DR SHANTI DIWAKARLA (Orcid ID : 0000-0003-2328-3528)
DR JOHN B FURNESS (Orcid ID : 0000-0002-0219-3438)

Article type : Original Article

Colokinetic effect of an insulin-like peptide 5 related agonist of the RXFP4 receptor

Shanti Diwakarla[1,2], Ross AD Bathgate[1,3], Xiaozhou Zhang[1], Mohammed Akhter Hossain*[1,4] and John B Furness*[1,2]

[1] Florey Institute of Neuroscience and Mental Health, [2]Department of Anatomy and Neuroscience, [3]Department of Biochemistry and Molecular Biology and [4]School of Chemistry, University of Melbourne, Parkville, VIC, 3010, Australia.
*Equal senior authors

Running title: Colokinetic action of INSL5 analogue

Corresponding Author:
Prof. John B Furness
Digestive Physiology and Nutrition Laboratories
Florey Institute of Neuroscience,
University of Melbourne
Parkville, VIC 3010
Australia
Email: j.furness@unimelb.edu.au

This is the author manuscript accepted for publication and has undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1111/NMO.13796

Phone (not for publication) (+61) 3 83448859

Abstract

Background: Insulin-like peptide 5 (INSL5) is a hormone stored in colonic enteroendocrine cells that also contain the unrelated hormones, GLP-1 and PYY. It acts at the relaxin family peptide 4, RXFP4, receptor. RXFP4 is expressed by enteric neurons in the colon and it has been speculated that INSL5, through its action on enteric neurons, might be involved in the control of colonic contractions. Similar to insulin and relaxin, INSL5 consists of A and B peptide chains linked by three disulfide bonds, two between the chains and one intrinsic to the A chain. Because of its complex structure, it is difficult to synthesize and to prepare peptide analogues to investigate its roles. We have recently developed a potent simplified peptide analogue, INSL5-A13 (INSL5 analogue 13).

Methods: In the present work, we have investigated the actions of INSL5-A13 in mice. We investigated the ability of INSL5-A13 to increase the speed of emptying of a bead from the colon, after expulsion had been slowed by the peripherally-restricted opioid agonist, loperamide (1 mg/kg).

Key Results: INSL5-A13 was a full agonist at the mouse RXFP4 expressed in HEK cells, with an EC50 of ~ 9 nM. INSL5-A13 caused an acceleration of colorectal bead propulsion in mice constipated by loperamide in the dose range 0.2 to 60 μg/kg, with an EC50 of ~ 6 μg/kg *in vivo*. It also accelerated bead propulsion in untreated mice. Bead expulsion was not accelerated in RXFP4-/- mice.

Conclusion and Inferences: Our data suggest that RXFP4 agonists could be useful in the treatment of constipation.

KEYWORDS: INSL5, RXFP4, colokinetics, constipation

Key Points

- INSL5 is expressed by enteroendocrine cells in the colon and its receptor, RXFP4 is expressed by enteric neurons.
- We designed and synthesized an INSL5 peptidomimetic, INSL5-A13, that was a full agonist at the mouse RXFP4 receptor.
- INSL5-A13 accelerated bead expulsion in mice and reversed the constipation caused by the opiate agonist, loperamide. It was ineffective in RXFP4 KO mice.

1 INTRODUCTION

Constipation is a common gastrointestinal motility disorder that is highly prevalent in the elderly, in children, and in individuals who suffer from certain neurodegenerative diseases (e.g. Parkinson's disease and multiple sclerosis). Unfortunately, there are limited therapeutic options for treatment of constipation, with the most common treatment being osmotic or stimulant laxatives,[1] which have limited efficacy and substantial side-effects, while newly tested drugs display little, or no, greater effect than placebo.[2] Thus, it would be valuable to identify new pharmacological tools.

Insulin-like peptide 5 (INSL5) is a gut hormone that is secreted by colonic enteroendocrine L-cells.[3-5] Its G protein-coupled receptor, RXFP4, also referred to as GPCR142 or GPR 100[6] is expressed by enteric neurons.[4] INSL5, similar to the related peptides, insulin and relaxin, consists of A and B peptide chains linked by disulfide bonds, two between the chains, and one intrinsic to the A chain.[7-9] Because of its complex structure, it is difficult to synthesize and to prepare peptide analogues to investigate roles of INSL5. We have recently developed a potent analogue in which the A-chain is truncated, the intra-A chain disulfide is not present, and the amino-acid at A15 is a lysine (as occurs in mouse, but not human, where this is threonine). This peptidomimetic, INSL5-A13 (INSL5 analogue 13), is approximately equipotent with INSL5 at RXFP4, is easier to synthesize with 17.4-fold higher yield compared with native human INSL5.[10]

In this study, we investigated the actions of INSL5-A13 at the mouse RXFP4 receptor and its ability to enhance colorectal propulsion in mice with the intact RXFP4 receptor and with RXFP4 receptor knockout.

