Oxytocin Analogs with Enhanced Craniofacial Antinociceptive Effects in Low Magnesium Formulations

Darryl Rideout, Ph.D.[1*], Bruce Daugherty, Ph.D.[1], David T. Hsu, Ph.D.[1], Xinmin Simon Xie, M.D., Ph.D.[2], David Yeomans, Ph.D.[3], Brent Stranix, Ph.D.[4], Daniel St-Cyr, Ph.D.[4], Jan-Felix Scholtes, Ph.D.[4], Cristobal Alhambra, Ph.D. [4], Luciana Leo, Ph.D.[5], Sam R.J. Hoare, Ph.D[5]., Seth Lederman, M.D.[1], Gregory M. Sullivan, M.D.[1]

[1]Tonix Pharmaceuticals Inc., 26 Main St, Suite 101 Chatham NJ 07928 [2]AfaSci Research Laboratories, 522 Second Avenue, Redwood City, CA 94063 [3]Stanford University Dept. of Anesthesiology, Perioperative and Pain Medicine 300 Pasteur Drive, Room H3580 MC 5640 Stanford, CA 94305 [4]X-Chem, Inc. 4800 Rue Levy-Suite 200, Montreal, QC, Canada H4S 1Z9 [5]Montana Molecular, 366 Gallatin Park Dr., Bozeman, MT 59715

[a] Presenter

INTRODUCTION

Introduction Intranasal oxytocin (OT) is a non-opioid analgesic for craniofacial pain in animal models. It is being considered for craniofacial pain, including trigeminal neuralgia, and is being tested clinically for binge-eating disorder, adolescent obesity and social anxiety disorder. However, OT binding to oxytocin receptor (OTR) and OT activity *in vitro* and *in vivo* exhibit Mg2+ dependency, with maximal activity observed in formulations with high Mg2+ concentration. Structural studies show that hydrated Mg2+ forms an essential bridge between OT and OTR in the active complex and plays a key role in the agonistic potency of OT.

Our goal is to identify OT analogs with decreased Mg2+ dependency to minimize Mg2+-related fluctuations in drug activity within and between patients. Electrophysiology (EPhys) studies in rodent trigeminal ganglia (TG) neurons were carried out on OT and analogs. The results herein will show that replacement of 7-proline in oxytocin with hydroxyalkyl and dihydroxyalkyl residues can sometimes increase Emax (hyperpolarization in mV) at low Mg2+ and decrease Mg2+ dependence significantly. The structure-activity relationship and the potential for antinociceptive drug discovery will be discussed.

EXPERIMENTAL METHODS-CHEMICAL SYNTHESIS

Chemical Design and Synthesis

Hydroxylated OT analogs P-2 and G-1 to G-5 (**Table 1, Fig. 2**) were designed with the objective of increasing the activity and decreasing the Mg2+ dependency relative to OT.

Protected N-hydroxyalkyl and N-dihydroxyalkyl glycine derivatives were synthesized (e.g. **Fig. 1**) or purchased as precursors for compounds G-1 to G-5. G1-G5 were then synthesized from protected amino acids and amino acid-like building blocks in a modified Fmoc solid-phase synthesis on CTC resin, followed by selective cleavage, deprotection, and macrocycle closure to form the oxytocin analogs (as in Ichinose et al., 2019). The resulting cyclic nonapeptides were purified using semipreparative reverse-phase HPLC and characterized by LC/MS and HNMR.

Biological assays *In vitro* agonism was determined in G-protein OTR-expressing cultured HEK-293T cells by measuring Gq (Emax and EC50) and DAG signaling.

***Ex vivo* electrophysiology (EPhys)** was measured in freshly isolated small diameter trigeminal ganglia (TG) neurons from male rats by measuring the impact on neuronal excitability (Bhadarwaj et al., 2022). The Ephys results were measured in mV. Standard deviations for Ephys E values at low and high Mg2+ range from 0.1 to 1.2 mV for 10 nM ligand, 0.3-1.8 mV for 100nM ligand , and 0.1 to 1.1 mV for 100-0 nM ligand for OT and the 6 analogs (**Table 1**).

Antinociception in Rats with craniofacial pain was measured intranasally in a model involving latency of response to laser heating stimulus (Bhadarwaj et al., 2022).

