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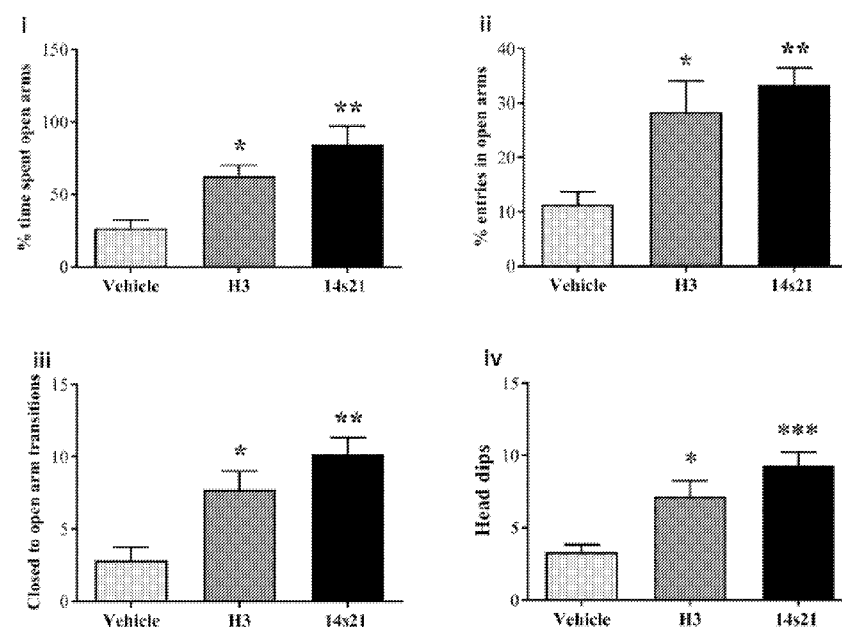
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(54) Title: STAPLED PEPTIDE AGONISTS AND THEIR USE IN TREATMENT OF BEHAVIORAL DISORDERS

Fig. 5B



(57) Abstract: The present invention relates to stapled relaxin-3 peptide agonists; to their functional analogues, derivatives, fragments, and/or their functional mimetics that comprise a chemical staple between at least two amino acid residues and exhibit increased stability and efficacy; and to uses of such peptides to treat eating or neuropsychiatric disorders, such as an anxiety, stress-related, eating or depressive disorder. In particular, a relaxin-3 B-chain peptide is stapled, i.e. covalently cross-linked, at amino acid positions 11 (*i*) and 18 (*i*+7) or at positions 14 (*i*) and 21 (*i*+4).

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STAPLED PEPTIDE AGONISTS AND THEIR USE IN TREATMENT OF BEHAVIORAL DISORDERS

FIELD OF THE INVENTION

The present invention relates to stapled relaxin-3 peptide agonists; to their
5 functional analogs, derivatives, fragments, and/or their functional mimetics that comprise
a chemical staple between at least two amino acid residues and exhibit increased
stability and efficacy; and to uses of such peptides to treat eating or neuropsychiatric
disorders.

BACKGROUND OF THE INVENTION

10 The search for novel drugs, with therapeutic translational strategies, for
combating psychiatric disorders is motivated by the emerging medical need to improve
on the effectiveness and side-effect profile of currently available therapeutics. Major
depressive disorder (MDD) continues to exert a tremendous socioeconomic cost
worldwide, with 2013 analysis of data obtained from the Global Burden of Diseases,
15 Injuries, and Risk Factors Study 2010 reporting that mental and substance abuse
disorders accounted for 7.4% of the global burden of disease; MDD alone represented
40% of this burden. Anxiety disorders, which include generalized anxiety disorder (GAD),
panic disorder, post-traumatic stress disorder (PTSD), social anxiety disorder, and
simple phobias, follow MDD and represent 14.6% of the burden of disease attributed to
20 mental health and substance abuse [Whiteford et al., *Lancet* 382: 1575-1586 (2013)].
From a productivity and innovation perspective, over the past two decades, new drugs
for psychiatric disorders have been both scarce in number and based more on already-
established, rather than novel, mechanisms. Given the wealth of supporting preclinical
data with a strong emphasis on the role of neuropeptides in modulating behavior,
25 pharmaceutical companies have been attempting to target neuropeptide receptors for
over two decades. Moreover, neuropeptide receptors have become one of the most
attractive therapeutic targets for the treatment of psychiatric disorders in the last decade,
witnessing the exciting and rapid progression in understanding the structure and
regulation of genes encoding neuropeptides, their transcription and translation, their
30 genetic manipulation and the successful synthesis of both peptide and non-peptide
receptor ligands. These characteristics suggests that therapeutic drugs which target

neuropeptide systems may be less susceptible to unwanted “non-specific” side effects compared to current drug treatments. Not surprisingly, the antidepressant potential of drugs which directly target neuropeptide Y (NPY) and corticotropin releasing factor (CRF) signaling is currently under investigation, while drugs that target receptor for neurotrophic factors and other neuropeptides, such as brain-derived neurotrophic factor (BDNF) and neuropeptide S, offer considerable promise as antidepressants and anxiolytics. Furthermore, among the 40 drugs that are currently being tested in Phase II/III trials for schizophrenia, MDD or anxiety disorders, very few are compounds that target neuropeptide receptors [Griebel and Holsboer, *Nat Rev Drug Discov* 11: 462-478 (2012)]. Thus, there is a growing need to explore the novel and relatively less identified ligand-receptor systems which may have huge therapeutic potential in the treatment of major psychiatric disorders.

The neuropeptide relaxin-3 (INSL7) is a recently identified member of the insulin superfamily, typified by the presence of an A and B-chain linked by inter-and intra-chain disulfide bonds [Hudson et al., *Nature* 301: 628-631 (1983)]. The endogenous receptor for relaxin-3 is the class A G protein-coupled receptor (GPCR) 135, which is classified by IUPHAR as RXFP3 [Liu et al., *J Biol Chem* 278: 50754-50764 (2003)]. Relaxin-3 is abundantly expressed in mammalian brain and acts as a neurotransmitter by activating its cognate receptor RXFP3 [Liu et al., *J Biol Chem* 278: 50754-50764 (2003)] revealing several key features to highlight the role of relaxin-3/RXFP3 system as an attractive target for the treatment of neuropsychiatric disorders, including anxiety and depression. Neuroanatomical studies revealed that relaxin-3 is mainly expressed within neurons of the pontine nucleus incertus [Burazin et al., *J Neurochem* 82: 1553-1557 (2002)], while smaller populations are present in the pontine raphe, periaqueductal grey, and a region dorsal to the substantia nigra. The distribution of relaxin-3 immunoreactive fibers and RXFP3 mRNA/binding sites within key midbrain, hypothalamic, limbic, and septohippocampal circuits of the rodent and primate brain suggests relaxin-3/RXFP3 neural networks represent an “arousal” system that modulates the behavioral outputs such as feeding and the responses to stress; and associated neuronal processes including spatial memory and hippocampal theta rhythms [Ganella et al., *Behav Pharmacol* 23: 516-525 (2012)]. In fact, the restricted localization of relaxin-3 (GABA) neurons and the broadly distributed relaxin-3 projections throughout the brain are

remarkably similar to those of the raphe/5-HT and locus coeruleus noradrenaline pathways/networks [Berridge et al., *Sleep Med Rev* 16: 187-197 (2012)], thus contributing to the central stress response implicated in the aetiology of anxiety and depression.

- 5 A stable agonist/antagonist of the RXFP3 receptor could be an excellent tool for understanding the mechanism behind these behaviors and for treating psychiatric disorders such as anxiety and depression. Recent advancements in developing the high-affinity agonist/antagonist of relaxin-3 has shown limited utility to develop them as a drug due to their large size and their half-life will likely be short in the circulation and brain.
- 10 Moreover, peptide ligands can only be administered via ICV injections to target the CNS, as systemically administered peptides may not cross the blood-brain barrier.

- Studies of relaxin-3 peptide mutants together with complementary studies on RXFP3 receptor mutants and modelling has showed that the primary binding and activation sites are present within the surface of the helical domain of the B-chain and
- 15 thus it's A-chain does not have any functional role. The single B-chain alone when isolated from A-chain lacks secondary structure and is thus not potent enough to activate the RXFP3 receptor. There remains a need and strong interest in the development of stable and potent relaxin-3 peptide agonists for potential therapeutic use.

SUMMARY OF THE INVENTION

- 20 A hydrocarbon *i, i+7* stapled relaxin-3 B-chain derived stapled peptide agonist that manifested stabilized α -helical structure and striking proteolytic resistance has been developed. *In vitro* binding affinity and receptor activation profiles of *i, i+7* stapled ligand confirmed the full agonist activity at RXFP3. Such composed monomolecular agonism proved to be superior to any existing best-in-class relaxin-3 agonist in enhancing food
- 25 intake and drinking behavior when it was centrally infused into the rat brain, confirming its constituent activity *in vivo*. To be clinically viable, it is herein demonstrated that intranasal delivery of a lead *i, i+7* stapled peptide and relaxin-3 can effectively harmonize the activities to govern the overall metabolic efficacy, which predominantly results in increased energy expenditure, and potentiate the orexigenic effects in rats. Further, we

validated the utility of an intranasal delivery method in anxiety and depression related behavior paradigms.

According to a first aspect, the present invention provides a single chain relaxin-3 polypeptide or analog, derivative, fragment or mimetic thereof having RXFP3 receptor agonist activity, comprising a relaxin B-chain polypeptide stapled at amino acid positions 11 and 18 or at positions 14 and 21 of the relaxin-3 B-chain; wherein the amino acids at positions 11 and 18 or at positions 14 and 21 are covalently cross-linked.

In preferred embodiments, the staple is an all-hydrocarbon staple.

In preferred embodiments, the staple is an 11-carbon cross-link.

10 In preferred embodiments, the B-chain polypeptide sequence has the Formula 1;

R A A P Y G V R L C G R E **X1** I R A V I F **X2** C G G S R W; (SEQ ID NO: 12),

wherein **X1** and **X2** are cross-linked amino acids.

In other embodiments, the B-chain polypeptide sequence has the Formula 2;

R A A P Y G V R L C **X1** R E F I R A **X2** I F T C G G S R W; (SEQ ID NO: 13)

15 wherein **X1** and **X2** are cross-linked amino acids.

In other embodiments, the stapled amino acids at positions 11 and 18 or at positions 14 and 21 are α , α -di-substituted amino acids. An example of a suitable amino acid is Alanine.