2 MATERIALS AND METHODS

2.1 Experimental animals

Male C57Bl6 mice (20–30g; ARC, WA, Australia), between the ages of 3 to 5 months, were used for loperamide and INSL5-A13 dose-response studies. Male wildtype (WT) littermate (n=11) and knockout (KO; n=13) mice[4] were used to assess the specificity of INSL5-A13. RXFP4 WT and KO littermates were generated by heterozygote breeding. All experiments were conducted in accordance with the National Health and Medical Research Council (NHMRC) guidelines for the care and use of animals and with approval from the Florey Institute of Neuroscience and Mental Health Animal Ethics Committee (#18-128 FINMH and #18-081 FINMH). All animals were group housed (2-6 per cage) and maintained in a humidity-controlled room at 22°C under a 12 h light/dark cycle with access to food and water ad libitum.

2.2 INSL5-A13 synthesis

The RXFP4 agonist, INSL5-A13, was chemically synthesized as previously described.[10] In summary, the INSL5 B-chain and truncated A-chain were prepared by continuous flow Fmoc (N-(9-fluorenyl)methoxycarbonyl) chemistry on a solid phase support. Directed disulfide bond formation was achieved by the use of appropriate S-protection groups, which were subsequently removed in a stepwise fashion.[10] The simplified two-chain peptide, INSL5-A13, was purified using RP-HPLC and its identity and purity confirmed by RP-HPLC, MALDI-TOF mass spectroscopy.

2.3 Forskolin-induced cAMP inhibition

INSL5-A13 was tested for its ability to activate mouse RXFP4 expressed in HEK-293T cells in parallel with the RXFP4 agonist R3/I5 which has previously been shown to bind strongly to mouse RXFP4.[11] INSL5-A13 was tested for its ability to inhibit forskolin induced cAMP activity in HEK-293T cells transfected with mouse RXFP4 and a pCRE (cAMP Response Element) β-galactosidase reporter plasmid.[12] HEK-293T cells transfected with the reporter gene alone were tested in parallel as a negative control. Cells were stimulated with 300nM forskolin plus or minus increasing concentrations of INSL5-A13 or R3/I5 for 6 hours and reporter activity was assessed as described.[13] Each treatment was tested in triplicate and each experiment was performed independently at least three times. Data was expressed as the % forskolin activity whereby 100% was defined as forskolin alone and 0% as maximum R3/I5 stimulation. Data were analysed and plotted using GraphPad PRISM 8 and are expressed as the mean ± SEM of the pooled data.

2.4 Loperamide-induced constipation and distal colonic propulsion studies

Loperamide (Sigma-Aldrich, St. Louis, MO) was prepared in 1% Tween-80 (Sigma-Aldrich) in distilled water and was administered subcutaneously (s.c.) at the back of the neck. For dose-response loperamide studies, mice were injected with either vehicle (1% Tween-80/water) or loperamide (0.3, 1, 3 mg/kg). At 30 min post-injection, mice were lightly anesthetized with 2% (v/v) isoflurane in O_2, administered at 1L/min for a maximum of 15 seconds following induction with 5% isoflurane in O_2 at 1L/min. A 3-mm round bead was inserted 2 cm into the distal colon using a flexible, plastic rod. After bead insertion, mice were placed in individual cages. The time taken from bead insertion to bead expulsion was recorded. A higher mean expulsion time indicated stronger inhibition of colonic propulsion. The maximum time allowed for bead expulsion before manual removal was 30 min. If bead expulsion took longer than 30 min, the bead was manually removed by gently massaging the bead down the colon until it was expelled. The time was scored

as 30 min. All experiments were performed in the afternoon (between 13:00 and 15:00pm) and all data was used for analysis.

