular Mg^{2+} levels in cells isolated from blood samples were significantly reduced compared with controls, and the levels were reported to exhibit an inverse relationship with duration and intensity of migraine attacks.[32] The incidence of Mg^{2+} deficiency was reported to be 45% during menstrual attacks and 14% during menstruation without migraine.[12] The possible contribution of Mg^{2+} in migraine is supported by reductions in MRM attack frequency with oral magnesium supplementation[12,32] as well as by the efficacy of magnesium infusion in terminating *status migrainosus* attacks.[33] Taken together, these clinical studies provide presumptive evidence that deficiency of Mg^{2+} plays a role in MRM.

Mg^{2+} deficiency may contribute to migraine attacks in multiple ways including regulation of calcium ion influx, serotonin receptor activity, platelet aggregation, cerebrovascular tone, and release of nitric oxide and inflammatory mediators such as neuropeptides, substance P, and cytokines.[14,34] These broad neurovascular, neuropeptide, and transmitter actions of Mg^{2+} clearly overlap with known migraine pathogenesis.[14,34,35] Nevertheless, a direct link between Mg^{2+} deficiency and MRM is yet to be demonstrated. Herein, we suggest that Mg^{2+} deficiency is a critical factor in modulating OT/OTR signaling that leads to an increased likelihood of MRM.

OXYTOCIN THEORY

Understanding migraine as a threshold disorder[36] suggests that reduction in inhibitory tone may increase the likelihood of future attacks. OT is a nonapeptide synthesized in the supraoptic and paraventricular nuclei (PVN) and accessory magnocellular nuclei of the hypothalamus.[37,38] The axon terminals of these cells promote the secretion of OT into the bloodstream from the posterior pituitary.[39] OT cells in the PVN also project throughout the central nervous system (CNS) including the amygdala, the striatum, and the superficial and deep lamina of the dorsal horn.[40–42] OT binds to OTRs that are widely localized in the different brain (neuronal and glial cells) and spinal cord regions as well as on peripheral tissues such as the uterus and breast.[43,44] Of key importance for the OT/MRM theory is the inhibitory effect on pain and nociception.[43,45–47] The robust analgesic effect of OT/OTR binding appears to occur via a potassium channel/nitric oxide/K_{ATP} pathway.[45]

Key clinical observation of OT in migraine

OT levels are lowest at days −2 to +3 of the menstrual cycle, the same time period associated with low estrogen levels and in which MRM attacks occur.[48,49] There is substantial correlative and direct evidence showing that OT can modulate migraine headache.[18,50–52] For instance, over the course of pregnancy, the levels of circulating OT increase,[50] and over the same period, the frequency of headaches decreases.[51] Likewise, women who breastfeed versus bottle-feed their babies have higher OT,[52] and migraine recurrence rates are lower in the breastfeeding group.[51] Furthermore, intranasal OT was shown to relieve headaches in patients in a dose-dependent manner.[18] A recent 40-patient pilot study showed analgesic efficacy of intranasal OT in chronic migraine headache patients but not in low-frequency episodic migraine.[16] Although the study did not meet its primary endpoint of significant pain reduction versus placebo at 2 h postdosing, a significant difference in pain relief was observed by 4 h after dosing in chronic migraine patients.[16] Interestingly, post hoc findings demonstrated that patients who had taken nonsteroidal anti-inflammatory drugs within 24 h were less likely to show OT analgesic efficacy.[16] One plausible theory for this observation is that inflammation can traffic the OTR to the membrane[16,53] allowing for increased efficacy of intranasal OT. A follow-on open-label study in patients with chronic and high-frequency episodic migraine showed that intranasal OT significantly reduced both the pain and the frequency of headache.[16] Although this study suffered from a very high placebo rate of response (74%), intranasal OT induced a striking decrease in frequency from baseline with an average of 14.1 headache days to an average of 5.9 headache days during treatment.[16] In total, these clinical studies provide presumptive evidence showing that OT can attenuate migraine pain in patients.

MECHANISM OF ACTION OF OXYTOCIN IN PAIN MODULATION

Activation of the trigeminovascular system is essential for migraine pain. Thus, the excitability of this system contributes to the probability of a migraine attack.[35,54] OT, binding to trigeminal OTR, decreases the excitability of these trigeminal neurons in rodents[46] and so could contribute to decreased probability of a migraine attack. In humans and animals, OT and OTR expression have been demonstrated in the TG and the brain.[19,20,40,46,55] Immunoreactivity of OT and OTR protein expression was significantly localized to calcitonin gene-related peptide (CGRP) positive and myelinated Aδ sensory neurons and fibers in the TG as well as in trigeminal satellite glial cells.[20,56] The CNS OTR has been demonstrated in areas known to be critical to migraine pathogenesis.[56] Distribution of [^{125}I]-OT using tissue counts and autoradiography after intranasal administration to rodents showed strong localization of OT label in the TG and in all three branches of the trigeminal nerve as well as in brain regions known to be rich in OTR,[20] including areas of the CNS associated with migraine, including the pons, medulla (particularly the dorsal horn of *nucleus caudalis*), hippocampus, thalamus, and midbrain.[16] Taken together, OTR distribution and distribution of OT following nasal application provide a substrate for OT actions on both peripheral and central mechanisms of migraine.

OT, acting at OTR, can have analgesic effects through multiple possible mechanisms (see Dussor et al.[57] for a review). There is strong evidence for direct inhibitory effects of OT on primary afferent nociceptors.[20,57,58] OT can act at OTR to hyperpolarize nociceptors and desensitize signaling at OTR particularly after inflammatory injury.[22,46] Relevant to migraine, activation of OTR by OT blocks

CGRP release from dural afferents in animals following induction of inflammation.[20] Furthermore, a vast majority of OTR immunoreactive neurons in the rat co-express CGRP following inflammation.[25] Interestingly, TG from noninflamed rats does not show inhibition of CGRP release by OT nor show CGRP/OTR colocalization, a finding that is consistent with the known upregulation of OTR and CGRP in the TG by inflammation.[20,59] The nociceptive response to the release of inflammatory cytokines is also thought to be a key component of migraine pathophysiology.[60] The same rapid upregulation of OTR is critical in uterine contraction and is thought to be driven primarily by interleukin-6 (IL-6), for which there are three response elements on the human OTR gene.[61,62]

OT can also influence pain through central actions within the TNC. Single-cell responses in the TNC of rats following noxious facial electrical shock were reduced by more than half by 10 min and by more than 90% by 45 min after intranasal OT administration.[16] Moreover, in a migraine rodent model of nitroglycerin infusion, increased c-Fos expression in TNC neurons was significantly reduced after intranasal OT treatment.[16] Additionally, topical spinal microinjection of OT dose dependently inhibited peripherally evoked activity (V1, ophthalmic) nociceptive transmission in the TNC[47] that was blocked by pretreatment with the selective OTR antagonist.[47] Taken together, these in vivo preclinical studies demonstrate that activation of central TNC neurons is reduced by exogenously applied OT, suggesting the role of OT in pain modulation by influences at the level of the TG and/or the TNC and point to a potential therapeutic direction. Other additional mechanisms by which OT/OTR may produce analgesic actions include postsynaptic effects of OT released from PVN projections to the spinal/trigeminal dorsal horn to inhibit nociception[57,63] and potential activation and desensitization of the transient receptor potential cation channel subfamily V member 1 channel.[57,64]

MODULATION OF OT/OTR ACTIVITY IN MIGRAINE

Several endogenous factors can, therefore, modulate trigeminal pain including endogenous OT levels, Mg^{2+}, estrogen, sodium ion (Na^{+}), cholesterol, and IL-6. Increased likelihood of a MRM attack can result from modulation of (a) endogenous OT levels, (b) OT affinity for the OTR, and (c) the expression of OTR; all of these occur during menstruation. Figure 1 is a concatenation of serial levels of OT, estrogen, Mg^{2+}, Na^{+}, estrogen, cholesterol, and IL-6 over the menstrual cycle that are extracted from published sources.[11,65–68]

Local or circulating OT levels

OT levels vary over the menstrual cycle and are lowest at days −2 to +3 of the cycle[67]—the same time period during which MRM attacks occur. What precipitates this drop? During the menstrual cycle, estrogen drops approximately at the same time as OT drops.[65,67] Estrogen can stimulate both OT synthesis[69] and its release in the systemic circulation[70,71] and CNS structures.[72,73] Thus, the drop in OT during menstruation may be driven, at least in part, by dropping levels of estrogen. Figure 2A illustrates this diagrammatically: decreases in estrogen levels may result in lower OT levels and decreasing OTR activity at trigeminal and other migraine-associated

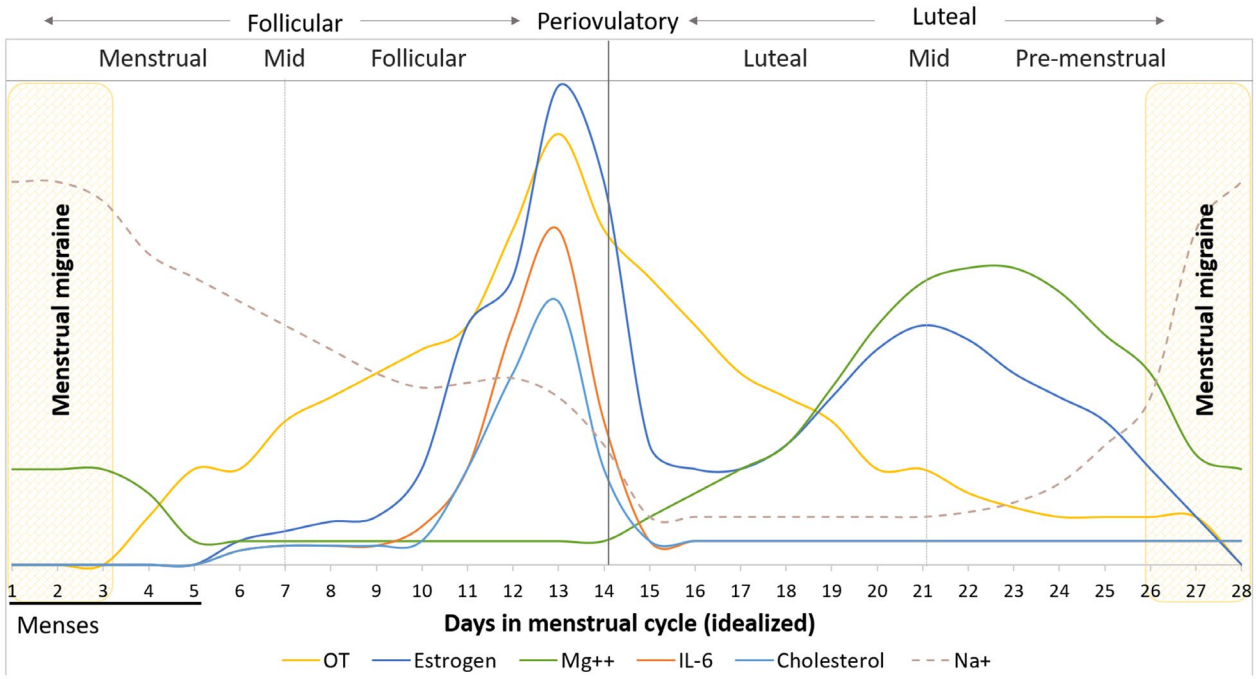

FIGURE 1 Composite illustration extracted from multiple literature sources showing measured and calculated fluctuation of the serum levels of OT, estrogen, Mg^{2+}, IL-6, cholesterol, and Na^{+} level during normal menstrual cycle[11,65–68]

neurons. Thus, the drop in estrogen during the menstrual cycle can increase the likelihood of a painful MRM attack. Future studies are needed to co-serially sample the levels of OT and estrogen in MRM patients. A strong positive correlation, particularly if the order of change could be established, would lend credence to the idea that estrogen levels can modulate OT levels and thus trigeminal OTR tone and nociceptive activity.