In preferred embodiments, the B-chain polypeptide sequence has the Formula 1.

20 In preferred embodiments, the cross-linking amino acids which generate $i+7$ stapled peptide are α -methyl, α -alkenyl amino acids, wherein the first amino acid contains a C5 alkenyl, preferably, a 4'-pentenyl. The C5 alkenyl, preferably a 4'-pentenyl, is advantageous in R configuration.

In other preferred embodiments, the cross-linking amino acids which generate $i+7$ stapled peptide are α -methyl, α -alkenyl amino acids, wherein the first amino acid contains a C8 alkenyl, preferably a 7'-octenyl, is advantageously in S configuration.

Such a type of stapled peptide, known as all-hydrocarbon stapled alpha-helical peptides, are disclosed in [Schafmeister et al., *J Am Chem Soc* 122: 5891-5892 (2000); Verdine and Hilinski, *Methods Enzymol* 503: 3-33 (2012)]. Other types of stapled peptides which are within the skill of one in the art can be used in the invention. In particular, a double hydrocarbon staple as well as multiple contiguous staples (recently called stitched peptides) [Hilinski et al., *J Am Chem Soc* 136: 12314-12322 (2014)] could be used.

In other embodiments, other cross-linking stabilizing secondary structure or α -helical conformation could be also inserted in the sequence such as lactam, disulphide, thioether, azobenzene, hydrazine, triazole, biphenyl, bis-triazoyl, oxime, perfluoroaryl and carbamate. In preferred embodiments the stapled polypeptide has α -helical conformation.

In another aspect of the present invention, the cross-linker may be proteogenic or non-proteogenic. The linker may be as simple as a covalent bond (e.g. carbon-carbon double bond, disulphide bond, carbon-heteroatom bond, etc.), or it may be more complicated such as a polymeric linker (e.g. polyethylene, polyethylene glycol, polyamide, polyester, etc.). In certain embodiments, the linker comprises a monomer, dimer, or polymer of aminoalkanoic acid (e.g. glycine, ethanoic acid, alanine, beta-alanine, 3-aminopropanoic acid, 4-aminobutanoic acid, 5-pentanoic acid, etc.). In certain embodiments, the linker comprises a monomer, dimer, or polymer of aminohexanoic acid (Ahx).

In other embodiments, the linker comprises amino acids and may include functionalized moieties to facilitate attachment of nucleophile (e.g. thiol, amino) from the peptide to linker.

In certain forms, the linker is N-terminus to C-terminus. In certain forms, the linker is from C-terminus to N-terminus. In still other forms, the linker may be through interior amino acids. As will be appreciated by one of skill in the art, the linker is typically

positioned in such a way as to avoid interfering with the binding activity of the peptide. The linker may also be positioned in such a way to avoid interfering with the stapling of the peptide.

5 It will be appreciated that the number of crosslinking moieties (i.e. linkers or staples) is not limited to one or two, rather the number of crosslinking moieties utilized can be varied in length of the targeting and/or effector domain as desired, and as compatible with the desired structure and activity to be generated.

In more preferred embodiments, the B-chain polypeptide sequence is from human relaxin-3.

10 In more preferred embodiments, the B-chain polypeptide comprises the amino acid sequence set forth in SEQ ID NO: 1.

In other embodiments, the B-chain sequence is from human relaxin-3 and the cysteine residues at positions 10 and 22 of the native human relaxin-3 sequence are replaced by serine residues.

15 In more preferred embodiments, the alpha carbon of the residue at position 14 has (R) stereochemistry and the alpha carbon of the residue at position 21 has (S) stereochemistry.

In more preferred embodiments, the B-chain polypeptide sequence is from human relaxin-3 and the amino acids at positions 14 and 21 are replaced by (R)-2-(4'-pentenyl) alanine and (S)-2-(7'-octenyl) alanine, respectively, as set forth in SEQ ID NO: 20 9 or SEQ ID NO: 10.

In more preferred embodiments, the polypeptide has alpha-helical and/or any helical secondary structure.

25 According to another aspect, the present invention provides a pharmaceutically acceptable salt form of the polypeptide of the invention.

In more preferred embodiments, the polypeptide decreases stress-related and/or anxiety-related and/or depressive-like behavior and/or increases feeding activity.

According to another aspect, the present invention provides a pharmaceutical composition comprising a stapled polypeptide according to any aspect of the invention or a pharmaceutically acceptable form, such as salt or solvate form, optionally together with one or more pharmaceutically acceptable carriers, excipients or diluents for the treatment of an anxiety, stress-related, eating or depressive disorder. In certain embodiments, the pharmaceutical compositions described herein include a therapeutically effective amount of stapled polypeptide or pharmaceutically acceptable salt thereof.

In preferred embodiments of the invention, the pharmaceutical composition is formulated for nasal delivery of a stapled polypeptide according to any aspect of the invention.

In preferred embodiments of the invention, the pharmaceutical composition comprises: (a) the said stapled polypeptide; (b) a bioavailability enhancing agent selected from the group consisting of a fatty acid, a sugar ester of a fatty acid and a mixture thereof and (c) a poly(alkylene oxide) chain, wherein the chain is a PEG (polyethylene glycol) or a poly(ethylene oxide) PEO.

Preferred poly(alkylene oxides) are selected from the group consisting of alpha-substituted poly(alkylene oxide) derivatives, PEG homopolymers and derivatives, poly(propylene glycol) (PPG) homopolymers and derivatives, poly(ethylene oxides) (PEO) polymers and derivatives, bis-poly(ethylene oxides) and derivatives, copolymers of poly(alkylene oxides), and block copolymers of poly(alkylene oxides), poly(lactide-co-glycolide) and derivatives, or activated derivatives. Preferably, the water-soluble polymer has a molecular weight of about 200 to about 40,000 Da. The preferred water-soluble polymers are poly(alkylene oxides), most preferably PEG or poly(ethylene oxide) (PEO).

In another preferred embodiment, the composition comprises: (a) the said peptide; (b) sucrose laurate; (c) a citrate-based bioavailability enhancing agent.

In another aspect of the invention, the intranasal composition in accordance with the present invention is an aqueous or dry powder composition.

According to another aspect, the present invention provides a method for the prophylaxis or treatment of a disorder, the method comprising administering to a subject in need thereof an effective amount of a polypeptide according to any aspect of the invention, or a pharmaceutical composition according to any aspect of the invention.

5 Exemplary disorders are generalized anxiety disorders, major depressive disorders, eating disorders or any stress-related neuropsychiatric disorders.

In other embodiments, the administration is by the intranasal or the intravenous route.

In more preferred embodiments the administration is by the intranasal route.

10 In a further aspect, the composition according to any aspect of the invention may be administered using a nasal metered dose spray, metered dose inhaler or measured dose inhaler.

According to another aspect, the present invention provides the use of a polypeptide according to any aspect of the invention, or a pharmaceutical composition
15 according to any aspect of the invention for the manufacture of a medicament for the treatment of an anxiety, stress-related, eating or depressive disorder.

BRIEF DESCRIPTION OF THE FIGURES

Figs. 1A-1D show the design, sequence composition and structural analysis of stapled peptides. Helix wheel diagram of (Fig. 1A) *i, i+4* stapled peptides (14s18 and
20 18s22) and (Fig. 1B) *i, i+7* stapled peptides (14s21 and 11s18). Different positions of the staples correspond to the analogs shown in the corresponding tables. Circular dichroism analysis of stapled peptides and variants, demonstrating the observed range of α -helical stabilization by hydrocarbon stapling. (Figs. 1C and 1D) *i, i+4* and (Figs. 1E and 1F) *i, i+7* hydrocarbon staples manifest substantial structural stabilization compared to the
25 unstapled H3 B-chain, lactam, disulfide variants of 14s18 hydrocarbon staple. (Figs. 1C and 1E) Only one *i, i+4* staple (14s18) and both *i, i+7* staples (14s21 and 11s18) of H3 B-chain sequence yielded peptides with marked α -helicities. The bar diagram shows the percent helicity of all the peptides compared to the H3 B-chain. The ribbon diagram

shows the reinforced α -helical structure of 14s18 (*i, i+4*) and 14s21 (*i, i+7*) peptide ligands.

Figs. 2A-2I show *in vitro* activity and stability profiles of H3 B-chain stapled peptide analogs. Competition binding curves for H3 B-chain stapled peptide analogs in HEK-RXFP3 cells using Eu-DTPA-R3B1-22R as the labelled ligand. Various concentrations of (Fig. 2A) H3 (1 pM -10 μ M H3), stapled peptides (1 pM to 100 μ M of 14s18, 18s22 and H3 B-chain), (Fig. 2B) full length 14s18 stapled peptide, 14s18 staple variants: 14s18-Lactam and 14s18-Disulfide peptides (1 pM to 100 μ M), N-terminus truncated Δ 14s18 and Δ 13s17 peptides (1 pM to 10 μ M), (Fig. 2C) binding affinity of *i, i+7* stapled peptide, 14s21, 14s21-Ser, 11s18 compared to (*i, i+4*) 14s18 peptide (1 pM to 10 μ M). Data are presented as mean $\pm$ s.e.m. of triplicates from three to four independent experiments. Inhibition of forskolin-induced cAMP assay in HEK-RXFP3 cells by (Fig. 2D) H3, H3 B-chain, 14s18, N-terminus truncated Δ 14s18 and Δ 13s17 peptide (0.1 pM to 10 μ M), (Fig. 2E) cAMP activation profiles of full length 14s18 stapled peptide, 14s18 staple variants: 14s18-Lactam and 14s18-Disulfide peptides (0.1 pM to 10 μ M) and (Fig. 2F) *i, i+7* stapled peptide: 14s21, 14s21-Ser, 11s18, H3 and H3 B-chain (0.1 pM to 10 μ M). cAMP concentration is represented as a percentage of the forskolin-induced response (mean $\pm$ s.e.m.; n=3), (Fig. 2G) Western blot shows that H3 and 14s21 induce dose-dependent increases of ERK1/2 phosphorylations. Representative dose-response curves for the effect of H3 and 14s21 peptide on ERK1/2 kinase phosphorylation assay in HEK-RXFP3 cells are shown. Data are presented as mean $\pm$ s.e.m. of triplicates from three independent experiments. (Fig. 2H) Thermodynamic stability of stapled peptides. Temperature-dependent thermal unfolding curves of *i, i+7* stapled peptide: 11s18, 14s21 and *i, i+4* stapled peptide, depicting the linear dependency and partially unfolded nature of the peptides upon heating. (Fig. 2I) Proteolytic stability of stapled peptides. Trypsin resistance profiles of H3 B-chain and 14s18 (*i, i+4*) and 14s21 (*i, i+7*) stapled peptides over a time period of 25 hours. Data (mean $\pm$ s.e.m.) represents percent fraction intact performed in experiments.