ID and Structure				Electrophysiology (Hyperpolarization) in mV			Gq Max in % of OT vaues		
Name	ID	roline-7 derivat	R2 (glycine-7 derivatives)	Low Mg2+: Hyperpolarizati on Emax at 1 uM ligand and 0.5 mM Mg2+ in	High Mg2+: Hyperpolarization Emax mV at 1 uM ligand and 1.75 mM Mg2+ in mV	Mg effect E(1.75 mM Mg)-E(0.5 mM Mg) at 1 uM ligand in mV	Low Mg2+: Emax for Gq at 0.5 mM Mg++ in %	High Mg2+: Emax for Gq at1.75 mM mM Mg++ in %	Mg effect for Gq E(1.75 mM Mg)-E(0.5 mM Mg) in % difference
oxytocin (OT)	P-1	··H	–	6.5	15.5	9.0	100	100	0
–	P-2	··OH	–	8.5	12.3	3.8	96	92	-4
–	G-1	–		5	6	1.0	73	89	16
R721	G-2	–		9.5	8.5	-1.0	38	66	29
–	G-3	–		7.5	13.5	6.0	----	----	----
–	G-4	–		0.5	7.7	7.2	92	94	2
–	G-5	–		6	8.8	2.8	----	----	----

Table 1

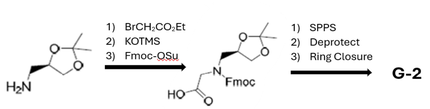

Fig. 1 Representative synthesis (compound G-2)

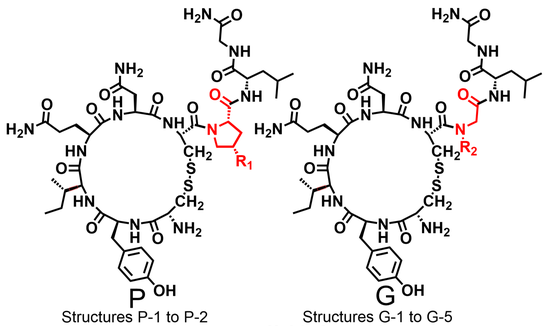

Fig. 2 Structure Scaffolds for Table 1

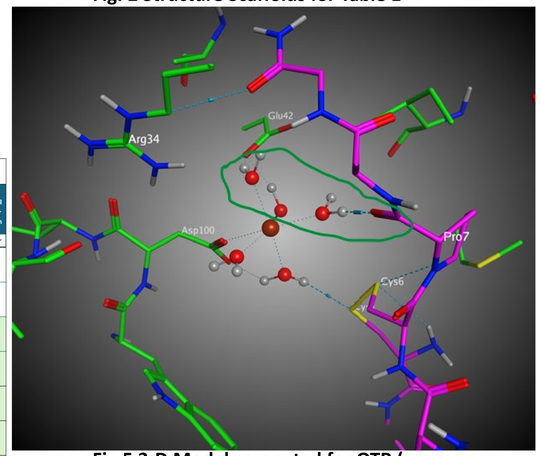

Fig.5 3-D Model computed for OTR (green carbons), Mg2+ (dark red), H2O (red, white) and OT (magenta carbons)

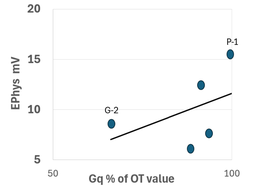

Fig. 3 Ephys vs. Gq at high Mg2+

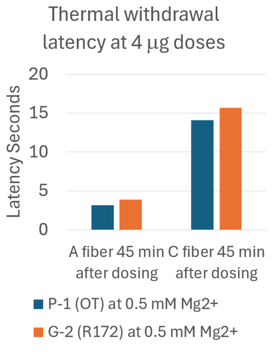

Fig. 4 P-1 and G-2 antinociception at 4 µg in male rats

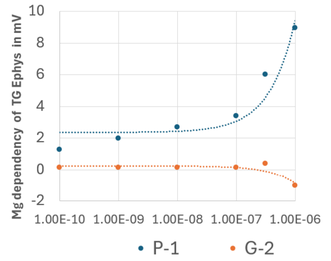

Fig. 6 Ephys Mg2+ Dependence for P-1 and G-2 (1.75 mM vs 0.5 mM)

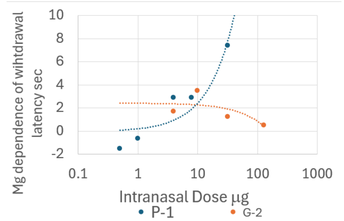

Fig. 7 C-fiber Antinociceptive Mg2+ Dependence for P-1 (300 mM vs 0 mM, Bharadwaj) and G-2 (1.75 mM vs 0.5 mM)