Modulation of OT affinity for OTR

The affinity of OT for its receptor is highly variable depending on the biochemical milieu in which the receptor exists (see Figure 2B). OTR affinity is positively allosterically modulated by Mg^{2+} (up to 1500-fold).[74] The local level of Mg^{2+} may, therefore, be involved in the regulation of OT-mediated signaling functions.[74,75] The OTR exists in both a high- and a low-affinity conformation; Mg^{2+} interacts with the ligand-binding site of OTR to stabilize the conformation favoring high affinity and specific binding of agonists[74,75] and can, in addition, increase the binding capacity of OTR.[76] Mg^{2+} levels are normally their highest during the mid-luteal phase of menstruation, falling just before the menstrual period (Figure 1).[11,77] Thus, elevated levels of Mg^{2+} can increase the affinity of available OT at OTR in TG and TNC neurons. However, women who experience MRM show lower levels of Mg^{2+} overall and an additional drop in Mg^{2+} during migraine attacks.[12,78] A drop in Mg^{2+}, which is associated with migraine in general[31] and MRM[12,32] in particular, should decrease the activity of endogenous OT. Consequently, the tonic inhibition of trigeminal and other OTR+neurons promote an increase in the likelihood of MRM attack (see Figure 2B). Thus, the low level of Mg^{2+} in women with MRM may be a precipitating factor in inducing their headaches. As mentioned above, magnesium supplementation is effective in decreasing the frequency of MRM attacks, supporting the idea that levels of this electrolyte are critical in the pathogenesis of MRM.[32]

To exist in a high-affinity state, OTR requires at least two factors, namely a divalent metal cation such as Mg^{2+} and a high-cholesterol environment.[76] In fact, depending on the presence of

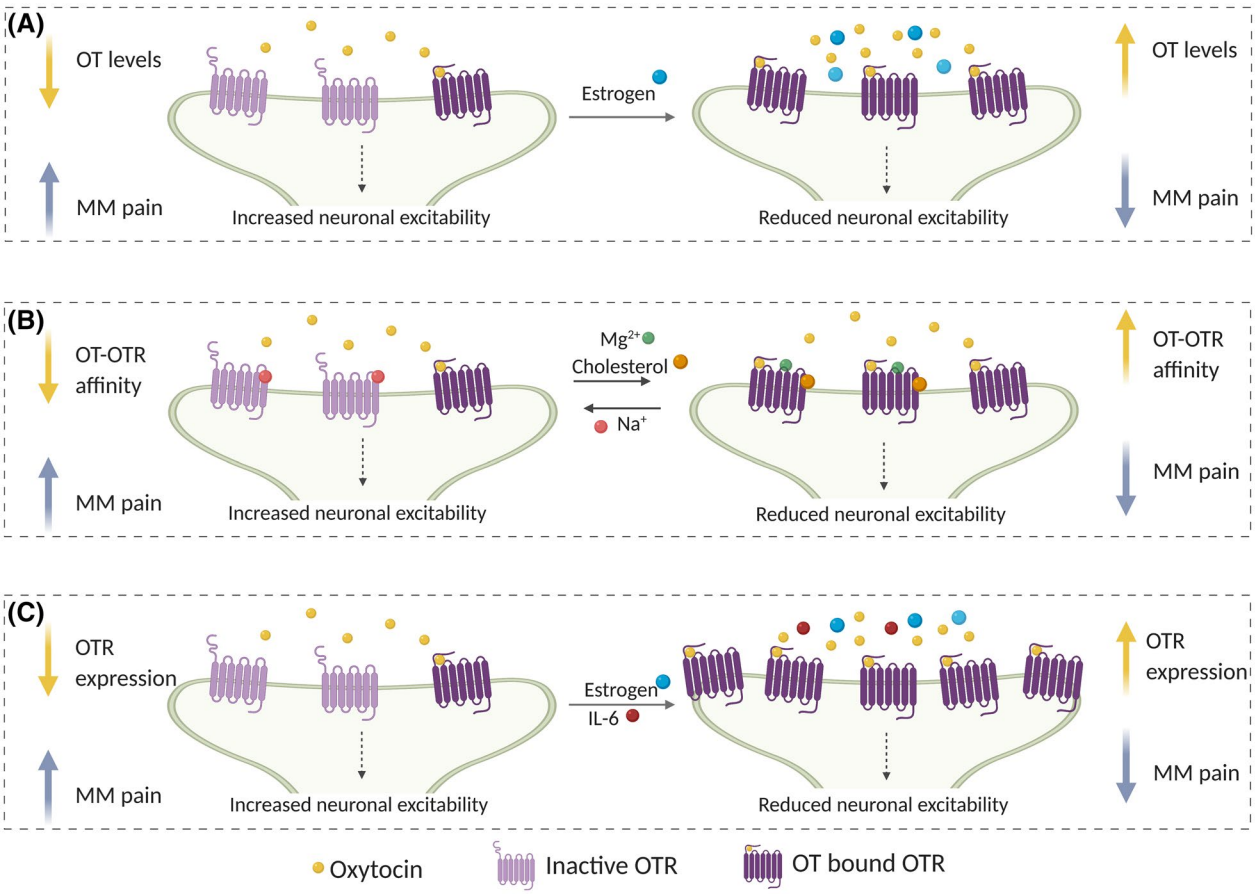

FIGURE 2 (A) Estrogen leads to increased OT levels thereby reducing excitability of the trigeminal pain system. The drop in OT during menstruation may be driven, at least in part, by the drop in estrogen. (B) Na^+ may lead to decrease in OTR-OT affinity and thereby increase excitability of the trigeminal pain system. Mg^{2+} and cholesterol increase in OTR-OT affinity and thereby reduce excitability of the trigeminal pain system. High levels of Na^+ and low levels of Mg^{2+} and cholesterol can contribute to the probability of onset of MRM attack by modulating OTR affinity for OT in the trigeminal pain pathway. (C) IL-6 and estrogen both increase OTR expression. Decreased IL-6 and estrogen levels during menstruation could decrease OTR expression, reducing the inhibitory effect of OT on trigeminal excitability and thus increasing the likelihood of painful MRM attacks. Created with BioRender.com

these factors, the receptor can reversibly change its conformation from low affinity ($K_d \sim 100$ nM) to high affinity ($K_d \sim 1$ nM) and vice versa[76] for OT. In the presence of Mg, cholesterol binding to its specific domain in the OTR causes a conformational change that both increases the affinity of OT for the OTR and stabilizes the receptor against proteolytical degradation.[79] Cholesterol (total cholesterol and low-density lipoprotein) levels are highest during the follicular phase and decline during the luteal phase reaching the lowest levels just before menstruation[68,80] corresponding to times associated with MRM attacks (see Figure 2B). Although some studies show elevated overall cholesterol levels in some migraine patients,[81–83] levels of cholesterol and OT have not been co-serially investigated in MRM patients.

In contrast to the positive modulating effects of Mg^{2+}, sodium has been shown to negatively modulate the affinity of OT for the OTR.[84] Specifically, sodium (Na^+) allosterically modulates the agonist-binding site stabilizing the receptor in a low-affinity receptor state.[84] The concentration of Na^+ in plasma is lowest during the luteal phase[85,86] and peaks at menstruation[11] corresponding to when MRM attacks occur. Moreover, Na^+ concentrations were reported to be significantly higher in the cerebrospinal fluid in patients with migraine than in healthy controls.[87,88] These clinical studies may suggest that peak levels of serum Na^+ during menstruation could negatively modulate the OT affinity for the OTR, leading to increased neuronal excitability in the trigeminal system (see Figure 2B). The levels of Na^+ and OT have not yet been co-serially investigated in MRM patients.

As shown in Figure 2B, magnesium and cholesterol, taken together, positively modulate OT binding to OTR and Na^+ negatively modulates OT-OTR affinity. Thus, during menstruation, the drop in Mg^{2+} and cholesterol, along with the increase in Na^+ should lead to reduced OTR affinity to OT. The reduced OTR affinity to OT should lead to an increase in neuronal excitability in the trigeminal system and the probability of an MRM attack.

OTR expression levels

OTR expression is highly variable and can rapidly change. Similar to the expression in the uterus, trigeminal OTR expression is strongly and rapidly enhanced in the presence of inflammatory mediators.[20,61] Inflammation rapidly upregulates OTR protein expression by more than 10-fold within 2 h postinflammation induction and potently enhances trigeminal antinociception in rats.[16] Circulating IL-6 is a likely candidate to promote changes in OTR expression as there are three response elements in the OTR.[53] Results from both clinical and preclinical studies demonstrate that OT analgesia is substantially enhanced in the presence of IL-6 and other inflammatory mediators.[16,20] IL-6 serum levels are significantly higher in migraine patients during an attack compared with control.[89] Additionally, serum IL-6 levels follow estrogen levels, increase during preovulation and drop to the lowest point during the luteal phase and menstruation[66] (see Figure 2C).

In addition to IL-6, there is an estrogen response element on the OTR promoter, and consequently, estrogen can cause a significant increase in OTR messenger ribonucleic acid (mRNA) in both peripheral and CNS tissues.[43,90,91] The estrogen-induced increase in OTR expression may in part be mediated by de novo synthesis of OTR mRNA or by alterations in the stability of OTR gene transcripts.[43,92] Specifically, preclinical studies show that estrogen treatment induced a fourfold increase in the hypothalamus, a threefold increase in the amygdala, a 1.7-fold increase in the hippocampus, and a threefold increase in the myometrium compared with ovariectomized female rats.[90–92] Additionally, estrogen can lead to increased number and immunostaining of OT fibers in regions of the hypothalamus.[90,91] Thus, in addition to modulating OT levels, serum estrogen can modulate expression levels of OTR. In summary, estrogen and inflammatory mediators including IL-6 can increase OTR expression. During menstruation, a drop in IL-6 and estrogen serum levels may decrease OTR expression collectively promoting an increased likelihood of a MRM attack (see Figure 2C).

We recognize that many other factors can influence OT/OTR tone and thus may influence MRM. It should also be noted that OT and the OTR show structural similarities with vasopressin and its receptors and OT agonists and antagonists can act at vasopressin receptors, especially at higher concentrations.[93,94] As such, a detailed examination of the effect of the same menstrually related factors, including magnesium, cholesterol, and sodium, on vasopressin and its receptor and its effect on OT/OTR tone is warranted.