Figs. 3A-3K show *in vivo* effects of H3 relaxin and 14s21 peptide administration through intracerebroventricular injection (ICV) on feeding, drinking and locomotor behavior in male SD rats. (Fig. 3A) Pictorial presentation showing rat with implanted cannula and (Fig. 3B) representative micrograph from Nissl-stained brain section

showing the track left by guide cannula (black line) and injection cannula (red line) position in the lateral ventricle (marked by arrow) at 1x (scale bar represents 1 mm). (Fig. 3C) Effect of ICV injection of H3 (0.1 nmol) and 14s21 peptide (1 nmol) on 1-h food intake in early light phase in satiated male SD rats. *** $p < 0.001$ vs vehicle. Effect of ICV administration of H3 (0.1 nmol) and 14s21 peptide (1 nmol) on cumulative food intake (Fig. 3D) and cumulative water intake (Fig. 3E) over 4 hours in early light phase. Effect of ICV administration of H3 relaxin and 14s21 peptide on the Laboratory Animal Behavior Observation Registration and Analysis System (LABORAS) home-cage activity. Data points represent the mean of the parameter at 10-minute intervals (Figs. 3F-3K). Columns represent the mean of values at 80-minute intervals and error bars represent the SEM (insets in Fig. 3F-3K). Activity recorded in the first 80 min after the drug administration is indicated by the shaded area. N = 6 rats/group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, as determined by regular one- or two-way analysis of variance (ANOVA) comparing vehicle with peptide administration. In both comparisons, ANOVA was followed by Tukey *post-hoc* multiple comparison analysis to determine the statistical significance.

Figs. 4A-4J show feeding and drinking behavior following intranasal administration. (Fig. 4A) Pictorial diagram showing rat position during intranasal administration. (Figs. 4B and 4C) Concentration-response curves for the intranasal administration effects of H3 and 14s21 peptide. Intranasal administration of H3 (1, 10, 100 and 1000 nmol) and 14s21 peptide (0.1, 1, 10 and 100 μ mol) have been demonstrated to be efficient in activating the receptor at doses equivalent to or higher than ICV. The changes in feeding and drinking behavior were recorded in LABORAS over 80 min immediately after the peptide administration. (Fig. 4B) 100 nmol and 1000 nmol of H3 relaxin treated group displayed a significant increase in food intake and drinking behavior in comparison with vehicle. (Fig. 4C) 14s21 peptide at 1 μ mol, 10 μ mol and 100 μ mol concentrations displayed the significant increase in food intake whereas only 10 μ mol dose of 14s21 peptide could elicit the drinking behavior. Effect of intranasal administration of H3 (0.1 μ mol) and 14s21 peptide (10 μ mol) in satiated SD rats analyzed over 4 h (240 min) on the LABORAS home-cage activity. Data points represent the mean of the parameters over the preceding 10 min (Figs. 4D-4I). Columns represent mean of values over 80 min and error bars represent SEM (insets in Figs. 4D-4I). (Fig. 4J)

Comparison of feeding and drinking behavior in male and female SD rats (N=6 rats/group) analyzed over 2 h in LABORAS immediately after the intranasal administration of H3 (0.1 μ mol) and 14s21 peptide (10 μ mol). Data represent mean $\pm$ s.e.m. N=6 rats/group. * p < 0.05, ** p < 0.01, *** p < 0.001 determined by one-way or two-way ANOVA followed by Tukey's multiple comparison analysis compared to the vehicle groups under the same sex in the same treatment conditions.

Figs. 5A-5H show the effect of intranasal treatment of H3 relaxin and 14s21 peptide on the Elevated Zero Maze (EZM), Light-Dark Box, Open field test and Novelty-suppressed feeding test. H3 (0.1 μ mol) and 14s21 peptide (10 μ mol) were intranasal administered to the rats and 30 min after the drug administration, behavior was recorded in anxiety behavior paradigms (Figs. 5A-5H). (Fig. 5A and 5B) Anxiolytic-like phenotype of H3 and 14s21 peptide in SD rats in the EZM, as indicated by the significantly increased percent time spent in the open exposed arms (i), total percent entries made in the open arms (ii) and total transitions made in closed to open arms (iii). Number of head dips over the side while the rat was in open part (iv). (Figs. 5C and 5D) Total time spent in the light zone (i) and increased counts in the light zone (ii) displaying the anxiolytic-like effects of H3 and 14s21 peptide in the light/dark exploration test. (iii) Representative activity traces in light-dark box for vehicle, H3 and 14s21 peptide. (Figs. 5E and 5F) In the open field test, both H3 and 14s21 peptide treated rats showed the increased time spent in center of arena (i), latency to reach to center (ii), but only 14s21 peptide displayed the significant increase in velocity (iii), and total distance travelled (iv), during the experimental paradigm. (Figs. 5G and 5H) In a Novelty-suppressed feeding test, both the H3 relaxin and 14s21 peptide significantly increased the time spent in the center (i), significantly reduced the time to reach the food in the center (ii), significantly increased the velocity travelled (iii), but had no significant difference in the mean distance travelled in the arena (iv), by either of the peptides. Heat maps represent the time spent in the respective behavioral paradigm (Figs. 5A, 5C, 5E and 5G), light grey patches (indicated by arrows) = more time, dark patches = less time. N=6 (Vehicle), N=7 (H3) and N=6 (14s21 peptide). Data in Figs. 5B, 5D, 5F and 5H represent mean $\pm$ s.e.m. * p < 0.05, ** p < 0.01, *** p < 0.001 determined by One-way ANOVA followed by Tukey's *post-hoc* multiple comparison analysis compared to vehicle rats.

Figs. 6A-6E show antidepressant behavior of H3 relaxin and 14s21 peptide analyzed following acute, subacute and chronic treatment in a repeat forced swim test (FST). (Fig. 6A) Schematic diagram of the experimental schedule of repeat rat FST. Rats were intranasally administered the vehicle, H3 relaxin, 14s21 peptide and 30 min after the drug administration, behavior assessment was undertaken in the forced swim test. Twenty-four hours after the pre-training period [Training day (day 1)], rats were given the intranasal treatment of vehicle, H3 and 14s21 peptide to assess the behavior on the actual day of test in FST [Test (day 2)]. Daily intranasal administration of vehicle, H3 and 14s21 peptide was then undertaken for 14 days and the effect of H3 (0.1 μ mol) and 14s21 peptide (10 μ mol) intranasal treatment were recorded in 5 min on immobility duration and time spent in climbing and swimming behavior in female SD rats. (Fig. 6B) The graphs show behavior analyzed on the first day of treatment during the 15 min of the training period followed by 5-min tests in acute, subacute and chronic behavior paradigms. (Fig. 6C) Behavior observed during the 5-min test on the next day [Test (acute)]. (Fig. 6D) Observations after 7 days of intranasal treatment of vehicle, H3 and 14s21 peptide [Retest 1 (subacute)]. (Fig. 6E) Observations on immobility, climbing and swimming behavior after 14 days of intranasal administration of vehicle, H3 and 14s21 peptide [Retest 2 (chronic)]. Bar represents the mean $\pm$ s.e.m. N = 6 rats/group for vehicle, H3 (0.1 μ mol) and 14s21 peptide (10 μ mol) treated animals. Time spent in immobility significantly decreased over the test and retest 1 while climbing behavior significantly increased over test, retest 1 and retest 2. No significant change in swimming behavior was observed during the test, retest 1 and retest 2. Significant difference between the groups are denoted as * p < 0.05, ** p < 0.01, *** p < 0.001, determined by One-way ANOVA compared to vehicle rats. In all comparisons, ANOVA was followed by Tukey's *post-hoc* multiple comparison analysis to determine the statistical significance.

Figs. 7A-7H show ribbon diagram structures of all H3 analogs. Image shows ribbon diagrams with α -helices and corresponding linker staple in the peptides. A $i, i+4$ linker staple is shown in Fig. 7A (14s18) and Fig. 7B (18s22) positions. (Fig. 7C) depicts the lactam bond at 14s18 positions and (Fig. 7D) shows the disulfide bond at 14s18 positions. (Fig. 7E) depicts the 14s18 stapled peptide, 7-residues truncated from N-terminus (Δ 14s18) and (Fig. 7F) 13s17 stapled peptide, 7 residues truncated from N-terminus (Δ 13s17). $i, i+7$ linker staple is depicted at (Fig. 7E) 11s18 and (Fig. 7F) 14s21

positions of H3 B-chain. Residues important in RXFP3 binding and activation are shown in the peptide ribbon structure.

Fig. 8 shows trypsin digestion profiles of the stapled peptides. Reverse-phase (RP)-high-performance liquid chromatography (HPLC) profiles of unstapled R3 B-chain and 14s18 (*i, i+4*) and 14s21 (*i, i+7*) stapled peptides over a period of 25 hours. LC-MS/MS was carried out on an Agilent Technologies 1200 Series module with a Vydac C-18, 250 9 4.6 mm; 5 lm, column (Grace Vydac, Hesperia, California) at a 1 mL/min flow rate using a solvent gradient ranging from 5 to 100 % acetonitrile/water/0.1 % TFA over 38 min (14s18) and 44 min (14s21). The elution was monitored by absorption at 220 nm.

10 DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to the development of a relaxin-3 peptide agonist and compositions thereof.

Definitions

Certain terms employed in the specification, examples and appended claims are collected here for convenience.

As used herein, the term “comprising” or “including” is to be interpreted as specifying the presence of the stated features, integers, steps or components as referred to, but does not preclude the presence or addition of one or more features, integers, steps or components, or groups thereof. However, in context with the present disclosure, the term “comprising” or “including” also includes “consisting of”. The variations of the word “comprising”, such as “comprise” and “comprises”, and “including”, such as “include” and “includes”, have correspondingly varied meanings.