For INSL5-A13 dose-response studies, INSL5-A13 was dissolved in distilled water and injected intraperitoneally (i.p.) at 0.2, 2, 6, 20 or 60 μg/kg, 10 min after loperamide was administered (1 mg/kg, s.c.). Bead expulsion testing was performed 20 min after INSL5-A13 administration. To ensure there was no desensitization following repeated exposure to loperamide, mice were treated with loperamide at the end of the experimental period to ensure a constipation phenotype could still be induced. Specificity studies in RXFP4 KO and matched WT littermate mice were performed in the same manner as INSL5-A13 dose-response studies, however constipation was induced using 0.5 mg/kg loperamide due to the already reduced colonic propulsion observed in RXFP4 KO mice. INSL5-A13 (20 μg/kg) was administered 10 min after loperamide injection (s.c.), and bead expulsion testing was performed 20 min later. To minimize the number of mice used per experiment, and so mice acted as their own controls, the same cohort of mice for each series of experiment were used with a 1 week recovery period.

2.5 Data Analysis

All data are expressed as the mean ± standard error of the mean (SEM) and analyzed using GraphPad Software version 8.2.0 for Windows (GraphPad Software Inc., San Diego, CA). Statistical differences between groups were determined using one-way ANOVA followed by Tukey's multiple comparisons test. Statistical significance was defined as $P < 0.05$.

3 RESULTS

3.1 Effect of INSL5-A13 on transfected cells

INSL5-A13 was a potent full agonist of forskolin induced cAMP inhibition in cells expressing mouse RXFP4 (Figure 1A) with a potency (pEC50) of 8.05 ± 0.19 (8.91 nM; n=3) slightly lower than the RXFP4 agonist R3/I5 (pEC50 = 9.2 ± 0.14; n=4). Importantly, INSL5-A13 had no effect in cells not expressing mouse RXFP4 (Figure 1B).

3.2 Effect of INSL5-A13 on distal colonic propulsion

To determine the dose-effect relation for INSL5-A13, we used mice in which colonic propulsion was slowed by loperamide. Prior to these experiments we showed that INSL5-A13, i.p., was effective in mice not treated with loperamide (Figure 2).

Subcutaneous administration of loperamide dose-dependently delayed colonic propulsion (Figure 3A). There was no difference in average bead expulsion time between control mice and

mice treated with the vehicle for loperamide (vehicle (lop)); expulsion times: 163 ± 17s vs. 172 ± 27s, respectively), indicating that vehicle injection had no effect on colonic motility. A dose of 0.3 mg/kg loperamide caused minimal delay in expulsion time (289 ± 35s), but 1 mg/kg significantly delayed expulsion to 1018±155s (P<0.0001 compared to vehicle control) and 3 mg/kg significantly delayed it to 1203±133s (P<0.0001), an approximately 6 fold increase when compared with vehicle-treated groups. A number of mice treated with 3 mg/kg required manual removal of the bead (n=8), because it was not expelled at 1800s, indicating a severe constipation.

Mice were administered intraperitoneal INSL5-A13 following loperamide-induced constipation (1 mg/kg, s.c.) at intervals of one week. This produced a dose-dependent acceleration of the colonic propulsion that had been slowed by loperamide (Figure 3B). The INSL5 analogue at 6 µg/kg significantly reduced the time taken for bead expulsion (424 ±142s; P<0.05) when compared with loperamide-only treated mice (1018±155s), and doses of 20 µg/kg (expulsion time 297±27s; P<0.05) and 60 µg/kg (expulsion time 282±82s; P<0.05) were also more effective when compared to loperamide treated mice that were administered the INSL5-A13 vehicle (vehicle (INSL5-A13)), distilled water; expulsion time 945±186s (Figure 3B). To ensure that repeated weekly testing of mice did not desensitize the action of loperamide, mice were re-administered loperamide (1mg/kg, s.c.) at the end of the experimental period (week 10). Loperamide slowed distal colonic propulsion to a similar extent when compared to the bead expulsion time at the beginning of the study (week 3:1018±154s) compared to the end of the study (week 10: 1253±236s), indicating that the reversal of distal colonic propulsion was dependent on the administration of INSL5-A13 (Figure 3B).