CONCLUSION: CLINICAL PRACTICE AND THERAPEUTIC IMPLICATIONS

Trigeminal neurons possess OTR and are inhibited by OT. The same neurons also often contain CGRP, and the release of CGRP is inhibited by OT. Blockade of CGRP or CGRP receptors with either monoclonal antibodies or small molecule receptor antagonists is effective in preventing migraine in many patients.[95–97] A recent study using telcagepant, a CGRP receptor antagonist, showed a reduction in on-drug premenstrual headaches.[98] Thus, OT-induced CGRP release inhibition could be a targeted mechanism for MRM therapeutics. Similarly, clinical studies have shown Mg^{2+} to be effective in attenuating MRM attacks.[32] Furthermore, the elevation of both OT levels and OTR expression likely provides the mechanism through which rapid changes in estrogen level can precipitate MRM as well as the mechanism through which MRM can be prevented by pharmacological estrogen.[28] More directly, elevating OT levels within the trigeminal system could be effective in preventing or aborting MRM.[16] Thus, OT, estrogen, and Mg^{2+}, either alone or in combination, should decrease the excitability of the migraine-associated trigeminovascular system that is activated in MRM providing a basis for novel therapeutics for MRM.

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CONFLICT OF INTEREST

David C. Yeomans and Shashidhar Kori are consultants for Tonix Pharmaceuticals. All other authors have no conflict of interest.

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Impact of Magnesium on Oxytocin Receptor Function

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Simple Summary: What is already known: Mg^{2+} levels modulate the affinity of oxytocin receptors for oxytocin in vitro, low serum Mg^{2+} is correlated with migraine headache onset. What this study adds: Electrophysiologic and behavioral assays demonstrate that Mg^{2+} increases the efficacy of oxytocin; oxytocin efficacy is limited by Mg^{2+} availability. Clinical significance: Modulating Mg^{2+} levels may enhance oxytocin efficacy for pain, other uses, and endogenous processes.

Abstract: Background and Purpose: The intranasal administration of oxytocin (OT) reduces migraine headaches through activation of the oxytocin receptor (OTR). Magnesium ion (Mg^{2+}) concentration is critical to the activation of the OTR, and a low serum Mg^{2+} concentration is predictive of a migraine headache. We, therefore, examined the functional impact of Mg^{2+} concentration on OT-OTR binding efficacy using two complimentary bioassays. Experimental Approach: Current clamp recordings of rat trigeminal ganglia (TG) neurons measured the impact of Mg^{2+} on an OT-induced reduction in excitability. In addition, we assessed the impact of Mg^{2+} on intranasal OT-induced craniofacial analgesia in rats. Key Results: While OT alone dose-dependently hyperpolarized TG neurons, decreasing their excitability, the addition of 1.75 mM Mg^{2+} significantly enhanced this effect. Similarly, while the intranasal application of OT produced dose-dependent craniofacial analgesia, Mg^{2+} significantly enhanced these effects. Conclusions and Implications: OT efficacy may be limited by low ambient Mg^{2+} levels. The addition of Mg^{2+} to OT formulations may improve its efficacy in reducing headache pain as well as for other OT-dependent processes.

Keywords: oxytocin; magnesium; pain; analgesia; headaches; agonist

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1. Introduction

Oxytocin (OT) is a nine-residue, cyclized peptide which, through to the oxytocin receptor (OTR), acts as both a hormone, regulating diverse processes such as glucose metabolism, social bonding, lactation, and uterine contraction, as well as a neurotransmitter, modulating neural processes including pain [1–4]. Inadequate OTR activity is associated with postpartum hemorrhage, inadequate milk production, autism spectrum disorder (ASD), Prader–Willi syndrome, and migraine headaches [5–8]. Studies in both animal models and patients have demonstrated that intranasal administration of OT can relieve core symptoms in ASD, schizophrenia, migraine, and anxiety disorders [9–11]. Several factors have been found to alter OTR activity including OT levels, OTR expression, and OTR affinity for its ligand [8]. The last of these, OTR affinity, is closely modulated by the local concentration of the magnesium cation (Mg^{2+}) which acts as an essential cofactor, dramatically increasing the affinity of OTR for OT [8,12,13]. Recently, Meyerowitz et al. [14] published the structure of the OT-OTR-Mg^{2+} coordination complex as identified using

cryo-electron microscopy, demonstrating the critical role that Mg^{2+} plays in OT binding to OTR. Thus, alterations of serum or exogenously applied Mg^{2+} levels should play a critical role in OTR activity and consequently the physiological and psychological functions listed above.

Our previous work showed that OT decreases craniofacial pain in rodent models and headache severity and frequency in patients with migraine [10]. Not surprisingly, low serum Mg^{2+} levels strongly correlate with the frequency of headache attacks in patients with migraine [15–17]. However, the functional consequence of Mg^{2+} control of OT binding has not been adequately examined, particularly with regard to the function of OTR activity and pain. The purpose of this study was, therefore, to examine the impact of Mg^{2+} on OT-OTR binding efficacy by examining these effects in neuronal electrophysiology and pain behavior. The tools selected to address our question included both current clamp experiments on freshly dissociated rat trigeminal ganglia (TG) neurons to determine OT effects on neuronal excitability as well as the in vivo impact of the addition of Mg^{2+} to intranasally administered OT in a rat model of craniofacial pain.

2. Materials and Methods

2.1. Experimental Design

The objective of this study was to examine the impact of Mg^{2+} on OT-OTR binding efficacy by examining the effects on two key physiologic assays. We have previously demonstrated that OTR levels in trigeminal neurons are dependent, in part, on the presence of inflammatory cytokines [18]. Therefore, in this study, rats were pretreated with a complete Freund's adjuvant (CFA) injection into the temporomandibular joint (TMJ) in order to produce a robust inflammation of trigeminally innervated tissue, inducing OTR upregulation approximately 24 h prior to harvesting and dissociating TG neurons. An electrophysiological recording was used to measure the change in membrane potential of TG neurons due to vehicle/OT treatment. In a separate group of animals, approximately 24 h after CFA injection, withdrawal latencies in response to noxious heat applied to the cheek were determined. Increased withdrawal latencies was indicative of analgesia/anti-nociception. Sample sizes were chosen on their basis to detect statistically significant results, with statistical analysis detailed throughout the study.

2.2. Animals

All animal care and experimental procedures complied with the laws of the United States and regulations of the Department of Agriculture and were approved by the Stanford University (Stanford, CA, USA) Institutional Animal Care and Use Committee, in accordance with the 2011 National Institute of Health Guide for the Care and Use of Laboratory Animals. Animal studies are reported in compliance with the ARRIVE guidelines (Kilkenny, Browne, Cuthill, Emerson, & Altman, 2010; McGrath & Lilley, 2015). Ethical permission (A3213-01 on 3 November 2020)

Rats (male, 250–330 g, Envigo, Indianapolis, IN, USA) were maintained two per cage in a controlled environment (temperature: 21.5 ± 4.5 °C/relative humidity: 35–55%) under a standard 12 h light/12 h dark lighting cycle (lights on at 7:00, no twilight). Cage changes occurred twice a week, using standard bedding. Food and water were provided ad libitum. Estrogen levels have been shown to drive OTR expression levels and so could drive substantial variability in response to OT treatment. Thus, we chose to use male animals for these studies to minimize this factor. We are currently pursuing separate experiments intended to explore this issue with regard to Mg^{2+} effects. Initial sample sizes were approximated by Power analysis, with animals assigned to groups randomly. Drug treatment experiments were conducted in a blinded fashion.

2.3. Sample Preparation

2.3.1. Solutions for Electrophysiological Recording

OT (Grindeks, Riga, Latvia) was dissolved in distilled water as 1 mM stock and diluted for external solutions. Different concentrations of OT (1.0, 3.0, 10, 30, 100, 300, or 1000 nM) were applied from the reservoir using gravity feeding through a 27G tip-blunted needle with an opening placed about 200 µm away from the recorded cell. The stream of solution covered the cell well when the switch was turned on. The cells were physically stable during perfusion.

2.3.2. Solutions for Behavior Studies

The appropriate amount of test compound was accurately weighed out using a calibrated electrical balance and placed into microcentrifuge tubes. Solutions were prepared for an administration volume of 50 µL containing vehicle or one solution for one of five doses of OT (0.5, 1.0, 4.0, 8.0, or 32.0 µg) plus or minus 300 mM magnesium citrate. These doses and concentrations were selected based on the results of prior studies [10,19,20]. Ten groups of 10 rats each were randomly assigned to receive a vehicle or one of the five doses of OT or OT plus 300 mM Mg^{2+}. Each solution was coded by the sponsor and the experimenter performed the experiment in a strictly blinded manner, including drug administration and data analysis.

2.4. Electrophysiology

2.4.1. Induction of Inflammation

Briefly, rats (male, 250–330 g, Envigo, n = 10) were placed in an anesthesia chamber and anesthetized with 2.5% isoflurane. Prior to TMJ injection, the rat's mouths were propped open to palpate the target area. In this position, an oval-shaped groove located in the center of the cheek and above the mandible can be distinctly felt. With the syringe positioned at a 30-degree angle from the rat's cheek, the tip of the needle was inserted just under the articular disc (approximately 1.5 mm in diameter and 1.0 mm deep). Thereafter, 50 µL of CFA (DIFCO; Sigma Aldrich, St. Louis, MO, USA) was injected (1 mL syringe with a 25G 5/8-inch needle) into the left TMJ to produce robust and prolonged orofacial inflammation. After CFA injection, rats were returned to home cages. Approximately 24 h later, rats were euthanized by decapitation after induction of deep anesthesia with isoflurane.

2.4.2. Tissue Processing

The rat's TG were carefully dissected from the surrounding connective tissues and minced into small pieces with an iris scissor. The TG were digested in 0.5 mL of mixed enzyme solution: (w/v, final concentration) 0.1% trypsin (Sigma, T9201), 0.1% collagenase Sigma, C1764), and 0.01% DNase (Sigma, D5025) diluted in Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F12) (Sigma, St. Louis, MO, USA). The tissue pieces were then incubated at 32 °C with a water bath for 55 min. Following digestion, tissue fragments were mechanically dissociated using a series of glass Pasteur pipettes with decreasing internal diameter. Dissociated cells were centrifuged at 180× g for 3 min, the supernatant was removed, and the cells were gently re-suspended in an external recording solution. Cells were then plated onto poly-L-lysine (Sigma, St. Louis, MO, USA) coated cover slips (Chemglass Life Sciences Vineland, NJ, USA.