As used herein, the term “amino acid” refers to one of the twenty naturally occurring amino acids, including, unless stated otherwise, L-amino acids and D-amino acids. The term amino acid also refers to compounds such as chemically modified amino acids including amino acid analogs, naturally occurring amino acids that are not usually incorporated into peptides such as norleucine, and chemically synthesized compounds having properties known in the art to be characteristic of an amino acid, provided that the compound can be substituted within a peptide such that it retains its biological

activity. Other examples of amino acids and amino acid analogs are listed in [Gross and Meienhofer, The peptides: Analysis, Synthesis, Biology, Academic Press Inc., New York (1983)]. An amino acid also can be an amino acid mimetic, which is a structure that exhibits substantially the same spatial arrangement of functional groups as an amino acid but does not necessarily have both the α -amino and α -carboxyl groups characteristic of an amino acid.

A "conservative amino acid substitution" as used herein is one in which the amino acid residue is replaced with an amino acid residue having a similar side chain. Families of amino acid residues having similar side chains have been defined in the art. These families include amino acids with basic side chains (e.g., lysine, arginine, histidine), acidic side chains (e.g., aspartic acid, glutamic acid), uncharged polar side chains (e.g., glycine, asparagine, glutamine, serine, threonine, tyrosine, cysteine), nonpolar side chains (e.g., alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan), beta-branched side chains (e.g., threonine, valine, isoleucine) and aromatic side chains (e.g., tyrosine, phenylalanine, tryptophan, histidine). There are over 700 known nonstandard amino acids any of which may be included in the peptide precursors for use in the present invention. See, for example, [S. Hunt, The Non-protein Amino acids: In Chemistry and Biochemistry of the Amino Acids, edited by G. C. Barrett, Chapman and hall, (1985)]. Some examples of nonstandard amino acids are β -alanine, D-alanine, 4-hydroxyproline, D-glutamic acid, N-methyl-L-leucine, β -cyanoalanine. Additionally, the amino acids suitable for use in the present invention may be derivatized to include amino acid residues that are hydroxylated, phosphorylated, sulfonated, and glycosylated, to name a few. Additionally, these amino acids may include functional groups including, but not limited to alcohol, thiol, ketone, aldehyde, ester, ether, amine, imine, amide, nitro acid, carboxylic acid, disulphide, carbonate, carboalkoxy acid, isocyanate, carbodiimide, carboalkoxy, and halogen functional groups. It will be appreciated by one of ordinary skill in the art, however, that certain amino acids are capable of promoting formation of alpha helix structures, or other desired secondary structures, and thus specific amino acids are particularly preferred in the present invention, depending on the desired secondary structures to be generated.

Once the desired amino acids are selected for the synthesis of a desired peptide according to the present invention, synthesis of the desired peptide can be achieved

using standard deprotection and coupling reactions. One of ordinary skill in the art will realize that the choice of a particular synthetic technique will depend upon the particular structures to be synthesized [Blackwell et al., *J Org Chem* 66: 5291-5302 (2001)].

Thus, one or more amino acid residues that are not in positions 14 and 21 in a
5 relaxin B-chain polypeptide comprising, essentially consisting of, or consisting of the amino acid sequence encoded by the nucleic acid sequence SEQ ID NO: 1, 9 or 10 or a fragment thereof, may be replaced with one or more other amino acid residues from the same side chain family without significantly reducing the activity of the stapled polypeptide or deviating significantly from the scope of the present invention. In the
10 present invention, the terms “functional” or “active” “analogs,” “derivatives,” or “fragments” are used interchangeably to mean a chemical substance that is related structurally and functionally to another substance. An analog, derivative, or fragment contains a modified structure from the parent substance, in this instance, the biological function or activity of relaxin-3 stapled peptides in cellular and animal models. The
15 biological activity of the stapled relaxin-3 analog, derivative, or fragment may include an improved desired activity or a decreased undesirable activity. Analogs, derivatives, or fragments of the instant invention, include, but are not limited to, analogs of the relaxin-3 peptide, that are homologous to other relaxins and/or insulin, and/or insulin like peptides.

In the present invention, the terms “functional”, “mimetic” means relaxin-3 B-chain
20 derived stapled peptide having a non-amino acid chemical structure that mimics the structure of relaxin-3 derived peptide. Such a mimetic generally is characterized as exhibiting similar physical characteristics such as size, charge or hydrophobicity in the same spatial arrangements found in relaxin-3 peptide. A specific example of a peptide mimetic is a compound in which the amide bond between one or more of the amino acids
25 is replaced, for example, by a carbon-carbon bond or other bond well known in the art [see, for example, Sawyer, *Peptide Based Drug Design*, ACS, Washington (1995)].

As used herein, the term “peptide,” is used in reference to a functional or active analog, derivative or fragment of relaxin-3 or relaxin-3 B-chain derived sequence containing naturally occurring amino acids, non-naturally occurring amino acids or
30 chemically modified amino acids, provided that the compound retains the bioactivity or function of relaxin-3 peptide.

The peptides described herein may exist in particular geometric or stereoisomeric forms. They may have one or more chiral centers and thus exist as one or more stereoisomers. Such stereoisomers as encompassed by the present disclosure can exist as a single enantiomer, a mixture of diastereomers or a racemic mixture.

5 The term “isomers” as used herein includes any and all geometric isomers and stereoisomers. For example, “isomers” include cis- and trans- isomers, E- and Z- isomers, R- and S-enantiomers, diastereomers, (D)-isomers, (L)-isomers, racemic mixtures thereof, and other mixtures thereof, as falling within the scope of the invention. In certain forms the compound of the present invention may made up of at least about
10 90% by weight of a preferred enantiomer. In other forms the compound of the present invention may made up of at least about 95%, 98%, or 99% by weight of a preferred enantiomer. Preferred enantiomers may be isolated from racemic mixtures by any method known to those skilled in the art, including chiral high pressure liquid chromatography (HPLC) and the formation and crystallization of chiral salts or prepared
15 by asymmetric syntheses. See, for example, Jacques et al., *Enantiomers, Racemates and Resolutions* (Wiley Interscience, New York, 1981); Wilen S. H., et al., *Tetrahedron* 33: 2725 (1977); Eliel, E. L. *Stereochemistry of Carbon compounds* (McGraw-Hill, N.Y., 1962); Wilen, S. H. *Tables of Resolving Agents and Optical Resolutions* p. 268 (E. L. Eliel, Ed., Univ of Notre Dame Press, Notre Dame, Ind. 1972).

20 The term “staple” as used herein refers to the intramolecular connection (also referred to cross-linking) of two peptide domains (e.g. two loops of a helical peptide). Generally, the staple comprises a hydrocarbon linkage requiring the use of modified amino acid residues and covalently joining olefin moieties (i.e. stapled together) using a ring-closing metathesis (RCM) reaction. When the peptide has a helical secondary
25 structure, the staple is a macrocyclic ring, which is exogenous (not part of) core or inherent (non-stapled) helical peptide structure. The macrocyclic ring is comprised of an alkene double bond and incorporates at least two amino acids. The hydrocarbon linkage can also be replaced by other chemical linkages.

After the desired peptide is synthesized using an appropriate technique, the
30 peptide is contacted with a specific reagent to promote carbon-carbon bond formation. In one particular embodiment, a metathesis catalyst is utilized to effect one or more olefin

metathesis reactions and subsequent generation of a cross-linker and stabilization of the alpha helix or other desired secondary structure. One of ordinary skill in the art will realize that a variety of metathesis catalysts can be utilized in the present invention. Selection of particular catalyst will vary with the reaction conditions utilized and functional groups present in the particular peptide. Exemplary catalysts include, but are not limited to stabilized, late transition metal carbene complex catalysts such as Group VIII transition metal carbene catalysts, most preferably Ru and Os metal centers having a +2 oxidation state, an electron count of 16 and penta-coordinated. For discussion on metathesis reactions (See, Grubbs et al., "Ring Closing Metathesis and Related Processes in Organic Synthesis" Acc. Chem. Res. 1995, 28, 446-452, and U.S. Pat. No. 5,811,515).

In general, the synthesis of these stabilized secondary structures involves (1) synthesizing the peptide from a selected number of natural or non-natural amino acids, wherein said peptide comprises at least two reactive moieties capable of undergoing a C-C bond forming reaction; and (2) contacting said peptide with a reagent to generate at least one cross-linker and to effect stabilization of a specific secondary structure motif; and (3) synthesizing a peptide from selected natural or non-natural amino acids, wherein said peptide comprises at least two vinyl amino acids capable of undergoing an olefin metathesis reactions; and (4) contacting said peptide with a metathesis catalyst to generate at least one cross-linker and to effect stabilization of an alpha helix structures. Alternatively, any combination of two vinyl amino acids may be incorporated to generate desired cross-linked structures.

A number of approaches for covalent helix stabilization in i+4 and i+7 stapled peptides have been reported. A few of them also include cross-linkers that are polar and pharmacologically labile, such as disulfides and lactam bridges. An important conceptual advance on this front is the development by Grubbs and co-workers of chemistry for olefin cross-linking of helices through O-allyl serine residues located on adjacent helical turns, via ruthenium catalysed ring-closing metathesis (RCM). The disulphide and lactam crosslinks analyzed in i+4 stapled peptide, however, showed no evidence of enhancing helical stability, highlighting the difficulty of this problem from a design standpoint. An alternate metathesis-based approach was employed herein, namely to screen multiple configurations of all-hydrocarbon crosslinks differing in position of attachment, stereochemistry, and cross-linker length. Where some configurations impart significant

helix stabilization, others actually destabilize an alpha helix. We have shown that stabilizing the alpha helix in this scrutinized approach leads to enhanced affinity and potency towards its cognate receptor and also markedly increased resistance to proteolysis.

5 References herein (in any aspect or embodiment of the invention) to stapled polypeptides of the invention, including those of formula 1 or 2, includes references to such compounds per se, as well as to pharmaceutically acceptable salts or solvates, or pharmaceutically functional derivatives of such compounds.

10 As used herein, the term "salt" or "pharmaceutically acceptable salt" refers to those salts which are within the scope of treatment, suitable for use in contact with the tissues of mammals without undue toxicity, irritation, allergic response and are commensurate with a reasonable benefit/risk ratio. Pharmaceutically acceptable salts are well known in the art. For example, Berge et al., describes pharmaceutically acceptable salts in detail in *J. Pharmaceutical Sciences* (1977) 66:1-19.