3.3 Specificity of INSL5-A13 action on distal colonic propulsion

The specificity of INSL5-A13 to reverse loperamide-induced constipation was assessed using RXFP4 KO and WT littermates. Interestingly, the average bead expulsion time in RXFP4 KO mice was approximately 2 times greater than in RXFP4 WT littermates (P < 0.02). Therefore, a dose of 0.5 mg/kg loperamide was used to ensure an overly severe constipation phenotype was not produced (i.e. a maximum bead expulsion time of > 30 min was seldom induced). Bead expulsion time was significantly increased in WT littermates administered loperamide (0.5 mg/kg) when compared to loperamide vehicle mice (vehicle (lop): 215±20s vs. loperamide: 860±91s), and INSL5-A13 (20 µg/kg) significantly reduced this time to 374±70s (Figure 4A). In contrast, INSL5-A13 had no effect on loperamide induced constipation in RXFP4 KO mice (Figure 4B), indicating that INSL5-A13 specifically acts on the RXFP4 receptor to enhance colonic propulsion.

4 DISCUSSION

INSL5 is a two chain peptide (A and B chains) with 45 residues. There is one intra-A-chain disulfide and two inter-chain disulfide bridges. INSL5 is one of the most difficult peptides among insulin/relaxin superfamily in terms of both synthesis and purification. The A-chain is difficult to assemble using native amino acids and B-chain is difficult to purify because of aggregation. These lead to poor assembly and recovery of the peptide.[14] We carried out SAR on INSL5 and a number of peptides based on the INSL5 structure. This SAR study resulted in the discovery of INSL5-A13, which exhibited native INSL5-like binding affinity together with potent cAMP inhibition and pERK activation at human RXFP4.[10] INSL5-A13 has a simpler structure than INSL5, with 38 amino acids and two disulfide bridges. It is easier to synthesize and overall yield is >17 fold higher than INSL5. In the present study, INSL5-A13 demonstrated potent cAMP inhibition at cells that express mouse RXFP4, but no effect on cAMP activity in cells that did not express mouse RXFP4.

We found that the INSL5 mimetic, INSL5-A13, when administered i.p., hastened colon propulsion in mice. The effect was observed in mice whose colonic propulsion had been slowed by loperamide and in mice not administered loperamide. INSL5-A13 was ineffective in RXFP4 knockout mice. INSL5 is the naturally occurring endogenous agonist for RXFP4, which is expressed by myenteric neurons. It is well established that the myenteric plexus contains the circuitry necessary to generate propulsive movements of the colon,[15] consistent with this being the site of action of INSL5-A13. INSL5 in the colon is stored in enteroendocrine cells that also contain GLP-1 and PYY, so-called colonic L cells. The results suggest that INSL5 released from L cells may initiate propulsion. While this could be by INSL5 diffusing to the myenteric ganglia, it is more likely to be by INSL5 acting on the endings in the mucosa of myenteric intrinsic primary afferent neurons (IPANs). IPANs are the first neurons in the nerve circuits through which propulsion of content is mediated.[16] In the mouse colon, as in other gut regions and other species, the processes of myenteric IPANs are in the mucosa, in close proximity to enteroendocrine cells. Interestingly, some colonic L cells in the mouse also contain serotonin,[17] which excites IPANs when it is applied to their mucosal endings.[18] Thus we can propose that a physiological role of INSL5 may be to initiate or augment propulsion. It is thus pertinent that colonic propulsion of a bead is slower in RXFP4-/- mice. We hypothesize that mechanical distortion of the mucosa by a bead causes INSL5 to be released and to activate enteric nerve circuits of propulsive reflexes.

Knockout of *Insl*5 has no effect on transit in the small intestine,[19] which is consistent with the almost complete absence of INSL5 from the small intestine.[4] On the other hand, our study suggests that the INSL5/ RXFP4 system has a role in control of colonic propulsion, including that *Rxfp*4 KO animals experience greater expulsion times versus WT mice. Billing et al.[20] found that *Insl*5 expression was very low and that the peptide was undetectable in the proximal colon, but both gene

and peptide were high in the distal colon, where they were colocalized with PYY in EEC. These data suggest that the INSL5/ RXFP4 system is involved in expulsion of content from the distal part of the large intestine, but has little or no role in motility control in other gut regions.

INSL5-A13 at doses of 20 and 60 μg/kg caused an approximately 4-fold reduction in the bead expulsion delay caused by loperamide. The effect of loperamide is due to its agonism of μ-opioid receptors that are expressed by enteric neurons, whose activation reduces acetylcholine release from the enteric nerve endings.[21] This suggests that INSL5 agonists could be useful in the treatment of constipation. RXFP4 is expressed by enteric neurons, but is not expressed in the CNS,[4, 11] so agonists of RXFP4 would be expected to have no central off-target effects. Thus INSL5-mimetics may provide a novel approach for the treatment of constipation or slowed colonic propulsion.