2.4.3. Current Clamp Recording

Whole-cell voltage-clamp recordings were performed using the MultiClamp 700B amplifier (Molecular Devices, San Jose, CA, USA) and analyzed offline with pCLAMP10.4 software (Molecular Devices, San Jose CA, USA). The external solution was composed of (in mM) NaCl (130), N-2-Hydroxyethylpiperazine-N'-2-Ethanesulfonic Acid (HEPES)-Na (10), KCl (5), $CaCl_2$ (1), and Glucose (10), pH adjusted to 7.3–7.4 using HCl, with or without 1.75 mM $MgCl_2$. The electrode internal solution was composed of (in mM) KF (120),

HEPES (10), ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA) (11), $CaCl_2$ (1), $MgCl_2$ (1), KCl (10), and KOH (11), pH adjusted to 7.3–7.4 using KOH. Patch-pipettes were fabricated from 1.5 mm outside diameter (OD) borosilicate capillary glass (Warner Instruments, Hamden, CT, USA) using a micropipette puller (Model P-87, Sutter Instrument, Novato, CA, USA). NaCl, HEPES-Na, KCl, CaCl2, Glucose, HCl, MgCl2, KF, HEPES, EGTA, KOH were purchased from Sigma (St. Louis, MO, USA). Glass pipettes filled this intracellular saline with a resistance of 3–5 MΩ. Whole-cell patch recordings had series resistances of <25 MΩ after whole-cell configuration and were periodically checked with the seal test voltage step (10 mV, 10 ms) to monitor series resistances throughout the recordings. Hyperpolarizing current pulses (about −0.3 nA, 500 ms) were delivered every 5 s throughout the experiment, unless otherwise specified, in order to monitor membrane input resistance and stabilize membrane potential in control external solution.

Measurement of change in membrane potential: After successful current clamp recording, the effect of the vehicle external solution application to cells on membrane potential was measured. After the membrane potential had stabilized for at least 10 s, a solution containing OT plus or minus 1.75 mM Mg^{2+} was applied for 2–5 min until the membrane potential stabilized further (on a new level) for at least 10 s.

2.5. Behavioral Analgesia

2.5.1. Withdrawal Latency

Rats (male, 250–330 g, Envigo, n = 10 per group) were used and treated with CFA injection into the TMJ as described above in order to produce a robust inflammation of trigeminally innervated tissue. Approximately 24 h after CFA injection, withdrawal latencies in response to noxious heat applied to the depilated (NAIR® hair removal cream; Church & Dwight Co., Ewing, NJ, USA) and blackened (with India ink (Chartpak Inc., Leeds, MA, USA)) cheek were determined. Latency to withdrawal response was used as an indicator of nociceptive responsiveness. We have previously demonstrated, using single-fiber peripheral nerve recordings, that low intensity (slow ramp) skin heating evokes withdrawal responses mediated by the activation of C- (unmyelinated) nociceptive fibers; higher intensity skin heating (rapid ramp) selectively elicits responses mediated by A-delta (myelinated) thermonociceptors [21,22]. Briefly, to assess C fiber mediated responses, heat intensity was adjusted by altering the supply voltage (35–55 V) of the focused lamp until a withdrawal response was observed to occur with latency between 7.5 and 8.5 s. In order to reduce the potential for tissue damage, a cut-off latency of 15 s was implemented after which the stimulus was terminated. Rats not responding (within 10 s) to a supply voltage of 55 V during baseline testing were excluded from the study. For A-delta fiber testing, the heat intensity was adjusted by altering the supply voltage (60–85 V) of the focused lamp until withdrawal responses were observed to occur with a latency of between 2.5 and 3.5 s. The intensity applied to achieve such latencies was noted for each animal and used to assess withdrawal latencies prior to and following nasal administration of the test agent. To reduce the potential for tissue damage, a cut-off latency of 5 s was implemented during A-delta fiber testing. Rats not responding (within 3.5 s) to a supply voltage of 85 V were excluded from the study. A total of five rats were excluded from further testing by not reaching these criteria. Baseline withdrawal latencies were determined for each fiber type prior to nasal application of the test agent.

To deliver intranasal OT or vehicle the rats were anesthetized in a chamber using isoflurane (2%). They were then placed on a heating pad in a supine position as the anesthesia was continued with a nose cone. This horizontal position of the head was maintained throughout the procedure preventing drainage of the drug solution to the trachea and esophagus. The total volume of 50 µL solutions was administered by pipette in 6–7 µL drops in alternating naris every two min, over a total of 14 min. The drop was placed at the naris opening while occluding the opposite naris allowing the animal to snort the drop into the nasal cavity. The rats were allowed to wake up in a separate cage on a heating pad. Rats were then returned to their home cage. Withdrawal latencies in response

to A-delta or C fiber cheek stimulation were then remeasured at 60 min following dosing. At the end of the testing session, rats were euthanized by CO_2 inhalation.

2.5.2. Efficacy Evaluation

Withdrawal latencies in response to thermal stimuli (noxious heat) were recorded as an index of thermal pain sensitivity. Increased withdrawal latencies were considered indicative of analgesia/anti-nociception.

2.6. Data and Statistical Analysis

All data are presented as means ±SD with significance set at $p < 0.05$. Statistical analysis was undertaken only for data sets where each group size was at least n = 5. All results were analyzed using the GraphPad Prism 9.0 software (GraphPad Software Inc., San Diego, CA, USA. RRID:SCR_008).

2.6.1. Electrophysiology

Data were acquired using Clampfit—V10.4, Molecular Devices (San Jose, CA, USA) and data sheets were constructed in Excel (Microsoft) and GraphPad Prism Software. Two-way ANOVA were performed to compare the overall significance of the difference between OT and OT + Mg^{2+}. Sidak's multiple comparison test was used to determine differences at individual concentrations. Significance was set at $p < 0.05$.

2.6.2. Withdrawal Latency

All data are expressed as mean withdrawal latency ±SE at 60 min post-dosing. Data generated during the testing of each fiber type (e.g., A-delta and C fibers) were analyzed, tabulated, and graphed separately. Statistical analyses were conducted using GraphPad Prism statistical software. All tests were conducted at the 0.05 level of statistical significance. Data generated were assessed using separate 2-way repeated-measures ANOVAs to determine if nasal OT produced dose-dependent significant increases in withdrawal latencies for A-delta or C fiber mediated responses and whether the addition of Mg^{2+} would significantly increase that response as determined by a significant difference between dose-response curves. Sidak's multiple comparison tests were used for subsequent pairwise comparisons to pretreatment latencies.

3. Results

3.1. Electrophysiology Recording: Effect of Different Doses of OT with and without Mg^{2+} on Membrane Potential

After a successful current clamp of TG neurons, the vehicle was applied as a control to the external solution. Both with or without 1.75 mM Mg^{2+}, perfusion of cells with vehicle increased membrane potential insignificantly from -59.4 ± 1.9 mV to -60.6 ± 1.9 mV ($p = 0.13$, n = 9, student paired *t*-test). Figure 1A,B shows an example of a typical current clamp trace recording of a TG neuron from a CFA-inflamed rat. OT dose (3 nM) alone did not induce any change in the membrane potential (Figure 1A). However, the addition of 1.75 mM Mg^{2+} resulted in the hyperpolarization of membrane potential (Figure 1B). While OT alone dose-dependently induced membrane hyperpolarization, a consistently larger hyperpolarization of membrane potential was concentration-dependently observed with the addition of 1.75 mM Mg^{2+} (Figure 1C). Two-way ANOVA analysis showed the effect of OT on hyperpolarization was significantly different between the OT and OT + Mg^{2+} groups overall (2-way ANOVA, ($p < 0.05$)). A post-hoc multiple comparison (Sidak's) test showed significant difference between OT and OT + Mg^{2+} groups at 1, 3, 10 ($p < 0.05$), and 1000 nM OT concentrations ($p < 0.05$).

addition of 1.75 mM $MgCl_2$ to the external solution induced a strong hyperpolarization, −8.1 ± 1.2 mV (n = 7 for 1 nM OT) and −9.3 ± 1.2 mV (n = 7 for 3 nM OT), respectively (Figure 1D). Two-way ANOVA analysis showed the overall effect of OT on hyperpolarization were significantly different between the OT and OT + Mg^{2+} groups (2-way ANOVA, ($p < 0.05$)). A post-hoc multiple comparison (Sidak's) test showed a significant difference between OT and OT + Mg^{2+} groups at 1 ($p < 0.05$) and 3 nM OT concentrations ($p < 0.05$).

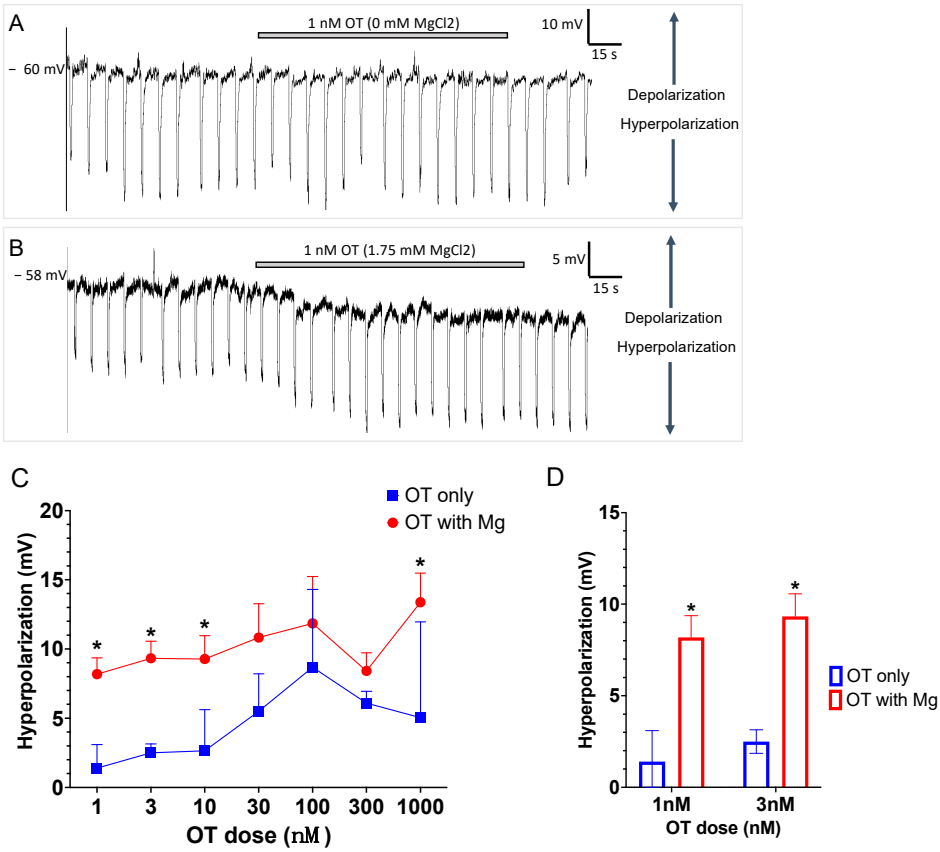

Figure 1. Effect of addition of Mg^{2+} to the OT-induced decrease in excitability of TG neurons from CFA-inflamed rats. (**A**,**B**) Examples of current-clamp traces of TG neurons from CFA-inflamed rat; (**A**) no change in membrane potential when treated with 3 nM OT in a 0 mM $MgCl_2$ buffer and hyperpolarization observed when treated with 3 nM OT in 1.75 mM $MgCl_2$ buffer. (**C**,**D**) OT dose-dependently hyperpolarizes TG cell membranes, decreasing excitability, the addition of 1.75 mM Mg^{2+} significantly (* $p < 0.05$, ANOVA) potentiates the capacity of OT to hyperpolarize TG cell membranes (**C**). A specific example of this is observed in (**D**), where the addition of Mg^{2+} significantly (* $p < 0.05$) increased the induced membrane hyperpolarization of 1 nM or 3 nM OT from −1.4 ± 1.6 mV (n = 6) and −2.5 ± 0.6 mV (n = 6) for OT alone to −8.1 ± 1.2 mV (n = 7) and −9.3 ± 1.2 mV (n = 7) for OT plus Mg^{2+}, respectively. Subsequent pairwise comparisons indicated significant (* $p < 0.05$) differences from OT alone for OT plus Mg^{2+} at 1, 3, 10, and 1000 nM OT concentrations. This enhanced hyperpolarization is emblematic of decreased excitability and thus, decreased capacity to carry pain signals to the central nervous system for pain perception. Error bars show ± S.D.