15 Pharmaceutically acceptable salts that may be mentioned include acid addition salts and base addition salts. Such salts may be formed by conventional means, for example by reaction of a free acid or a free base form of a stapled polypeptide of the invention with one or more equivalents of an appropriate acid or base, optionally in a solvent, or in a medium in which the salt is insoluble, followed by removal of said solvent,
20 or said medium, using standard techniques (e.g. in vacuo, by freeze-drying or by filtration). Salts may also be prepared by exchanging a counter-ion of a stapled polypeptide of the invention in the form of a salt with another counter-ion, for example using a suitable ion exchange resin.

25 Examples of pharmaceutically acceptable salts include acid addition salts derived from mineral acids and organic acids, and salts derived from metals such as sodium, magnesium, or preferably, potassium and calcium.

30 Examples of acid addition salts include acid addition salts formed with acetic, 2,2-dichloroacetic, adipic, alginic, aryl sulphonic acids (e.g. benzenesulphonic, naphthalene-2-sulphonic, naphthalene-1,5-disulphonic and p-toluenesulphonic), ascorbic (e.g. L-ascorbic), L-aspartic, benzoic, 4-acetamidobenzoic, butanoic, (+) camphoric, camphor-

5 sulphonic, (+)-(1S)-camphor-10-sulphonic, capric, caproic, caprylic, cinnamic, citric, cyclamic, dodecylsulphuric, ethane-1,2-disulphonic, ethanesulphonic, 2-hydroxyethanesulphonic, formic, fumaric, galactaric, gentisic, glucoheptonic, gluconic (e.g. D-gluconic), glucuronic (e.g. D-glucuronic), glutamic (e.g. L-glutamic), α -oxoglutaric, glycolic, hippuric, hydrobromic, hydrochloric, hydriodic, isethionic, lactic (e.g. (+)-L-lactic and ($\pm$)-DL-lactic), lactobionic, maleic, malic (e.g. (-)-L-malic), malonic, ($\pm$)-DL-mandelic, metaphosphoric, methanesulphonic, 1-hydroxy-2-naphthoic, nicotinic, nitric, oleic, orotic, oxalic, palmitic, pamoic, phosphoric, propionic, L-pyroglutamic, salicylic, 4-amino-salicylic, sebacic, stearic, succinic, sulphuric, tannic, tartaric (e.g. (+)-L-tartaric), thiocyanic, undecylenic and valeric acids.

15 Particular examples of salts are salts derived from mineral acids such as hydrochloric, hydrobromic, phosphoric, metaphosphoric, nitric and sulphuric acids; from organic acids, such as tartaric, acetic, citric, malic, lactic, fumaric, benzoic, glycolic, gluconic, succinic, arylsulphonic acids; and from metals such as sodium, magnesium, or preferably, potassium and calcium.

20 As mentioned above, also encompassed by stapled polypeptides of the invention are any solvates of the stapled polypeptides and their salts. Preferred solvates are solvates formed by the incorporation into the solid state structure (e.g. crystal structure) of the stapled polypeptides of the invention of molecules of a non-toxic pharmaceutically acceptable solvent (referred to below as the solvating solvent). Examples of such solvents include water, alcohols (such as ethanol, isopropanol and butanol) and dimethylsulphoxide. Solvates can be prepared by recrystallising the stapled polypeptides of the invention with a solvent or mixture of solvents containing the solvating solvent. Whether or not a solvate has been formed in any given instance can be determined by 25 subjecting crystals of the compound to analysis using well known and standard techniques such as thermogravimetric analysis (TGE), differential scanning calorimetry (DSC) and X-ray crystallography.

30 For a more detailed discussion of solvates and the methods used to make and characterise them, see Bryn *et al.*, *Solid-State Chemistry of Drugs*, Second Edition, published by SSCI, Inc of West Lafayette, IN, USA, 1999, ISBN 0-967-06710-3.

The amount of stapled polypeptide in any pharmaceutical formulation used in accordance with the present invention will depend on various factors, such as the severity of the condition to be treated, the particular patient to be treated, as well as the compound(s) which is/are employed. In any event, the amount of stapled polypeptide in the formulation may be determined routinely by the skilled person.

The dose administered to a mammal, particularly a human, in the context of the present invention should be sufficient to effect a therapeutic response in the mammal over a reasonable timeframe. One skilled in the art will recognize that the selection of the exact dose and composition and the most appropriate delivery regimen will also be influenced by inter alia the pharmacological properties of the formulation, the nature and severity of the condition being treated, and the physical condition and mental acuity of the recipient, as well as the potency of the specific compound, the age, condition, body weight, sex and response of the patient to be treated, and the stage/severity of the disease.

15

"Therapeutic" as used herein means the amelioration of, protection, in whole or in part, against further, nervous system or neuropsychiatric disorders, eating disorders and conditions associated with stress-related neuropsychiatric disorders.

The terms "administer", "administering", or "administration", as used herein refers to implantation, ingesting, injecting, or inhaling, the inventive polypeptide or compound.

The term "treatment", as used in the context of the invention refers to prophylactic, ameliorating, therapeutic or curative treatment.

The term 'variant' as used in the context of the present invention, is intended to describe variations to the amino acid sequence of the stapled relaxin B-chain polypeptide that do not reduce the activity of the polypeptide in terms of binding to RXFP3. Variants include conservative amino acid substitutions, and additions or deletions of amino acids that are not in positions 14 and 21 of the relaxin B-chain polypeptide and do not affect binding.

Having now generally described the invention, the same will be more readily understood through reference to the following examples which are provided by way of illustration, and are not intended to be limiting of the present invention.

EXAMPLES

- 5 Standard molecular biology techniques known in the art and not specifically described were generally followed as described in Sambrook and Russel, Molecular Cloning: A Laboratory Manual, Cold Springs Harbor Laboratory, New York (2001).

Example 1

Designing of stapled peptides and variants

- 10 A series of stapled peptides using in-silico modelling and more comprehensive staple scanning approach were designed to derive an optimized stapled peptides and variants for testing. To illustrate the nature of H3 relaxin-RXFP3 complex, we used previously reported structure-activity relationships of H3 relaxin to generate a molecular model. Additional structure-activity studies on relaxin-3 have identified the key residues
- 15 involved in binding and activation of the RXFP3 receptor comprising ArgB12, IleB15, ArgB16 and PheB20, located in the B-chain helical region, and the two C-terminal residues ArgB26 and TrpB27 at the end of a flexible tail were reported to be critical for activation of the receptors and thus stapling sites were chosen so that they do not interfere with the residues critical for binding and activation of RXFP3 receptor. Based
- 20 on this knowledge and with in-silico modelling prediction, the inventors designed stapled peptides which were stapled at $i, i+4$ [18th and 22nd positions (18s22) and 14th and 18th positions (14s18)] positions and $i, i+7$ [11th and 18th positions (11s18) and 14th and 21st positions (14s21)] positions. The inventors found that stapling at a more central position stabilized the alpha helix, thus the lactam and disulfide variants of previously published
- 25 ($i, i+4$)14s18 stapled peptide were also synthesized. In order to study the effective minimized active structure of relaxin-3 B-chain stapled peptide, we also attempted to truncate the 7 residues from N-terminus of relaxin-3 B-chain [R3B8-27 (14s18)- Δ 14s18]. We compared the binding affinity and activation profile of truncated (Δ 14s18) peptide to the full length [R3B (14s18)] 14s18 stapled peptide. Further, the (Δ 14s18) was compared

with the previously reported high-affinity agonist 13s17 stapled peptide [R3B8-27 (Δ 13s17)] [Hojo et al., *J Med Chem* 59: 7445-7456 (2016)].

Molecular modelling

The nuclear magnetic resonance (NMR) structure of relaxin-3 [Rosengren et al.,
5 *J Biol Chem* 281: 5845-5851 (2006)] was prepared using the Protein Preparation Wizard
in Maestro version 10.3 (Schrodinger LLC, New York, USA) to ensure structural
correctness. NMR analysis produces a set of estimates of constraints on distances
between atoms resulting in an ensemble of models rather than a single structure. The
2FHW structural ensemble contains 20 such models for relaxin-3. The preparation
10 process included setting the tautomer and protonation state of the peptide and subjecting
each of the 20 structures to restrained minimization using an optimized potentials for
liquid simulations (OPLS) force field [Jayakody et al., *Peptides* 84: 44-57 (2016)],
specifically the OPLS3 force field optimized to provide broad coverage of drug-like small
molecules and proteins, and a generalized Born model of solvation augmented with the
15 hydrophobic solvent accessible surface area term (GB/SA solvation model). Structures
1, 10 and 20 were selected for molecular dynamics (MD) simulations using the B-chain
only. Both the wild type relaxin-3 B-chain and a relaxin-3 B-chain Cys10Ser + Cys22Ser
double mutant (double mutant) were investigated. The Desmond System Builder version
4.3 (Schrodinger LLC) was used to build a solvated system for the simulation including
20 the solute peptide and the solvent water molecules with counter ions. We used that
flexible simple point charge (SPC) water model for the solvent with orthorhombic
boundary conditions and a 10Å buffer. The systems were neutralized with Cl⁻ ions and
0.15 M NaCl salt was added. The systems were then relaxed and subjected to 12 ns MD
using the OPLS3 force field and a substance (N), pressure (P) and temperature (T) [NPT]
25 ensemble class with T = 300 K and P = 1.01325 bar. The stapled peptides were built
using the relaxin-3 B-chain Cys10Ser + Cys22Ser double mutant of structures 1, 10 and
20 of the 2FHW structural ensemble and prepared as described above. The staple and
the two amino acids involved in stapling were relaxed using 200 steps of Polak-Ribiere
conjugate gradient (PRCG) minimization, the OPLS3 force field and the GB/SA solvation
30 model while the remaining residues were constrained. Systems were then built and these
were subjected to 12 ns MD as described above.

Peptide synthesis and characterization

Hydrocarbon-stapled peptides and variants were purchased from Biosynthesis Inc, USA. Staples were incorporated by placing the 8-carbon metathesized cross-link with (S)-configuration at *i*(14), *i*+4(18) and *i*(18), *i*+4(22) positions with (S)2-(4'-pentenyl)alanine (S₅). Lactam bond formation was undertaken at 14(*i*) and 18(*i*+4) positions by replacing the residues with K(Lys) and D(Asp). Disulfide bond formation was held by replacing the residues with C(Cys) at 14(*i*) and 18(*i*+4) positions. Hydrocarbon stapling for *i*, *i*+7 peptides was carried out with 11-carbon cross-link by using the combination of R-2-(4'-pentenyl) alanine (R₅) and S-2-(7'-octenyl) alanine (S₈) at *i*, *i*+7 positions for 14s21 and 11s18 stapled peptides. Peptides were purified by the manufacturer using the high-performance liquid chromatography to >95% purity. The molecular weight and purity of the peptides were further re-analyzed by mass spectrometry and RP- HPLC.