ACKNOWLEDGEMENTS

These studies were supported by ab NHMRC ideas grant (APP1194425). RADB is the recipient of an NHMRC Research Fellowship (#1135837). Studies at the Florey Institute were supported by the Victorian Government's Operational Infrastructure Support Program

DISCLOSURES

The authors have no disclosures

AUTHOR CONTRIBUTIONS

SD, JBF and MAH conceptualized and designed the study. MAH designed and synthesized INSL5-A13. XZ assisted with peptide synthesis. SD performed animal studies and analysed the data. RADB conducted the cell-based assays. JBF and SD integrated data and wrote the manuscript. All authors contributed to discussion, to development of the manuscript and approved the manuscript

REFERENCES

1. Ford AC, Talley NJ. Laxatives for chronic constipation in adults. Br. Med. J. 2012;345:e6168.
2. Nelson AD, Camilleri M, Chirapongsathorn S, et al. Comparison of efficacy of pharmacological treatments for chronic idiopathic constipation: a systematic review and network meta-analysis. Gut 2017;66:1611-1622.
3. Mashima H, Ohno H, Yamada Y, et al. INSL5 may be a unique marker of colorectal endocrine cells and neuroendocrine tumors. Biochem. Biophys. Res. Commun. 2013;432:586-592.

4. Grosse J, Heffron H, Burling K, et al. Insulin-like peptide 5 is an orexigenic gastrointestinal hormone. Proc. Natl. Acad. Sci. USA 2014;111:11133-11138.
5. Billing LJ, Smith CA, Larraufie P, et al. Co-storage and release of insulin-like peptide-5, glucagon-like peptide-1 and peptideYY from murine and human colonic enteroendocrine cells. Mol. Metab. 2018;16:65-75.
6. Liu C, Kuei C, Sutton S, et al. INSL5 is a high affinity specific agonist for GPCR142 (GPR100). The Journal of Biological Chemistry 2005;280:292-300.
7. Conklin D, Lofton-Day CE, Haldeman BA, et al. Identification of INSL5, a new member of the insulin superfamily. Genomics 1999;60:50-56.
8. Haugaard-Jönsson LM, Hossain MA, Daly NL, et al. Structure of human insulin-like peptide 5 and characterization of conserved hydrogen bonds and electrostatic interactions within the relaxin framework. Biochem. J. 2009;419:619-627.
9. Hossain MA, Bathgate RAD. Challenges in the design of insulin and relaxin/insulin-like peptide mimetics. Bioorg. & Med. Chem. 2018;26:2827-2841.
10. Patil NA, Hughes RA, Rosengren KJ, et al. Engineering of a novel simplified human insulin-like peptide 5 agonist. J. Med. Chem. 2016;59:2118-2125.
11. Sutton SW, Bonaventure P, Kuei C, Nepomuceno D, Wu J, Zhu J, Lovenberg TW, Liu C. G-protein-coupled receptor (GPCR)-142 does not contribute to relaxin-3 binding in the mouse brain: further support that relaxin-3 is the physiological ligand for GPCR135. Neuroendocrinology. 2005;82:139-50.
12. Chen W, Shields TS, Stork PJ, Cone RD. A colorimetric assay for measuring activation of Gs- and Gq-coupled signaling pathways. Anal. Biochem. 1995;226:349–354.
13. Belgi, A. Hossain, MA, Shabanpoor, F, Chan, L. Zhang, S, Bathgate, RAD, Tregear, GW, Wade, JD. Structure and function relationship of murine insulin-like peptide 5 (INSL5): free C-terminus is essential for RXFP4 receptor binding and activation. Biochemistry 2011; 50, 8352-8361.
14. Hossain MA, Bathgate RA, Kong CK, Shabanpoor F, Zhang S, Haugaard-Jönsson LM, Rosengren KJ, Tregear GW, Wade JD. Synthesis, conformation, and activity of human insulin-like peptide 5 (INSL5). ChemBioChem. 2008;9:1816-22.
15. Spencer NJ, Dinning PG, Brookes SJ, et al. Insights into the mechanisms underlying colonic motor patterns. J. Physiol. 2016;594:4099-4116.
16. Furness JB, Jones C, Nurgali K, et al. Intrinsic primary afferent neurons and nerve circuits within the intestine. Prog. Neurobiol. 2004;72:143-164.