Figure 1. Effect of addition of Mg^{2+} to the OT-induced decrease in excitability of TG neurons from CFA-inflamed rats. (**A**,**B**) Examples of current-clamp traces of TG neurons from CFA-inflamed rat; (**A**) no change in membrane potential when treated with 3 nM OT in a 0 mM $MgCl_2$ buffer and hyperpolarization observed when treated with 3 nM OT in 1.75 mM $MgCl_2$ buffer. (**C**,**D**) OT dose-dependently hyperpolarizes TG cell membranes, decreasing excitability, the addition of 1.75 mM Mg^{2+} significantly (* $p < 0.05$, ANOVA) potentiates the capacity of OT to hyperpolarize TG cell membranes (**C**). A specific example of this is observed in (**D**), where the addition of Mg^{2+} significantly (* $p < 0.05$) increased the induced membrane hyperpolarization of 1 nM or 3 nM OT from −1.4 ± 1.6 mV (n = 6) and −2.5 ± 0.6 mV (n = 6) for OT alone to −8.1 ± 1.2 mV (n = 7) and −9.3 ± 1.2 mV (n = 7) for OT plus Mg^{2+}, respectively. Subsequent pairwise comparisons indicated significant (* $p < 0.05$) differences from OT alone for OT plus Mg^{2+} at 1, 3, 10, and 1000 nM OT concentrations. This enhanced hyperpolarization is emblematic of decreased excitability and thus, decreased capacity to carry pain signals to the central nervous system for pain perception. Error bars show ± S.D.

3.2. Behavioral Analgesia: Effect of Different Doses of OT with and without Mg^{2+} on Withdrawal Latency

Baseline testing revealed stable head withdrawal response latencies for A-delta (2.6–3.2 s) and C-fiber (7.5–8.1 s) radiant heat stimulation of the cheek (Figure 2A,B). The intranasal application of OT produced a dose-dependent increase in withdrawal latency up to the highest dose (32 µg), where efficacy was seen to dramatically decrease for both A-delta and C fiber mediated pain responses. Application of the same doses of OT in the presence of Mg^{2+} however, produced a significant ($p < 0.0001$, 2-way ANOVA) increase in withdrawal latency compared to efficacy in the absence of Mg^{2+} for both A-delta (Figure 2A) and C-fiber (Figure 2B) testing. Follow-on analysis revealed significant differences (p

3.2. Behavioral Analgesia: Effect of Different Doses of OT with and without Mg^{2+} on Withdrawal Latency

In fact, while 1 or 3 nM OT had a minimal membrane potential effect (−1.4 ± 1.6 mV (n = 6) and −2.5 ± 0.6 mV (n = 6), hyperpolarization for 1 and 3 nM OT, respectively), the addition of 1.75 mM $MgCl_2$ to the external solution induced a strong hyperpolarization, −8.1 ± 1.2 mV (n = 7 for 1 nM OT) and −9.3 ± 1.2 mV (n = 7 for 3 nM OT), respectively (Figure 1D). Two-way ANOVA analysis showed the overall effect of OT on hyperpolarization were significantly different between the OT and OT + Mg^{2+} groups (2-way ANOVA, ($p < 0.05$)). A post-hoc multiple comparison (Sidak's) test showed a significant difference between OT and OT + Mg^{2+} groups at 1 ($p < 0.05$) and 3 nM OT concentrations ($p < 0.05$).

Baseline testing revealed stable head withdrawal response latencies for A-delta (2.6–3.2 s) and C-fiber (7.5–8.1 s) radiant heat stimulation of the cheek (Figure 2A,B). The intranasal application of OT produced a dose-dependent increase in withdrawal latency up to the highest dose (32 µg), where efficacy was seen to dramatically decrease for both A-delta and C fiber mediated pain responses. Application of the same doses of OT in the presence of Mg^{2+} however, produced a significant ($p < 0.0001$, 2-way ANOVA) increase in withdrawal latency compared to efficacy in the absence of Mg^{2+} for both A-delta (Figure 2A) and C-fiber (Figure 2B) testing. Follow-on analysis revealed significant differences (p

application of OT produced a dose-dependent increase in withdrawal latency up to the highest dose (32 µg), where efficacy was seen to dramatically decrease for both A-delta and C-fiber mediated pain responses. Application of the same doses of OT in the presence of Mg^{2+} however, produced a significant ($p < 0.0001$, 2-way ANOVA) increase in withdrawal latency compared to efficacy in the absence of Mg^{2+} for both A-delta (Figure 2A) and C-fiber (Figure 2B) testing. Follow-on analysis revealed significant differences ($p < 0.05$) between OT and OT + Mg^{2+} for A-delta testing at OT doses of 0.5, 1.0, and 32 µg OT; C-fiber responses were significantly different at 0.5, 4.0, 8.0, and 32 µg OT.

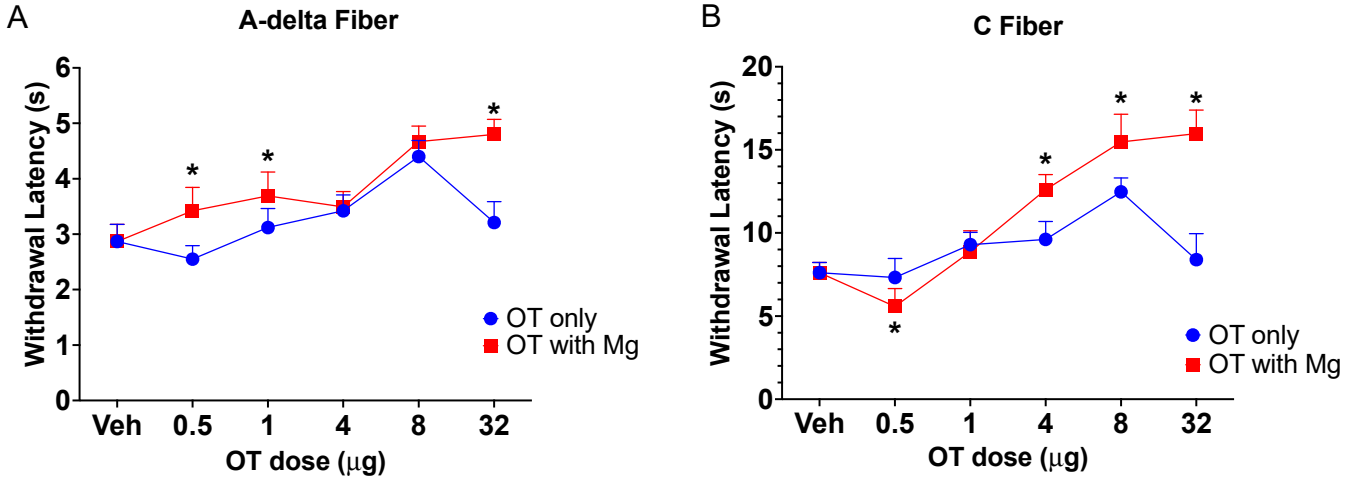

Figure 2. Effect of the addition of Mg^{2+} on intranasal OT-induced craniofacial analgesia. For both A-delta (**A**) and C-fiber (**B**) mediated withdrawal responses to noxious heat stimulation of the cheek of pre-inflamed rats, intranasally applied OT produced a dose-dependent analgesic effect as evidenced by significant ($p < 0.05$, ANOVA) increases in withdrawal latency at the 60 min time point after administration, n = 10. However, the addition of 300 mM Mg^{2+} to the treatment significantly increased OT analgesia for both stimulus types ($p < 0.05$). Subsequent pairwise comparisons indicated significant (* = $p < 0.05$) differences from OT alone for OT plus Mg^{2+} for A-delta testing at OT doses of 0.5, 1.0, and 32 µg ($p < 0.05$); C-fiber responses were significantly different (* = $p < 0.05$) at 0.5, 4.0, 8.0, and 32 µg OT. Interestingly, while the efficacy of the highest OT dose (32 µg) demonstrated a decrease in efficacy when compared to lower doses, this dose-response inversion was prevented by the addition of Mg^{2+}.

4. Discussion and Conclusions

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The requirement of Mg^{2+} for OT's high affinity for its receptor has long been known [13]. More recently, the precise architecture of this Mg^{2+} coordination site has been elucidated in the high-resolution cryo-electron microscopy structure of OT bound to its receptor, revealing that OT and OTR together form an octahedral Mg^{2+} coordination site between the receptor and ligand [14]. However, the functional consequence of Mg^{2+} control of OT binding has not been adequately examined, particularly with regard to how Mg^{2+} levels could affect the impact of OT-OTR binding on pain as well as other phenomena. The results of the second messenger study described in [14] demonstrate that Mg^{2+} is an essential cofactor for full OT-OTR agonism and that Mg^{2+} concentration-dependently increases G_q and G_{11} activation in OTR-transfected HEK cells. Interestingly, this study also demonstrated that OT-OTR binding did not reach maximal efficacy at physiologically relevant Mg^{2+} concentrations [14]. Consistent with these findings, while OT alone dose-dependently hyperpolarized TG neurons, decreasing their excitability, the addition of a supraphysiological (1.75 mM) concentration of Mg^{2+} significantly enhanced this effect, implying a supportive impact of Mg^{2+} on OT craniofacial analgesia. In a demonstration of this support, while intranasal application of OT produced dose-dependent craniofacial analgesia, the addition of 300 mM Mg^{2+} to the administered OT significantly enhanced this analgesia.