Concentration of G-2 (R721)	Withdrawal Latency (s) 0.5 mM $MgCl_2$	Withdrawal Latency (s) 1.75 mM $MgCl_2$
4 µg	1.725	1.175
10 µg	1.55	1.325
32 µg	2.6	2.275
128 µg	2.3125	2.6875

Table 2 Comparison of A-fiber antinociceptive effect in low and high Mg2+ concentrations

RESULTS

In vitro Gq assays. These were carried out in OTR-expressing cultured HEK-293T cells by determining Emax and EC50 for rate of initial increase in DAG and for maximum achievable DAG. We focused on Emax for the maximum achievable DAG for OT. **Table 1** shows the Emax for the maximum achievable DAG for OT and 4 analogs. At high (1.75 mM) Mg2+ the EPhys results for OT and 4 analogs trend upward with increasing Gq active (**Fig. 3**), and Gq can distinguish agonists from antagonists (Meyerowitz et al., 2022). Gq was useful for weeding out antagonists and weak agonists prior to the more low-throughput EPhys assays, but not for identifying OT analogs with the desired smaller magnesium dependence in EPhys.

EPhys. Freshly isolated small diameter rodent trigeminal ganglia (TG) neurons from male rats by measuring impact on neuronal excitability. Some analogs (notably G-2) that demonstrated Gq agonism produced robust hyperpolarization of TG neurons with substantially less Mg2+ dependence compared to native OT. Six candidates were identified based on Gq results and structure (the presence of hydroxyalkyl groups at the 7-residue—see P-2 and G-1 to G-5 in **Fig. 2** and **Table 1**). Even 7-position mono-alcohols such as P-2 and G-5 show significantly lower Mg2+ dependencies (Mg2+ dependencies 2.8 and 3.8 mV) than oxytocin with no hydroxyl at position 7 (Mg2+ dependency = 9mV). The diols (G-1-G4) show a larger range of Mg2+ dependency values. In the OT analog diols the proline at position 7 is replaced with a derivatives of N- 3-hydroxypropyl glycine (see **Fig. 2** and **Table 1**). The position of the second hydroxy groups in these 4 diols is critical. This is most likely because it affects the position of Mg2+ relative to OTR. For example, EPhys values for G-4 (Mg2+ dependency 7.2 mV and no agonism at 0.5 mM Mg2+) is much less active and more dependent on Mg2+ than G-2 (Mg2+ dependency -1 mV and 9.5 mV agonism at 0.5 mM Mg2+), even though they differ by only a single carbon atom. Computer modelling studies of OT analog/ Mg2+/OTR complexes are currently underway to better understand the SAR.

Craniofacial Antinociception. Analog R721, which showed negligeable Mg2+ dependency and the strongest activity at low Mg2+ in Ephys (**Table 1**), also produced potent analgesia *in vivo* when administered intranasally in a rat model of craniofacial pain mediated by the activation of either A-delta or C fiber nociceptors (**Fig. 4**)). P-1 (OT) and G-2 (R721) had analgesic effect for both C and A-delta fiber responses in a dose dependent manner. G-2 (R721) showed little or no antinociceptive Mg2+ dependence (**Fig. 7, Table 2**). At low doses of OT or analog (4 ug), the G-2 (R721) is somewhat more effective than is P-1 (OT) at low Mg2+ (**Fig. 4**).

DISCUSSION AND CONCLUSIONS

Discussion Structural studies show that Mg2+ sits in the interface of an OT-Mg2+-OTR complex, providing a mechanistic explanation for the Mg2+ dependencies. In water, the most stable Mg2+ coordination sphere consists of 6 inner sphere water molecules coordinated directly to Mg, and 3 outer sphere water molecules (Adrian-Scotto et al., 2005). Similarly, the coordination sphere in **Fig. 5** (a preliminary model based on the cyro-em-based OT-Mg2+-OTR PDB structure 7RYC and Fig. 4b in Meyerowitz et al., 2022) was recalculated with MOE, using 3D-RISM methodology to place the water molecules. The result consists of an inner sphere of 6 waters and the OT receptor residue D100 I**Fig. 5)**, with the outer ring consisting of OT residue P7, and OTR residues E42 and possibly R34 (see also Fig. 4b in Meyerowitz et al., 2022). Notably, the Mg2+ ion and its associated water molecules form an essential bridge between OT and OTR in the active complex.