Circular dichroism

The solution conformation properties of the peptides were evaluated using circular dichroism (CD) spectroscopy obtained on a JASCO 815 spectrometer (Tokyo, Japan). The final peptide concentration was kept constant (0.20 mg/ml) for all the peptides and CD spectra were collected using the following parameters (e.g. temperature, 20 °C; wavelength, 190–260 nm; step resolution, 0.5 nm; speed, 20 nm/s; response time 0.5 s; bandwidth, 1 nm; path length, 0.1 cm). Curves shown were smoothed with standard parameters. All spectra were converted to a uniform scale of molar ellipticity after background subtraction. The mean α -helical content of each peptide was calculated using the mean residual ellipticity at 222 nm as previously described [Jayakody et al., *Peptides* 84: 44-57 (2016)].

Reagents

Cell lines and culture

Stable RXFP3-expressing HEK293T cells (HEK-RXFP3) were maintained in 75 cm² flasks at 37°C in a humidified atmosphere in DMEM. DMEM was supplemented with 10% (v/v) FBS, 1X penicillin/streptomycin (10,000 U/ml). Cells were harvested at 90%

cell confluency for plating onto PLL pre-coated well plates for receptor-ligand binding and receptor activation assays.

Eu(III)-based ligand binding assay

Cells were plated in CoStar 96-well plates (#3596) at a density of 25,000
5 cells/well and were allowed to grow for 3 days. On the day of experiment, media was aspirated from all wells prior to the addition of the ligands to be tested. Ligands were diluted in binding media (DELFLIA L*R binding buffer concentrate 10X, diluted 1:10 containing the following as final concentrations: 50 mmol/L Tris-HCl, 5 mmol MgCl₂, 25µmol/L EDTA, 0.2% BSA) and samples were tested in triplicates, unless otherwise
10 noted. Saturation binding assay were carried out as previously described [Jayakody et al., *Peptides* 84: 44-57 (2016)], increasing concentration of Eu-DTPA-R3B1-22R (0.5-128 nM) peptide [Jayakody et al., *Peptides* 84: 44-57 (2016)] were added to the cells and incubated for 2.5 hours with gentle shaking (~50 rpm) at room temperature. Non-specific binding was determined in the presence of 1 µM unlabeled R3B1-22R. After
15 binding at room temperature for 2.5 hours, the binding solution was removed and cells were washed twice rapidly (~0.5 s) with ice cold PBS. The media was replaced manually with care. To make certain that cells are not released during the washing, microscopic analysis of the aspirate was done as a simple evaluation of cell release as a vehicle experiment. Following the washing step, the enhancement solution (Perkin Elmer; 1244-
20 104) was added directly to the wells (150 µL/well) and the plates were incubated for 30 min at 37°C prior to reading. The plates were read on a Wallac VICTOR instrument using the standard Eu (III) TRL measurement (340 nm excitation, 400 µsec delay, and emission collection for 400 µsec at 615 nm). For competition binding, variable concentrations of non-labelled R3B1-22R, R3 relaxin (A+B) chain, R3 relaxin B-chain
25 and stapled peptides and variants (1 pM to 100 µM) and a fixed concentration of Eu-DTPA-R3B1-22R (8 nM) was utilized using otherwise identical procedure and conditions provided above. Each experiment was repeated at least three times and one data point represents the mean of triplicates.

Inhibition of cAMP accumulation assay

The peptides were tested for their ability to inhibit cAMP activity in HEK-RXFP3 cells as previously described. Cells (1×10^5) were plated in PLL coated 24 well plates and were grown for 24h in complete DMEM media and then serum starved for 6 h before
5 experimentation. Cells were treated with relaxin-3 peptides and all analogs in triplicates at different concentrations (1 pM to 100 μ M) for 5 min and then for 15 min with forskolin (5 μ M) in 37°C incubator. Cells were lysed by shaking with 50 μ l 0.1 M HCl for 20 min. Cell lysate were thawed, mixed thoroughly, scraped and centrifuged at 1000g for 10 min. 50 μ l of supernatant solution was then applied on the mouse anti-rabbit IgG pre-coated
10 plate and assayed as per manufacturer's instructions (Cyclic AMP EIA Kit, Cayman Chemicals).

ERK 1/2 phosphorylation assay

Estimation of the level of phosphorylated ERK was performed using a western blot based technique as previously described [Shaul and Seger, *Curr Protoc Mol Biol*
15 Chapter 18, Unit 18 12 (2006)]. Cells were plated into 12 well plates with complete DMEM and then serum starved for 4 h before experimentation and assayed for phospho-ERK1/2. Cells were treated with H3 relaxin and varying concentration of 14s21 stapled peptide (1 pM to 100 μ M) for 5 min. After the reaction, cells were immediately lysed with 2X SDS loading buffer. Cell lysate was boiled at 100°C for 10 min and electrophoresed
20 through 8% SDS-Polyacrylamide gels for 2 h at 100V. After this, proteins were transferred to a PVDF membrane for 75 min at 110 V followed by blocking with 5% blocking solution in TBS/Tween 20 and immunoblotted for total ERK (tERK1/2) using Anti-p44/p42 MAPK (ERK1/2) diluted 1:1000 in 1% BSA in TBS/Tween 20 and goat anti-rabbit HRP conjugated IgG secondary antibody diluted 1:5000. Protein bands were
25 visualized by chemiluminescence using Luminata Forte HRP substrate. After visualization, primary and secondary antibodies were stripped using Restore™ western blot stripping buffer and reprobed using Anti-phospho p44/p42 MAPK (ERK1/2) diluted 1:1000 and visualized as described above.

Thermal stability assay

Temperature-dependent CD spectra of each peptide (50-65 μ M) were recorded at varying temperatures (every 5°C from 20°C to 90°C) from 190 to 260 nm. Thermal unfolding curves were generated by measuring the ellipticity at 222 nm at every 5°C from 20°C to 90°C with a temperature slope of 1°C/min. To obtain the melting temperatures, we analyzed the thermal unfolding curves using a two-state model with a 95% confidence interval as previously described [Jayakody et al., *Peptides* 84: 44-57 (2016)].

Protease resistance assay and LC-MS/MS analysis

Peptides (3 mg/ml) were digested with Trypsin (1 mg/ml) in water to the peptide solution. Twenty-five μ l of digestion mixture was taken at each time-point (0, 1, 3, 5, 7, 9 and 25 hours) and then the amount of intact peptide was quantitated by serial injection over time. The digested products were quantified by LC/MS-based peak detection at 220 nm. Each experiment was performed in triplicate. A plot of percent fraction intact (of peptide) versus time yielded an exponential decay curve, and half-lives were determined by non-linear regression analysis using GraphPad Prism software. LC-MS analysis was performed on a Thermo Scientific LCQ Fleet™ ion trap mass spectrometer coupled to a Thermo Scientific Accela 600 HPLC pump (Waltham, MA, USA). Chromatographic separation was performed using an Accucore RP-MS column (2.1 x 100 mm; 2.6 μ m) and column temperature was maintained at 40°C. Gradient separation was performed using a mixture of 0.1% formic acid and acetonitrile in 0.1% formic acid. Mass spectrometry was conducted on a Thermo Scientific LCQ Fleet™ ion trap mass spectrometer with an electrospray ionization interface in positive ion mode. An LC-MS method with scan range 50-2000 m/z was used for detection of the peptides. Peptides were quantified using peak area analysis with extracted-ion chromatogram (XIC) on Thermo Xcalibur™ software (Waltham, MA, USA). The following mass ranges were used for XIC: 600.3-606.3 m/z for R3 B-chain; XIC: 751.8-757.8 m/z for 14s18; XIC: 769.97-775.97 m/z for 14s21.

Source parameters were as follows: sheath gas flow rate 30 l/min; auxiliary gas flow rate 5 l/min; spray voltage 4.5 kV; capillary temperature 350 °C; capillary voltage 40 V; tube lens 95 V. LC-MS grade acetonitrile and Accucore RP-MS column was from

Thermo Scientific (Waltham, MA, USA). LC-MS grade formic acid was from Sigma-Aldrich (St. Louis, MO, USA).

Stereotaxic surgery

Animals

5 Experimentally naive male Sprague-Dawley rats (Laboratory Animals Center, National University of Singapore), weighing 200- 230 g at the time of arrival (for ICV studies) and female Sprague-Dawley rats, weighing 150-180g, (for intranasal drug administration) were utilized in this study. The procedures conducted followed the National Advisory Committee for Laboratory Animal Research (NACLAR), Singapore,
10 and in accordance with the Guide for the Care and Use of Laboratory Animals, National Research Council of the National Academies, USA. The animals were initially housed in pairs in individually ventilated cages and were given a 1-week acclimatization period to the vivarium prior to intracerebroventricular cannula implantation. All animals had free access to food and water throughout the experiment. The animal colony was maintained
15 at $22 \pm 2^{\circ}\text{C}$ during a 12-h light/12-h dark cycle with light on from 7:00 a.m. to 7:00 p.m. All behavioral testing occurred during the light phase between 8:00 a.m. and 1:00 p.m.

Stereotaxic implantation of cannula into lateral ventricle

Following the acclimatization period, the rats were first anesthetized with a cocktail of ketamine (75mg/kg) and xylazine (10 mg/kg) injected intraperitoneally and
20 mounted on to a stereotaxic frame. The scalp was shaved and thoroughly disinfected with iodine solution and ethanol. After a midline sagittal incision was made, the skull was cleaned and burr holes were drilled on the skull at the coordinates corresponding to the lateral ventricle. A guide cannula (Plastics One, USA) was unilaterally implanted using the following coordinates relative to the bregma (flat skull): AP: -0.8 mm, ML: -1.4 mm
25 and DV: -3.6 mm (Paxinos and Watson, 2007). One screw mounted in the skull and covered with dental cement served as an anchor for the guide cannula. The scalp was sutured and cleaned with iodine solution to prevent any infection. A dust cap was inserted into guide cannula to prevent blockage when not in use. The rats were then single-housed and allowed a rehabilitation period of 1 week, with carprofen (5 mg/kg) and
30 enrofloxacin (10 mg/kg) treatments injected subcutaneously for the first 5 days, during

which they were handled and weighed daily to habituate them to the experimenter. A stylet of stainless steel wire was inserted into each cannula to maintain patency. Correct positioning of cannula can also be verified in each rat by injecting 5 μ L of a 4 ng/ μ L solution of Angiotensin II and observing if this produced a positive dipsogenic response, defined as repeated drinking episodes of >5s that commenced within 1 min of angiotensin II administration. Only those animals with correct cannula placement were included in the data analysis. Similar surgery procedure techniques targeted at nucleus incertus were also established and conducted in several studies [Rajkumar et al., *Brain Res* 1508: 34-43 (2013)].