The trigeminal nerve provides pain signaling from the head to the central nervous system for the perception of craniofacial pain. Thus, decreases in the excitability of these neurons should produce decreases in craniofacial pain sensitivity. The results of the current study are consistent with our previous finding [23] that OT decreases the excitability of TG neurons in vitro as evidenced by a robust increased (hyperpolarized) cell membrane

The trigeminal nerve provides pain signaling from the head to the central nervous system for the perception of craniofacial pain. Thus, decreases in the excitability of these neurons should produce decreases in craniofacial pain sensitivity. The results of the current study are consistent with our previous finding [23] that OT decreases the excitability of TG neurons in vitro as evidenced by a robust increased (hyperpolarized) cell membrane potential. This work also suggested that this decrease in neuronal excitability is likely mediated, at least in part, by an increase in voltage-gated K^+ channel (Kv) current density [23]. As with the second messenger findings, the addition of 1.75 mM Mg^{2+} to the applied OT in the same concentration range used in the second messenger study produced a significant increase in the degree of hyperpolarization of the membrane. In the absence of Mg^{2+}, the maximal efficacy of OT is not reached, indicating the necessity of Mg^{2+} for the full agonism of OT. Interestingly, while the efficacy of the highest OT dose (32 µg) demonstrated a decrease in efficacy when compared to lower doses, this inversion was prevented by the addition of Mg^{2+}. These findings indicate that the addition of Mg^{2+} produces a more robust decrease in cell excitability, consistent with a stronger analgesic effect than that observed with OT alone.

Using autoradiography and tissue scintillation counts, we have previously demonstrated that radiolabeled OT, when applied nasally, concentrates in the trigeminal nerve and ganglia [20,24]; an approximately 10–20 times higher concentration of radiolabeled OT was detected in the trigeminal system compared to other tissue regions [20,24]. We have also shown that intranasal OT inhibits the transmission of pain messages to the central nervous system [10], inducing analgesia in rodent craniofacial pain models [10,19] and relief from headaches in patients with migraine [10]. The current study demonstrates that, as with the in vitro assays, the addition of Mg^{2+} to OT significantly enhances these analgesic effects. Previously, intravenous Mg^{2+} has been shown to abort continuous migraines and, when given as an oral supplement, reduce their frequency [25]. We have hypothesized that these effects might be mediated, in part, by a Mg^{2+} induced increase in the affinity of OTR for endogenous OT, thereby decreasing the excitability of trigeminal nociceptive neurons. Similarly, we have hypothesized that the decrease in serum Mg^{2+} during pre-menstruation and menstruation might help explain the phenomena of menstrual migraine [8].

In addition to the menstrual cycle effects on Mg^{2+} and migraine, serum estrogen levels, which vary over the menstrual cycle, have been shown to drive OTR expression levels [26,27] and have been hypothesized to underly, in part, the pathogenesis of menstrual migraine [8]. The variability of serum estrogen in females could also drive substantial variability in response to intranasal OT treatment. Thus, we chose to use male animals in these studies to minimize this factor. Because of the variability of serum Mg^{2+} and estrogen, it is likely that the effects of OT in females may vary significantly from those in males [8]. We are currently pursuing separate experiments intended to explore this issue with regard to the impact of Mg^{2+} on OT analgesia in females across the menstrual cycle.

The lowest OT dose (0.5 µg), supplemented with Mg^{2+} produced shorter withdrawal latencies compared to the OT group without Mg^{2+}. One explanation for this observation is based on our preliminary electrophysiological studies that show 1.75 mM Mg^{2+}, in the absence of OT, is in fact, depolarizing in some cells. Thus, with a very low concentration of OT, it is likely that this depolarizing effect overwhelms any minimal hyperpolarizing effect of the OT. Interestingly, while the efficacy of the highest OT dose (32 µg) demonstrated a decrease in efficacy when compared to lower doses, this inversion was prevented by the addition of Mg^{2+}. In a separate ongoing study (and so not directly comparable), we have similarly found that 128 µg demonstrates a significant drop in efficacy compared to lower doses that were preventable by the addition of Mg^{2+}. Inverted U dose responses have been widely reported for various systems, including social recognition [28], opioid-induced respiratory depression [29], and, very recently, in an autism spectrum clinical trial [30]. Although it is not unknown for peptide neurotransmitters to have an inverted U dose-response, the specific reason for a decrease in withdrawal latency at a higher OT dose is still unclear. One explanation could be off-target effects where OT, at high

enough concentrations, begins acting on a different receptor. For example, OT has a very high affinity for the V1a receptor and so it is possible that the effects of OT on this or other receptors might counteract those on the OT receptor. This decrease in efficacy at higher doses of OT may be instrumental in the difficulty in demonstrating clear efficacy of intranasal OT in many clinical studies, where a moderate effect is seen with low doses, but higher doses do not show an improvement. The addition of Mg^{2+} to an OT formulation should allow the use of higher doses, overcoming this barrier for a number of indications. The second messenger study by Meyerowitz et al. [14] showed that the inversion of OT dose-response was not observed, which is likely due to the simplified milieu of the transfected cells versus that of whole neurons or in vivo.

Taken together, the results of these two sets of experiments suggest that Mg^{2+} is required for the full agonism of OT and that, for pain and in many other therapeutic or disease settings, the efficacy of OT may be limited by the availability of Mg^{2+}. Thus, the addition of Mg^{2+} to OT formulations or the development of novel OT analogs based on recently elucidated OTR structural biology [14] that obviate the need for Mg^{2+} may enable enhanced OTR efficacy.

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Informed Consent Statement: Not applicable.

Data Availability Statement: The data that support the findings of this study are available from the corresponding author upon request.

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Review

Intranasal Administration for Pain: Oxytocin and Other Polypeptides

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Abstract: Pain, particularly chronic pain, remains one of the most debilitating and difficult-to-treat conditions in medicine. Chronic pain is difficult to treat, in part because it is associated with plastic changes in the peripheral and central nervous systems. Polypeptides are linear organic polymers that are highly selective molecules for neurotransmitter and other nervous system receptors sites, including those associated with pain and analgesia, and so have tremendous potential in pain therapeutics. However, delivery of polypeptides to the nervous system is largely limited due to rapid degradation within the peripheral circulation as well as the blood–brain barrier. One strategy that has been shown to be successful in nervous system deposition of polypeptides is intranasal (IN) delivery. In this narrative review, we discuss the delivery of polypeptides to the peripheral and central nervous systems following IN administration. We briefly discuss the mechanism of delivery via the nasal–cerebral pathway. We review recent studies that demonstrate that polypeptides such as oxytocin, delivered IN, not only reach key pain-modulating regions in the nervous system but, in doing so, evoke significant analgesic effects. IN administration of polypeptides has tremendous potential to provide a non-invasive, rapid and effective method of delivery to the nervous system for chronic pain treatment and management.

Keywords: intranasal delivery; pain; craniofacial pain; oxytocin; polypeptides; trigeminal system; nasal–cerebral pathway; migraine; nasal administration; neuralgia

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1. Introduction

Recent developments in biochemistry and molecular biology have contributed to improved understanding of polypeptides as key signal transmitters in the central nervous system [1,2]. Polypeptides such as oxytocin are linear organic polymers consisting of two or more amino acids. The use of peptides as pharmacological agents is attractive due to low toxicity of their metabolites and strong potency [3–5]. Although peptides show potential to treat neurological diseases and disorders, they are largely limited as pharmaceuticals for treatments due to the inadequate deposition of functional peptides to specific brain regions. Under physiological conditions, peptide delivery to the brain is limited by the presence of the blood–brain barrier (BBB), which inhibits most therapeutic peptides from entering the brain from blood [6]. In addition, peptides administered orally have generally poor bioavailability and short half-lives due to enzymatic metabolism [7,8]. Parenteral administration routes, such as intravenous, subcutaneous or intramuscular injections, often cannot reach meaningful effect-site concentrations within the central nervous system secondary to the BBB. Although brain-specific delivery strategies, e.g., intraparenchymal and intrathecal infusions, are available and capable of delivering drugs directly to the brain parenchyma or cerebrospinal fluid (CSF) for pain management, these options are very invasive and not always practical [7–9], and generally not accepted by

patients. One non-invasive strategy to allow efficient brain delivery of polypeptides is intranasal (IN) administration. Peptides delivered by the IN route are absorbed into the mucus membrane of the nasal cavity and reach both the brain and the systemic blood circulation [10]. Moreover, peptides can be specifically formulated for IN delivery to improve bioavailability in the brain [10]. Similarly, devices have been developed for the purpose of improving nose-to-brain delivery [11–14]. Intranasal delivery has tremendous potential to allow brain delivery of therapeutic polypeptides that are otherwise impossible to deliver.

Pain is a major public clinical concern with significant social and economic impact worldwide [15]. Of all chronic conditions, pain is the most disabling and has the most negative impact on quality of life [15]. Usually, acute pain conditions are well managed [16]. However, chronic pain conditions including migraine, trigeminal neuralgia, neuropathic and orthopedic pain conditions are especially difficult to treat [17–20]. Chronic pain is associated with altered activity in multiple networks in the central nervous system (CNS) [21,22]. In addition, chronic pain may result in changes in afferent inputs to the brain, brain structure and modulatory pathways [22–24]. Therefore, analgesics for many chronic pain conditions need to reach the peripheral and/or central nervous system at sufficient concentrations for effective treatment.

While recognizing the wide range of chronic pain conditions that could be included, in this narrative review we focus on IN delivery of therapeutic polypeptides for chronic pain associated with the trigeminal system, including headache, migraine and trigeminal neuralgia and other chronic craniofacial pain conditions. We first focus on the studies demonstrating that polypeptides reach the CSF and brain tissue and briefly discuss the nasal–cerebral pathway. Next, we review our studies and the relevant recent literature on IN delivery of oxytocin for trigeminal and chronic pain. Lastly, we review the literature on other IN applied polypeptides that work as analgesics.

2. IN Polypeptides Reach the CSF and Brain

Multiple studies show that polypeptides such as oxytocin reach the trigeminal nerve, cerebrospinal fluid (CSF) and the brain after IN delivery. Specifically, our and other groups [25–27] using a radiolabeling approach in rodent models have shown that IN applied radiolabeled oxytocin accumulates in the respiratory and olfactory epithelium, trigeminal ganglion, olfactory bulb, and brain regions such as the thalamus, hypothalamus, midbrain and pons (Table 1). In addition, recent studies using rodents and non-human primates provide direct evidence that IN delivery of labeled oxytocin reaches the brain via olfactory and/or trigeminal pathways, depositing in target tissues, including the amygdala and hippocampus [28,29]. IN administration of exogenous polypeptides such as oxytocin has been shown to result in functionally relevant increases in CSF concentrations [30,31]. A recent study by Lee et al. provides direct evidence for substantial penetrance of IN administered labeled oxytocin into the CSF in non-human primates [30]. Additionally, CSF samples measured before and after IN administration of oxytocin in pigs show oxytocin levels in CSF sufficient to influence neural activity [31]. These studies provide evidence that intranasally applied polypeptides can reach the nerves and brain regions involved in pain pathogenesis.