Peptides that contain glycine residues and hydroxylated residues serine and threonine are >100 times more flexible than the corresponding proline-containing peptides (Huang and Nau, 2003). This suggests that replacing the OT proline 7-N-dihydroxyalkyl glycine in oxytocin analogs (as in G-1 to G-4) will introduce enough flexibility for the diol oxygens to enter the green circled region between OT P7 and Mg2+ (see **Fig. 5**). Furthermore, studies of the bidentate inhibitor batimastat binding to the Zn2+ ion in MMP-12 (PDB 1JK3) show that the two oxygens in the ligand displace two Zn2+-bound waters. (Chen et al.,, 2019). This suggests that with the possibility that the diols G-1 to G-4 may be acting as bidentate ligands binding directly to Mg2+.

A single OH in P-2 and G-5 has little effect on Emax values at 1 uM (1000nM) ligand in low (0.5 mM) Mg2+: 6.5 mV for A (OT), 8.5 mV for mono-ol B, and 6.0 mV for mono-ol G-5 (see **Table 1**). However, for 10 nM ligand at 0.5 mM Mg2+, the effect is more pronounced. A pair of OH groups in a 1,2- or 1,3-diol (G-1 through G-4) results in a much broader Emax range at 0.5 mM Mg2+ (0.5 to 10 mV), despite the small differences in structure. This suggests that specific interactions between the two hydroxyl oxygens on the OTA and Mg2+ can enhance or interfere with Mg2+ /OTR interactions, depending on their locations.

Structure-activity-relationship assessments suggest that direct complexation of Mg2+ by the altered residues in G-2 and related OT analogues are responsible for their diminished Mg2+ dependence and increased activity relative to OT. For oxytocin, the EPhys Mg dependency (**Fig. 6**) and the craniofacial nociception Mg dependency (**Fig. 7**) increase with OT concentration In craniofacial nociception, this increase in Mg dependency with ligand dose results in the ∩- shaped (inverted U-shaped) dose response curve at low Mg. Bharadwaj, Yeomans et al., 2022) This ∩- shaped dose response curves have been observed in rat studies for reversal of opiate-induced respiratory depression and clinical studies on autism (Yamasue, Kojima, Kuwabara et al., 2022). Our most promising compound, R721 (G-2), is not expected to show ∩- shaped dose response curves in antinociception due to its decrease in Mg dependency with increasing dose (**Fig. 7**).

Conclusion

In conclusion, intranasal R721 and related OT analogues with N-hydroxyalkyl and N-dihydroxyalkyl glycine at position 7 may be candidate non-opioid analgesics for craniofacial pain. These OT analogs would not need the Mg2+ supplementation required by intranasal OT for maximal potency, avoiding the complexities of dose determination for two components (ligand and Mg2+) that have distinct PK and biodistribution behavior.

References:
A drian-Scottoet al., 2005 J. Mol. Structure THEOCHEM V728 (2005) pp 231–242
Bharadwaj, Yeomans et al., 2022 Pharmaceutics V14, article 1105, Impact of Magnesium on Oxytocin Receptor Function
Brackley and Toney, 2021, J Pharmacol Exp Ther V 378 pp 96–107, Oxytocin Receptor Activation Rescues Opioid-Induced Respiratory Depression by Systemic Fentanyl in the Rats
Chen et al., Chem Rev. 2019 V119 pp 1323–1455, Targeting Metalloenzymes for Therapeutic Intervention

References, Continued: Huang and Nau, 2003, Angewandte Chemie Int. Ed. V 42, pp 2269-2272. A Conformational Flexibility Scale for Amino Acids in Peptides**
Ichinose et al., 2019 J. Med. Chem. V62, pp 3297–3310 Development of a Highly Potent Analogue and a Long-Acting Analogue of Oxytocin for the Treatment of Social Impairment-Like Behaviors
Meyerowitz et al., 2022, Nature Structural & Molecular Biology V 29, pp 274–281 (2022) Cryo em of OT-OTR-Mg++ complex supplementary information
Yamasue, Kojima, Kuwabara et al., 2022 BRAIN 2022, pp 1- 10 doi 10.1093-brain-awab291 Effect of a novel nasal oxytocin spray with enhanced bioavailability on autism-- a randomized trial