10 Procedure

Animals were gently restrained and relaxin-3 (540 pmol, 100 pmol and 1 nmol), 14s21 stapled peptide (1 nmol) and vehicle (equivalent amount of sterile isotonic saline) was infused into the lateral ventricle over a period of 30s. All solutions were infused in a 5- μ L volume and the administration was done using an infusion cannula connected to a 10- μ L Hamilton microsyringe by polyethylene tubing. The peptide administered through the infusion cannula was left in place for 1 min before re-capping and placing the rats for the experimental trial in the LABORAS cages. Animals were placed immediately into the cages (containing no bedding) after the drug infusion. Food crumbs detected on the floor of the apparatus were included in the determination of food weights.

Data was recorded (a) by manual food and water intake measurement after every 1 hr, (b) by the LABORAS software, 4 hours data was processed and extracted in 10 min bin size up to 4 hours and was exported and tabulated in Excel. Thereafter, statistical analysis was carried out using GraphPad Prism 5 software. Statistical significance was routinely calculated for the behavior of (relaxin-3 + saline)/(14s21 stapled peptide + saline)/(relaxin-3 + 14s21 stapled peptide) grouped animals in LABORAS cages. Results are expressed as mean $\pm$ SEM. Statistical significance was evaluated by one-way ANOVA with Tukey's post-test: * p < 0.05, significant; ** p < 0.01, highly significant and *** p < 0.001 very highly significant.

Intranasal drug administration

Animals

Female and male Sprague-Dawley rats weighing 150–180 g at arrival were housed on 12 h light/dark cycle at $23 \pm 2^{\circ}\text{C}$ with ad libitum access to food and water. Animals were acclimated for 14 days, two per cage, and then randomly assigned to the experimental groups (8–12 animals each) and housed one per cage. The body weight was monitored during the experimental procedures. All efforts were made to minimize the number of animals used and their discomfort. All animals had free access to food and water throughout the experiment. The animal colony was maintained at $22 \pm 2^{\circ}\text{C}$ during a 12-h light/12-h dark cycle with light on from 7:00 a.m. to 7:00 p.m. All behavioral testing occurred during the light phase between 8:00 a.m. and 1:00 p.m.

LABORAS

Following intranasal drug administration, the rats were individually placed in the Laboratory Animal Behavior Observation Registration and Analysis System (LABORAS, Metris, Netherlands) home cages, which were similar to the regular home cages in which they were housed. Behavior parameters such as locomotion, distance travelled, feeding, drinking, rearing and grooming were then continuously assessed for 4 h by the LABORAS software.

Determination of dose-response using female SD rats

Intranasal delivery in Sprague-Dawley rats was carried out manually without anesthesia as previously described [Lukas and Neumann, *Neuropharmacology* 62: 398-405 (2012); Thorne et al., *Neuroscience* 127: 481-496 (2004)] with slight modifications. To minimize the non-specific stress response during the application procedure, rats were habituated to the handler 10 min daily prior the day of experiment. In detail, the conscious rat head was restrained in a supine position and two pads were placed under the dorsal neck to extend the head back towards the supporting surface. All rats were treated with relaxin-3, 14s21 peptide or saline/vehicle which was delivered over both nares alternatively using a 100 μl pipette. To minimize respiratory distress and swallowing of the dose, the total volume of 50 μl dose solution as 8-10 μl drops, alternating between

nares every 1-2 min, over a total of 6.5 to 7 min was delivered till the drugs were naturally sniffed in by rat. The rat was held for an additional 30-60 seconds to ensure the fluid was inhaled. Rats were returned to their home cage thereafter for 25-35 min for behavioral testing.

5 *Elevated Zero Maze (EZM)*

Apparatus

The EZM design was based on that originally proposed by Shepherd et al. and constructed of black acrylic in a circular track 10 cm wide, 105 cm in diameter, and elevated 65 cm above the floor (San Diego Instruments, San Diego, CA). The maze was divided in four quadrants of equal length with two opposing open quadrants with 1 cm high clear acrylic curbs to prevent falls and two opposing closed quadrants with black acrylic walls 27 cm in height. A 5 min trial under the dim lighting conditions with the animal placed in the center of a closed quadrant. Dependent measures were: latency to open, time in open, open entries, and head dips. Time in open was defined as the animal having both front legs past the boundary of the closed area extending into an open quadrant.

Procedure

All testing was conducted between 0800 and 1400 hours. Before the start of experiment trial, rats were singly housed and acclimatized to the behavior room for 1 h before the experiments. One animal was selected and transferred in a covered box and the intranasal administration of the drug and behavior experiment was performed under dim light. The animal remained in the cage for 20 minutes in order to allow adequate absorption of the drug, after which it was placed on the zero maze at a junction between an open and closed arm, facing into the closed area. All testing was conducted under dim lighting condition. After 5 minutes, the animal was removed from the maze and the maze was washed using an ethanol/water solution to avoid olfactory cues transferring from one experimental session to the next. Once testing was complete, computer-based event recording and ethological analysis software (Ethovision, version XT11, Noldus, Wageningen, Netherlands) was used to register and analyze the relevant experimental variables.

Light-Dark Box Testing

This test was performed to assess anxiety behavior [Crawley, *Pharmacol Biochem Behav* 15: 695-699 (1981)]. The apparatus is a glass box divided into two compartments; one compartment “light” (30 × 30 × 50 cm) is transparent; the other one “dark” is painted black (20 × 20 × 50 cm); a hole in the partition separating the two compartments allows access between compartments. This system is based on the internal conflict between the approach and avoidance of anxiety-provoking areas (here, the light compartment). At the beginning of the experiment, the rat was placed in the light compartment of the box head oriented to the hole and was let free to explore it for 5 min. During this time, the number of entries to each compartment and the time spent in the illuminated compartment were recorded; the apparatus was cleaned with a 30 % ethanol solution between trials. At the end of each test, rats were returned to their home cages and the apparatus were thoroughly cleaned to remove the smell from the previous one.

Open field Test

The rats were placed in an open field to evaluate locomotor activity. The open field was a 1 m² plastic sheet with 30-cm high walls. The central area was defined as central zone, in which animals’ activity was regarded as a measure of anxiety. On the test day, the rats were transported to the dimly illuminated (20 lx) test room and remained undisturbed in their home cage for 1 h. During each open field test session, the rats were placed on one of the corners facing the wall of the apparatus and was let free to explore for 10 min and behavior was recorded. The variables observed were: (a) the first latency to enter the central zone of the open field arena, (b) the amount of the time spent in the central zone as defined by all forepaws being in the central four squares of the apparatus, (c) the number of squares crossed (i.e., locomotor activity), (d) total distance travelled during the 5-min trial.

Novelty induced suppression of feeding

The behavioral assessment was carried out as previously described [Caldji et al., *Neuropsychopharmacology* 22: 219-229 (2000)] with slight modifications. The Sprague-Dawley rats were fasted for 18 h and were acclimatized for 1 h in a dimly light experiment

room. Feed pallets were placed in the center of the circular arena (diameter 120 cm and height 50 cm) with the dark floor and white coloured walls. The observation area was virtually divided into zones, namely feed area, center and periphery in the acquisition software (Ethovision XT11). Rats were administered intranasal with vehicle, relaxin-3 (H3) or 14s21 peptide, placed in the periphery of the arena and behavior was monitored for 10 min. The latency to enter the feed area and the durations of time spent in the feed area and periphery was measured with the Ethovision behavior tracking and analysis software. The quantity of food consumed and the number of faecal pellets left after each trial was also noted during the trial.

10 *Acute and Chronic Forced Swim Test (FST) and Procedure*

The repeated FST was conducted using ForcedSwimScan (Clever Sys Inc, USA) and consisted of individually placing the rats into a cylindrical tank (transparent acrylic, 45.7 cm height x 20.3 cm width), containing clean water at 25°C on four different occasions (pretest, test, retest 1, retest 2). These conditions of the test have been already described [Mezadri et al., *J Neurosci Methods* 195: 200-205 (2011)]. On the first day (Pretest), the rats were submitted to 15 min of FST on Day 1, and 5 min on day 2 (test), day 7 (retest 1) and day 14 (retest 2). After each session, the rats were taken out of the water and allowed to dry with paper towels for 10 min before being returned to their home cages. All the test sessions were videotaped using an infrared video camera positioned in front of the plastic tanks enabling the subsequent evaluation of animal behavior. A rule of thumb for Multivariate analyzes indicated that at least 3 cases are required for every variable added to the analysis. Animals received a daily intranasal administration of vehicle group, H3 relaxin (0.1 µmol) and 14s21 peptide (10 µmol), respectively, until retest 1 (Day 7) and retest 2 (Day 14). The experimental schedule is presented in Fig. 6A.

Immobility was defined as the lack of motion of the whole body consisting only of the small movements necessary to keep the animal's head above the water. Swimming was recorded when large forepaw movements displaced the body around the cylinder, more than necessary to merely keep the head above the water. Climbing was registered when vigorous movements with forepaws in and out of the water, usually directed against the wall of the tank, were observed. Diving was observed when the whole body of the

animal, including the head, was submersed. The behavioral distribution and behavioral categories were considered distinct and separated in time.

Statistics

Statistical analyses were performed on data using a regular one-way or two-way analysis of variance (ANOVA) followed by Tukey's post-hoc multiple comparison analysis to determine statistical significance between group treatments. A *P* value less than 0.05 was considered significant.

Example 2

Design, synthesis and structural analysis of stapled peptides.