17. Reynaud Y, Fakhry J, Fothergill L, et al. The chemical coding of 5-hydroxytryptamine containing enteroendocrine cells in the mouse gastrointestinal tract. Cell Tissue Res. 2016;364:489-497.
18. Bertrand PP, Kunze WAA, Bornstein JC, et al. Analysis of the responses of myenteric neurons in the small intestine to chemical stimulation of the mucosa. Am. J. Physiol. 1997;273:G422-G435.
19. Lee YS, De Vadder F, Tremaroli V, Wichmann A, Mithieux G, Bäckhed F. Insulin-like peptide 5 is a microbially regulated peptide that promotes hepatic glucose production. Molecular metabolism. 2016;5:263-70.
20. Reimann F, Billing L, Larraufie P, Lewis J, Leiter A, Li J, Lam B, Yeo G, Goldspink D, Kay R, Gribble F. Single cell transcriptomic profiling of large intestinal enteroendocrine cells in mice–identification of selective stimuli for Insulin-like peptide-5 and Glucagonlike peptide-1 co-expressing cells. Molecular metabolism 2019;29:158-169
21. Awouters F, Megens A, Verlinden M, et al. Loperamide: survey of studies on mechanism of its antidiarrheal activity. Dig. Dis. Sci. 1993;38:977-995.

Figure Descriptions

Figure 1 Inhibition of cAMP activity by INSL5-A13 in RXFP4 expressing cells

Agonist activity of INSL5-A13 compared with that of the RXFP4 agonist, R3/I5, in HEK-293T cells transfected with (A) Mouse RXFP4 or (B) pcDNA control. Agonist action was measured as inhibition of forskolin induced cAMP reporter gene activity. The data are each from 3-4 independent experiments and are expressed as mean ± SEM, n = 5 mice.

Figure 2 Bead expulsion time in mice that were injected 20 min before bead insertion with vehicle (distilled water) or INSL5-A13, 80 µg/kg, i.p. Bead expulsion time was significantly less when INSL5-A13 was administered, **P*<0.05, paired t-test.

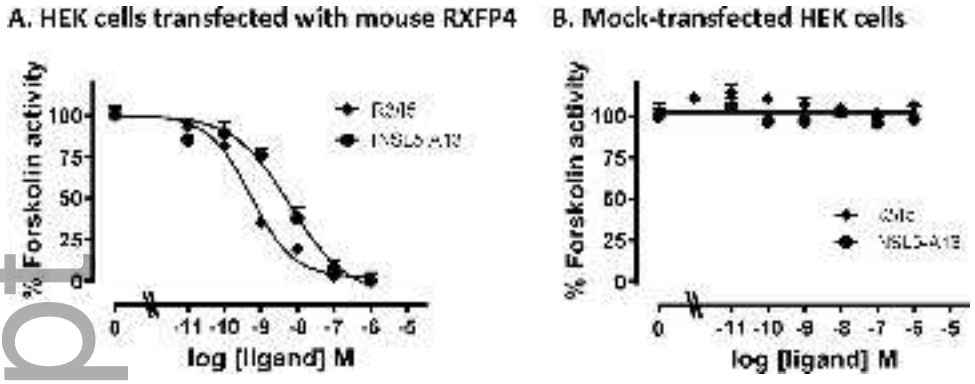
Figure 3 Loperamide-induced constipation and effect of INSL5-A13

A: Effect of loperamide on distal colon propulsion in mice. Doses of 1 and 3 mg/kg loperamide (s.c., 30 min prior) significantly delayed bead expulsion when compared to vehicle-treated mice. B: Effect of INSL5-A13 on the loperamide-induced delay of colonic propulsion in mice. INSL5-A13, administered 10 min after loperamide, was tested in ascending doses given to the same mice at weekly intervals. It was effective in reducing bead expulsion times at 6, 20, and 60 µg/kg (i.p.). Loperamide given one week after the final test of INSL5-A13 reversal maintained its effectiveness