Polypeptides administered subcutaneously or intravenously have very short half-lives (3–5 min for oxytocin) likely due to rapid intravascular catabolism, renal elimination and/or degradation in the liver [32,33]. In addition, only tiny fractions of peripherally applied polypeptide reach the CNS (approximately 0.002% for oxytocin) [32,33]. By contrast, IN application of polypeptides leads to much higher brain concentrations; for some peptides, more than 95% is directly transported from the nasal cavity into the CNS [33]. Thus, neural and physiological effects of polypeptides can sometimes be observed after IN delivery and not after intravenous injections [33,34]. However, a recent study provides evidence that a high dose of continuous intravenous infusion (with consistently high plasmatic concentration) of oxytocin was able to induce changes in regional cerebral blood flow in

the amygdala, a region rich in oxytocin receptors [35]. One possible explanation for this discrepancy is that oxytocin may reach hypothalamic sites of partial BBB leakiness, allowing access to oxytocinergic cells which, through a positive feedback, cause an increase in brain oxytocin [14]. However, high peripheral oxytocin concentrations can potentially lead to unforeseen side effects via peripheral oxytocin or vasopressin receptor activation [36].

Table 1. Uptake of radiolabeled oxytocin after intranasal administration in rats. Intranasal I-125-oxytocin is initially concentrated in the respiratory and olfactory epithelium. Labeled oxytocin is preferentially taken up by the trigeminal system and is also present in the hippocampus, thalamus midbrain and pons, key regions in the pain processing pathway. Note that a relatively high value in the blood is likely reflective of oxytocin fragments as the compound is rapidly degraded in the blood.

Tissue	**Mean (nM) ± SE**
Respiratory epithelium	731,147 ± 76,889
Olfactory epithelium	19,348 ± 8141
Trigeminal ganglion	574 ± 181
Trigeminal maxillary N.	471 ± 117
Trigeminal mandibular N.	676 ± 235
Trigeminal ophthalmic N.	424 ± 235
Dorsal dura	152 ± 11.6
Ventral dura	271 ± 43.4
Spinal dura	31 ± 7.9
Olfactory bulbs	33 ± 13
Ant. olfactory nucleus	34 ± 10
Caudate-putamen	39 ± 10
Septal nucleus	24 ± 6
Parietal cortex	29 ± 6
Hippocampus	15 ± 3
Thalamus	21 ± 4
Hypothalamus	21 ± 4
Midbrain	23 ± 12
Pons	26 ± 11
Cerebellum	20 ± 8
Blood	63 ± 4

Multiple studies show that IN delivery of some polypeptides, including oxytocin, produce analgesic effects, at least in part due to their effects on CNS pain circuitry. For example, our group, using electrophysiological and immediate-early gene expression experiments in rodents, showed that IN oxytocin can drastically inhibit responses to craniofacial painful stimulation in a specific brainstem region (trigeminal nucleus caudalis (TNC) [26]. In addition, our group provided evidence that IN oxytocin greatly reduces the number of activated neurons in the TNC in a rodent migraine model [26]. Similarly, IN application of neuropeptide S has been reported to inhibit arthritis pain-related behaviors via changes in amygdalar activity [37]. Consistent with these animal studies, a human study showed that oxytocin specifically modulates neural processes contributing to pain perception [38]. This study observed an association between the analgesic effect of oxytocin and oxytocin-induced modulation of cortical activity after noxious stimulation [38]. These studies clearly show that IN administration of polypeptides such as oxytocin not only reaches the brain but has significant effect on a variety of pain conditions.

applied drugs. The respiratory zone consists of mucus-producing goblet cells (20%) and ciliated cells (80%) and the cells are connected via tight junctions. These cells together perform a cleansing mechanism by trapping and transporting particulates in the mucus, termed as mucociliary clearance (MCC). The MCC is approximately 20 min and has thus become an important consideration for effective intranasal drug delivery. In addition, the trigeminal nerve innervates the respiratory epithelium in the nasal passage, suggesting a key role in the IN transport of compounds to the brain [40,41].

3. Nasal–Cerebral Mechanism

The olfactory region is in the deep upper part of the nasal cavity under the cribriform plate that has high perforations providing access to the CNS. The olfactory region corresponds to ~10% of the total surface area of the nasal cavity (~15 cm^2) and is highly vascularized. The olfactory epithelium is innervated by both the olfactory and trigeminal nerves. The passage of compounds from the nose to the brain via the olfactory zone might occur by various pathways/mechanisms, as discussed below.

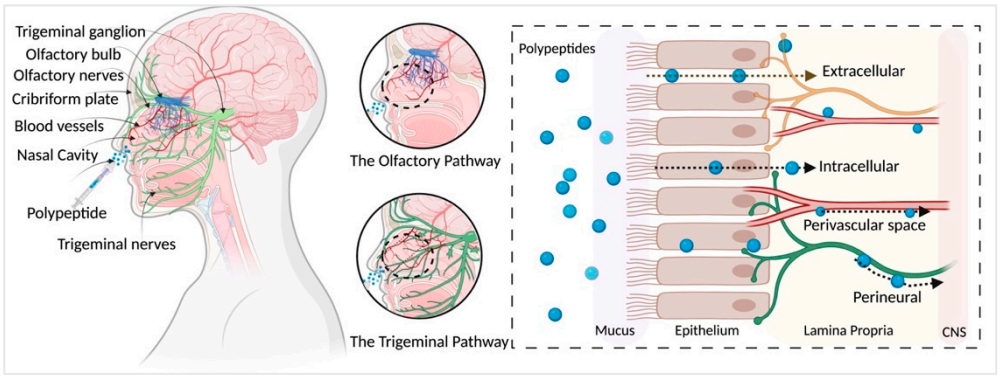

Figure 1. Pathways of polypeptide distribution after IN administration. Olfactory and trigeminal pathways are major contributors to intranasal delivery of polypeptides to the nervous system. Compounds pass through the nasal epithelium to reach the lamina propria largely via extracellular transport and reach the CNS mainly via bulk flow through the perineural, perivascular spaces. Created with BioRender.com (accessed on 21 May 2021).

Previous radiolabeled tracer studies [42–45] using polypeptides and proteins provide evidence that the olfactory and trigeminal nerve pathways are major contributors to intranasal delivery. Intranasally administered compounds first cross the surface of the nasal epithelium and reach the lamina propria, located under the basement membrane of the epithelial surface [10,39]. The lamina propria contains components of the olfactory nerves and the trigeminal nerves that provide the anatomical connections between the nasal passage to the CNS.

Compounds have been shown to be rapidly transported from the nasal passages to the olfactory bulb via extracellular pathways [39,46]. Extracellular transport likely involves diffusion along peripheral olfactory or trigeminal nerves [10,39]. By contrast, intracellular

pathway mechanisms are shown to be a slow process and are not likely to provide a significant mode of transport for IN compounds [10,39]. In addition to extracellular and intracellular transport, IN compounds are shown to distribute through the perineural spaces of the olfactory and trigeminal nerve bundles, via bulk flow [47,48].

There are vascular connections between the nasal passages and the brain that provide a potential mode of transport for IN compounds [10,41,49]. For example, there are blood vessel connections between the cribriform plate and nasal lamina propria [10,41,49]. Also, the nasal–olfactory artery sends branches from the olfactory bulb into the lamina propria [10,41,49]. Although not clearly understood, the perivascular spaces of these blood vessels are considered a potential extracellular pathway to enter the brain [39,50–52]. After reaching the brain, compounds can be distributed throughout the CNS via bulk flow mechanisms and/or more rapidly via the perivascular spaces [39,53]. For example, IN studies using [^{125}I]-labeled IGF-1 show rapid distribution towards the CNS in about 30 min [42]. Such rapid distribution is thought to occur due to extracellular convection rather than diffusion or intracellular transport. One hypothesis for the fast extracellular transport is via bulk flow in the perivascular spaces in the nose-to-brain pathway [42,46].

4. Analgesic Effects of Oxytocin

IN administered oxytocin has been investigated by several groups for relief of migraine and other pain types [26]. For example, analgesic effects of IN applied oxytocin have also been shown for pain after mild traumatic brain injury [54], wound pain [55], chronic low back pain [56] and chronic pelvic pain [57]. Modulation of neuronal activity of the trigeminal nerve, limbic and cortical brain regions as well as ascending and descending pain pathways in the spinal cord have been suggested as potential mechanisms for oxytocin's pain-modulating effects [58]. For example, a recent study in chronic low back pain patients using functional magnetic resonance imaging suggests that striatum plays a key role in the underlying pain-modulating effects of oxytocin in patients [59]. In addition to chronic back pain, oxytocin plays an analgesic role in migraine. Recently, Garcia-Boll and colleagues have demonstrated that oxytocin reduces trigeminocervical complex neuronal firing evoked by meningeal electrical stimulation, a well-established electrophysiological model of migraine [60]. Other potential mechanisms of migraine relief include blockade of CGRP release, which plays an important role in migraine. For instance, intranasal treatment with oxytocin has been shown to decrease the frequency of headaches in both chronic and high-frequency episodic migraineurs [26]. IN oxytocin has been studied in highly standardized experimental pain protocols. For example, Paloyelis et al. demonstrated that IN oxytocin reduced subjective pain ratings and attenuation of the amplitude of N1, N2 and P2 components in a double-blind, placebo-controlled cross-over study in healthy volunteers using laser-evoked potentials [38].

Interestingly, sex-specific effects of intranasal oxytocin on pain perception have been observed. For example, Tracy et al. showed that intranasal oxytocin increased the perceived intensity of noxious heat stimuli in women with chronic neck and shoulder pain, but not in men [61]. Similarly, a recent study on the perception of wound pain showed that intranasal oxytocin reduced wound pain in men, but not in women [55]. These studies on sex-specific effects suggest that oxytocin and endogenous sex hormones may interact to influence pain perception. Clinical studies using IN oxytocin for pain is summarized in Table 2.

Previously, Tzabazis et al. have shown that nasally applied oxytocin concentrates predominantly in the trigeminal nerve, ganglia and nucleus, as well as the dura mater—a key terminal field for the trigeminal nerve—and have shown that nasal oxytocin inhibits the firing of peripheral and central trigeminal nociceptive neurons [26]. In a recent study presented here for the first time, the authors demonstrate the analgesic effect of intranasal oxytocin in a rat model of trigeminal neuralgia. In this study, polymer crystals were stereotaxically applied between the trigeminal nerve root and the crista petrosal bone in order to produce chronic compression of the nerve root [62], which results in a behavioral phenotype highly reminiscent of human trigeminal neuralgia, including exquisite peri-oral

reotaxically applied between the trigeminal nerve root and the crista petrosal bone in order to produce chronic compression of the nerve root [62], which results in a behavioral phenotype highly reminiscent of human trigeminal neuralgia, including exquisite perioral hypersensitivity to brush and grimacing. Results from this original study showed that daily treatment with carbamazepine for 4 days or a single dose of IN oxytocin significantly decrease responsiveness to brush stimulation of the peri-oral face (Figure 2).

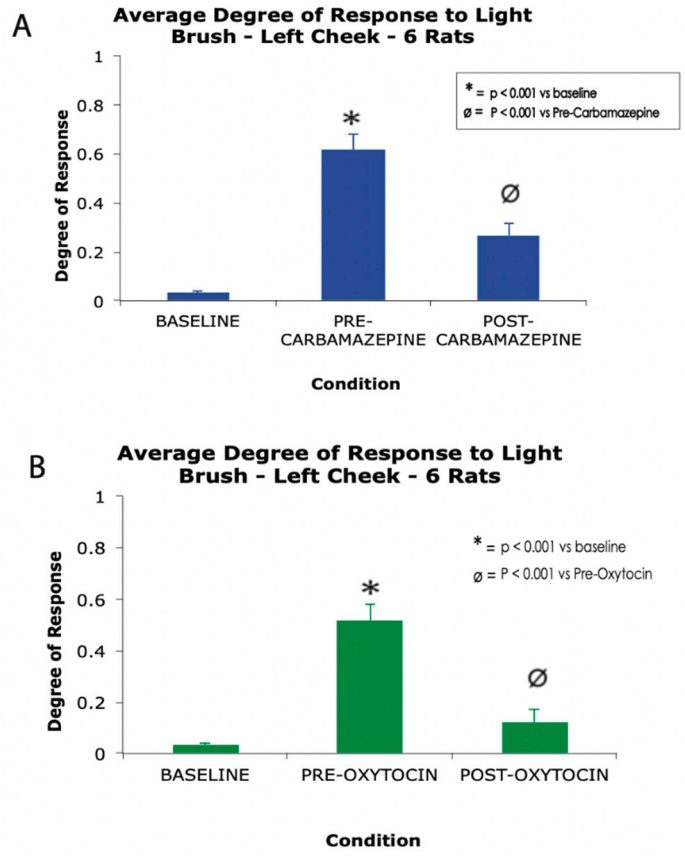

Figure 2. A high dose carbamazepine (**A**) and single dose of nasal oxytocin (**B**) produce robust analgesia in a rat model of trigeminal neuralgia. $^{*}p < 0.001$ vs. baseline, $^{ø}p < 0.001$ vs. oxytocin, one-way ANOVA. Error bars represent standard error of the mean.

Overall, preclinical and clinical studies demonstrate that intranasal delivery of oxytocin works well as an analgesic for craniofacial pain. However, future studies investigating the general clinical utility of this treatment as well as more precise explorations into the mechanism of delivery and mechanisms of analgesic action are warranted.

5. Other Polypeptides That Work as Analgesics

Intranasally administered polypeptides have been investigated by several groups for potential analgesic effects. Candidate compounds include but are not limited to oxytocin, vasopressin, desmopressin, calcitonin, enkephalins, dermorphin analogue, insulin, neuropeptide S, conotoxins and others. Clinical studies using IN polypeptides for pain is summarized in Table 2.

There are several publications that have investigated the analgesic effect of intranasally administered desmopressin in patients with acute renal colic. Constantinides et al. [63] reported complete resolution of colic pain 30 min after IN application of desmopressin in 54% of patients. Several other groups reported similar analgesic effects of desmopressin alone or in combination with diclofenac [64], tramadol [65] or ketorolac [66]. Although the analgesic effects of desmopressin were, in general, lower compared to systemically applied traditional analgesic drugs, most studies concluded that the ease of administration and the favorable side-effect profile of IN desmopressin make it an interesting option for selected patients.

Intranasally administered calcitonin has been used as an analgesic in a variety of patient populations, including patients with McCune-Albright syndrome-associated bone pain [67]. This treatment has also been successful with other bone-associated pain, such as the pain associated with distal radial fractures [68] and vertebral crush fractures, especially when osteoporosis-related [69]. Beneficial effects have also been postulated for trigeminal neuralgia [70], complex regional pain syndrome [71] and other difficult-to-treat pain syndromes [72,73].

Enkephalins are endogenous opioid pentapeptides, binding to both μ- and δ-opioid receptors, which are found in high concentrations in the brain. The intranasal delivery route has been postulated to allow for bypassing the BBB and hence yield maximum concentrations in the target areas, producing robust analgesia while limiting systemic side-effects such as constipation. Our group has investigated the analgesic effects of intranasal administration of a herpes-based viral vector encoding for human proenkephalin in a rodent model of traumatic brain injury (TBI) [74]. Two days after inducing mild TBI, rats received either the vector encoding for human proenkephalin (SHPE) or a control vector encoding for lacZ (SHZ.1). Control vector-treated rats developed facial allodynia post TBI, but those treated with the enkephalin vector did not. This effect lasted for at least 45 days, which was the latest time point investigated. Following intranasal administration of the viral vectors, robust expression of human proenkephalin was demonstrated in the trigeminal ganglia of rats treated with SHPE, but not after SHZ.1 treatment. Another group [75] has shown that intranasal administration of enkephalins yields an analgesic effect in rodent pain models and that the analgesic effects could be enhanced by co-administration of enzyme inhibitors and/or absorption enhancers to reduce rapid destruction by extracellular peptidases. Another interesting approach is to design enkephalin derivatives that are more resistant against these peptidases and extend their half-lives [76].

A relatively new development is intranasal application of conotoxin derivatives to alleviate pain. Clinical use of omega-conotoxin MVIIA (ziconotide) is severely limited by its poor ability to cross the BBB and hence needs to be administered intrathecally. However, robust analgesic effects have been reported for both IN administered ziconotide [77] and a biochemically modified version of the cone snail peptide [78].

Table 2. Summary of clinical trials using intranasal polypeptides for pain.

Pain Model	Polypeptide	Outcome	References
Chronic pelvic pain	Intranasal oxytocin	Twice-daily administration of oxytocin may represent an adjuvant analgesic for refractory pelvic pain	[57]
Thermal pain perception	Intranasal oxytocin	Sex-specific effects of intranasal oxytocin on thermal pain perception, suggesting that oxytocin and endogenous sex hormones may interact to influence noxious stimuli	[61]
Migraine	Intranasal oxytocin	Intranasal treatment with oxytocin decreases the frequency of headaches in both chronic and high-frequency episodic migraineurs	[26]
Colic pain	Intranasal desmopressin	Complete resolution of colic pain 30 min after IN application of desmopressin in 54% of patients	[63]

6. Therapeutic Considerations and Delivery Devices

The nasal anatomy and physiology including nasal mucosa, MCC, humidity and airflow may influence the intranasal administration. In addition, factors such as lipophilicity, molecular weight, dose per spray puff, volume per spray puff, pH and osmolality of the compound all play a role in optimal internasal delivery.

Strategies to improve drug uptake and prolong resistance/stability is an important consideration for optimal nasal deposition and delivery. Mucoadhesive polymers such as

chitosan and polyacrylic acid have been used as excipients for intranasal formulations [79]. These polymers interact with the mucins to prolong residence of the drug in the mucosa and thus improve drug uptake [80]. Mucoadhesive polymers can also modify the trajectory of the formulations to reach the nasal cavity and thus reduce drug loss [81]. In addition, preservatives such as lipophilic chlorobutanol help prolong the stability of the nasal drug formulation [79]. Dose volume of the sprays is also related to the nasal deposition of the formulation. Generally, the dose volume on the market is 50 µL to 100 µL [82,83] and volumes larger than 100 µL are known to run down the posterior pharynx [83].

In addition, the spray pattern, droplet size distribution and viscosity of the formulation all influence the nasal deposition and thus the delivery to the brain. Nasal spray pattern is largely influenced by the formulation of the compound, and it is speculated that a narrow plume angle might enable the spray to penetrate deeper into the nasal cavity and result in large deposition area [82]. In addition, droplet size influences nasal deposition, where larger droplets tend to deposit at the anterior area, whereas smaller droplets deposit in the inner area of the nasal cavity [82]. Physiological properties such as viscosity of the formulation influence the droplet size of the nasal spray. Results from Gua et al.'s study suggest that low-viscosity formulations (producing smaller droplets) significantly enhance middle and posterior coverage of the nasal cavity compared to higher viscosity formulations [84].

For efficient nose-to-brain delivery, intranasally administered compounds should reach the olfactory region [85]. In this context, significant efforts are made to optimize polypeptide delivery via nose-to-brain transport by enhancing drug distribution and absorption through the olfactory epithelium. For example, a breath-powered device has been used to deliver low-dose oxytocin and has been reported to enhance deposition in the intranasal sites for direct nose-to-brain delivery [34,86]. In addition, the Precision Olfactory Delivery (POD®) device targets the delivery of drugs into the upper nasal cavity operated by pressure [87]. Furthermore, therapeutic strategies to incorporate polypeptides into a vehicle system that provides prolonged drug stability and supports optimal drug delivery need to be considered. For example, liposomes, nanoparticles and micelles have recently gained potential as useful tools for targeting the brain with reduced toxicity in nasal mucosa and the CNS [88,89].

7. Limitations of Intranasal Delivery

Many compounds that are useful to treat chronic pain are limited by their transport to the brain due to the BBB. As reviewed here, IN delivery provides a non-invasive strategy to deliver polypeptides to the brain. Nasal–olfactory and trigeminal pathways are reliable pathways to deliver compounds to the brain for chronic pain while minimizing side effects.

There are some limitations of the nose–brain delivery method. For example, the volume of the compound that can be IN administered is relatively small (~100 µL). In addition, the surface area of the olfactory epithelium critical for nose–brain delivery, short retention time for drug absorption and the influence of mucosal secretion all limit the drug delivery to the brain. Furthermore, the limitations of nasal delivery of liquid formulations and long-term use of compounds are the limited microbiological stability and the presence of preservatives, which may lead to irritation and allergic effects [90]. Indication of nasal congestion due to cold or allergies may interfere with this method of delivery. Pumps with a shorter tip to avoid contact with sensitive mucosal surfaces and side actuation have been designed to aid during allergic conditions [91]. Overall, strategies to combat these limitations are constantly developing and remain critical for the development of new nasal delivery devices.

8. Future Directions

Future directions for intranasal pain management include identifying and investigating potential drug candidates, improving delivery strategies and optimizing central nervous target concentrations. Drug candidates for (co-)analgesia using an intranasal administration route under investigation include NK1-receptor antagonists [92], ketamine [93]

and esketamine [94], nalbuphine [95], ketorolac [96], dexmedetomidine [97] and many more. There is also a wide variety of research on permeation-enhancing agents, mucolytic agents, muco-adhesive agents, in situ gelling agents and enzyme-inhibiting agents in the formulation of nasal drug delivery systems [98]. In addition, nanoemulsions [99] and liposomal formulations [89] have also been used for intranasal drug delivery. In general, all of these pharmacological modifications and approaches intend to standardize and optimize drug delivery across the BBB into the CNS.

9. Conclusions

Chronic pain is difficult to treat, in part because it is associated with altered activity in multiple networks and changes in the pain pathways in the peripheral and central nervous systems. IN delivery is a proven strategy to allow targeted delivery of polypeptides to the trigeminal nerve and ganglia as well as pain-associated brain sites for the treatment of pain. After IN administration, radiolabeled oxytocin has been shown to be preferentially deposited in the trigeminal system and is also present in the hippocampus, thalamus, midbrain and pons, key regions in pain processing. The results described in this review demonstrate that there is overwhelming evidence for peripheral and central nervous system effects due to intranasally applied polypeptides. While it is not completely understood how these peptides are deposited into the peripheral and central nervous systems, it has become clear that nasal application of polypeptides has tremendous potential to provide analgesia in conditions where systemic application is impossible or has significant limitations.

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