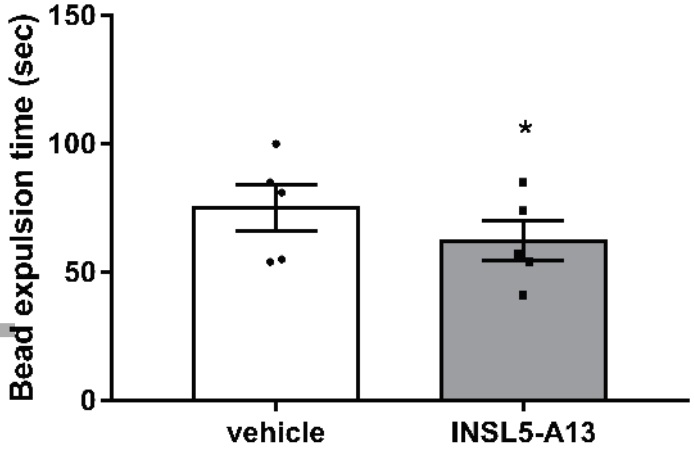
(final column at right). All values are presented as the mean ± SEM. Data were analyzed by one-way ANOVA followed by Tukey's multiple comparisons test. For loperamide dose-response studies (n=19-20), *** $P < 0.0001$ compared with loperamide vehicle (1%Tween-80/distilled water). For INSL5-A13 dose-response studies (n=10-20), * $P < 0.05$ compared with loperamide; # $P < 0.02$ compared with loperamide.

Figure 4 Effect of INSL5-A13 on the loperamide-induced delay of colonic propulsion in KO mice and WT littermates

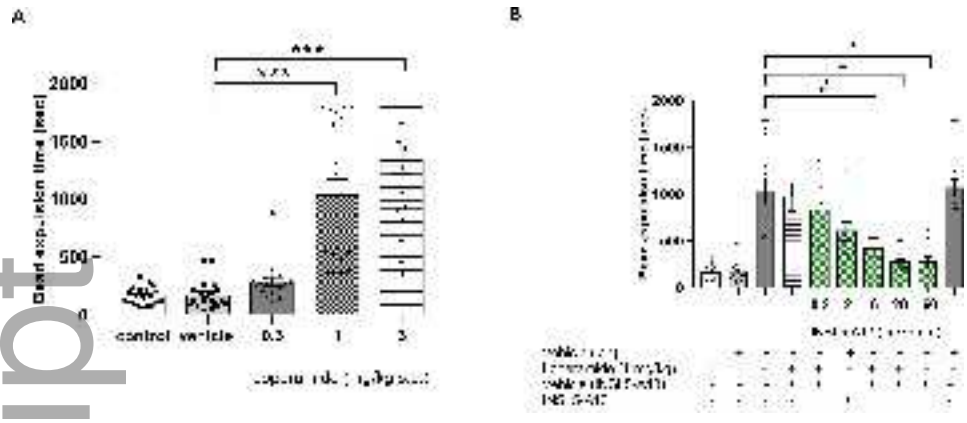
A: INSL5-A13 significantly reversed loperamide-induced constipation in RXFP4 WT littermate mice. B: INSL5-A13 had no effect on loperamide-induced constipation in RXFP4 KO mice. All values are presented as means ± SEM (n = 11-13). Data were analyzed by repeated measures one-way ANOVA followed by Tukey's multiple comparisons test. * $P < 0.05$ and ***P < 0.0001.



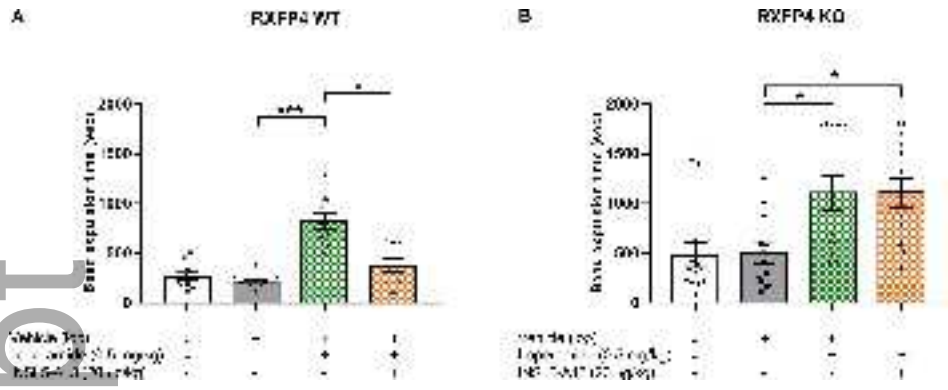
nmo_13796_f1.tif



nmo_13796_f2.tif



nmo_13796_f3.tif



nmo_13796_f4.tif