We previously explored the utility of inserting *i, i+4* staples, which span one-turn of an α -helix, into H3 B-chain of relaxin-3 (H3) peptide, yielding a lead 14s18 (*i, i+4*) stapled peptide with optimized α -helical structure, proteolytic resistance, and enhanced biological activity [Jayakody et al., *Peptides* 84: 44-57 (2016)]. Constraining the H3 B-chain peptide via side chain cross-linking with (S)2-(4'-pentenyl)alanine at (*i, i+4*) position served as an effective means to reinforce peptide helices. To compare the utility of hydrocarbon linker to other stapling strategies, we installed the corresponding (*i, i+4*) 14s18-Lactam and (*i, i+4*) 14s18-Disulfide bridge cross-linkers at respective positions (Fig. 1A, Figs. 7C and 7D). Circular dichroism analysis revealed that the insertion of a 14s18 hydrocarbon linker enhanced α -helical content up to 18.52% compared to 1.81% of unmodified H3 B-chain peptide whereas the spectra of corresponding 14s18-Lactam and 14s18-Disulfide bridged peptides displayed the random coil structure similar to the linear variant of H3 B-chain peptide (Fig. 1C). These N-terminus 7 residues were reported not to participate in binding and activation of the RXFP3 receptor. So, in an attempt to study the minimized effective structure of 14s18 stapled peptide, the 7-residues from the N-terminus of 14s18 stapled peptides (Δ 14s18) were truncated, and also in the control H3 B-chain peptide (Δ H3 B-chain). Conformational analysis of Δ 14s18 peptide revealed the preservation of α -helicity with 12.80% enhancement compared to 1.81% of unmodified H3 B-chain peptide, but overall helical content was reduced when compared with full length 14s18 stapled peptide (Fig. 1C), which suggests that despite the significant effect of hydrocarbon stapling in maintaining the α -helicity in Δ 14s18

peptide, N-terminus residues might have some influence in achieving the optimal α -helicity seen in full length 14s18 stapled peptide. Previously, an alternative $i, i+4$ stapling position within the H3 B-chain, e.g. at 18s22 position, did not demonstrate the enhanced α -helical profile and produced only a 6.17% increment of α -helical content compared to the unmodified peptide [Jayakody et al., *Peptides* 84: 44-57 (2016)]. Studies reported by others on alternative stapling positions within the H3 B-chain sequence were also unsuccessful, emphasizing the rational design and important consideration of both peptide sequence and stapling methodology for optimal outcomes [Hojo et al., *J Med Chem* 59: 7445-7456 (2016)]. Further, in an attempt to understand the impact of different hydrocarbon stapling strategies, the inventors explored the effect of structural stabilization by use of $i, i+7$ staples, which cross-link a two-turn span of α -helical peptide. With the reported structure-activity relationship studies of relaxin-3 and molecular modelling of various staple combination scans over the entire H3 B-chain sequence, the inventors identified optimal positions for $i, i+7$ staples at 14s21 and 11s18 positions in the H3 B-chain (Fig. 1B; Figs. 7G and 7H). Stapling sites were chosen so that the presence of staple in the sequence did not interfere with the residues critical for binding and activation for the RXFP3 receptor. Additionally, in order to understand the effect of Cys10/22 to Ser substitution in the H3 B-chain, the $i, i+7$ peptides (14s21 and 11s18) were kept in the natural sequence of H3 B-chain i.e. without mutating the Cys 10/22 to Serine and with its control peptide where Cys10/22 were mutated to Serine (14s21-Ser). Both of the Cys containing $i, i+7$ stapled peptides, 14s21 and 11s18, were successfully synthesized (Fig. 7, Table 2) and exhibited up to 23.51-fold and 21.92-fold stabilization of α -helix respectively, as determined by circular dichroism (Fig. 1E). In contrast to the $i, i+4$ series, both $i, i+7$ stapled peptides showed notably enhanced α -helicity compared with the unmodified peptide, with 14s21 having slightly higher helicity (Fig. 1E). Figure 1A and 1B show the sequences of $i, i+4$ and $i, i+7$ stapled peptides in a helix wheel projection, with different positions of the staples corresponding to all the analogs used in the study. Since, $i+7$ is a long hydrophobic hydrocarbon staple that cross-linked over the critical hydrophobic binding surfaces in relaxin-3 B-chain, it can serve as the powerful nucleator of α -helical structure. The incorporation of a two-turn $i, i+7$ staple can thus also induce helical structure throughout a much longer sequence [Bird et al., *Proc Natl Acad Sci U S A* 107: 14093-14098 (2010)]. The increase in hydrophobicities and hydrophobic moments of the stapled analogs is reflected in the increment of their RP-HPLC retention

times with respect to the unmodified H3 B-chain. All peptides listed in Table 1 were readily synthesized by solid-phase peptide synthesis using Fmoc chemistry and were used for bioassay (Table 1 and Table 2).

Table 1 shows stapled H3 B-chain analogs synthesized and characterized in the current study.

SEQ ID NO:	Analog	Sequence
1	H3 B-chain	RAAPYGVRLSGREFIRAVIFTSGGSRW
2	14s18	RAAPYGVRLSGRE- S5 IRA- S5 IFTSGGSRW
3	18s22	RAAPYGVRLSGREFIRA- S5 IFT- S5 GGSRW
4	Δ 14s18	RLSGRE- S5 IRA- S5 IFTSGGSRW
5	Δ 13s17	RLSGR- S5 FIR- S5 VIFTSGGSRW
6	Δ H3 B-chain	RLSGREFIRAVIFTSGGSRW
7	14s18-Lactam	RAAPYGVRLSGRE- K IRA- D IFTSGGSRW
8	14s18-Disulfide	RAAPYGVRLSGRE- C IRA- C IFTSGGSRW
9	14s21	RAAPYGVRLCGRE- R5 IRAVIF- S8 CGGSRW
10	14s21-Ser	RAAPYGVRLSGRE- R5 IRAVIF- S8 SGGSRW
11	11s18	RAAPYGVRLC- R5 REFIRA- S8 IFTGCGGSRW

S5: (S)-2-(4-pentenyl) alanine, **R5**: (R)-2-(4'-pentenyl) alanine, **S8**: (S)-2-(7'-octenyl) alanine

Table 2 shows analytical Data and Mass profiles of the synthesized peptides.

SEQ ID NO:	Analog	HPLC retention time (min)	TOF-MS	
			Theoretical Mass	Observed mass
1	H3 B-chain	10.63	3010.48	3010.65
2	14s18	13.683	3014.17	3014.51
3	18s22	12.150	3074.61	3074.67
4	Δ 14s18	15.83	2299.68	2299.65
5	Δ 13s17	16.34	2345.80	2345.74
6	Δ H3 B-chain	14.17	2295.65	2295.61

7	14s18-Lactam	9.567	2989.43	2990.27
8	14s18-Disulfide	7.850	2968.46	2968.26
9	14s21	12.567	3086.75	3086.37
10	14s21-Ser	9.933	3054.62	3054.07
11	11s18	12.700	3178.85	3178.13

Theoretical and observed masses of each analog. The purity of each peptide was confirmed to be > 95% by using analytical RP-HPLC peak area integration. Peptide molecular weights were confirmed by Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry for all peptides except Δ 14s18, Δ 13s17 and Δ H3 B-chain which were re-analyzed on reverse-phase HPLC and liquid chromatography mass spectrometry (LC-MS).

EXAMPLE 3

In vitro activity and stability of stapled peptides.

The properties of H3 B-chain analogs for their ability to bind RXFP3 receptor in competition binding assays and ability to activate the RXFP3 receptor in cAMP activation assays were evaluated. Well established ligand binding assays and a cAMP activation assay [Jayakody et al., *Peptides* 84: 44-57 (2016)] were used to compare the stapled peptides to H3 relaxin peptide as well as a linear H3 B-chain peptide in RXFP3 expressing cell lines. Saturation binding studies conducted with Eu-DTPA-R3 B1-22R in HEK-RXFP3 showed a single population of sites with a density of 1061 ± 46.68 fmol/mg with apparent binding affinity of 35 nM [Jayakody et al., *Peptides* 84: 44-57 (2016)]. Competition binding for Eu-DTPA-R3B1-22R observed with unlabeled test stapled peptides revealed that *i*, *i*+4 stapled 14s18 peptide exhibited weaker binding affinity of 380 nM, as compared to H3 relaxin (42 nM) but it was found to be increased compared to the unmodified H3 B-chain (1778 nM) (Fig. 2A, Table 3) [Jayakody et al., *Peptides* 84: 44-57 (2016)]. Further analysis on the effect of N-terminus 7 residue truncation of 14s18 stapled peptide (Δ 14s18) revealed a binding affinity of 479 nM which was slightly weaker compared to full length 14s18 stapled peptide (Fig. 2B), the result being consistent with helicity studies. Also utilized was a highly potent, minimized agonist of H3 B-chain, N-

terminus 7-residue truncated 13s17 stapled peptide (Δ 13s17) [Hojo et al., *J Med Chem* 59: 7445-7456 (2016)], as a control-test peptide to compare the binding affinity of truncated Δ 14s18 peptide. Δ 13s17 peptide exhibited the improved binding affinity of 151 nM as reported previously and thus Δ 14s18 peptide was not able to mimic the effect of improved binding affinity on RXFP3 receptor when compared to Δ 13s17 peptide (Fig. 2B, Table 3). Inhibition of forskolin-stimulated cAMP activation by H3 relaxin at RXFP3 receptor has been reported earlier [Liu et al., *J Biol Chem* 278: 50754-50764 (2003)]. To determine whether an equipotent binder can also activate the RXFP3 receptor, we tested the H3 B-chain analogs for further analysis of the concentration-response relationship with inhibition of forskolin-induced cAMP levels. H3 relaxin dose-dependently inhibited forskolin-induced cAMP levels with an EC_{50} value of 0.52 nM. H3 B-chain was significantly less potent than H3 relaxin with an EC_{50} of 955 nM. 14s18 peptide was significantly more potent than H3 B-chain with an EC_{50} value of 17 nM ($p < 0.05$ vs H3 B-chain). Δ 14s18 also dose-dependently inhibited the forskolin-induced cAMP levels with an EC_{50} of 43 nM which was slightly less potent compared to the 14s18 (17 nM) and Δ 13s17 (6 nM) stapled peptides (Fig. 2D, Table 3).

Table 3 shows competitive binding activity (pK_i) and functional in-vitro cAMP activity (pEC_{50}) values for H3 B-chain analogs.

Analog	Eu-DTPA-R3(B1-22R) pK_i		cAMP pEC_{50}	
H3 relaxin	7.38 $\pm$ 0.11	[41.69 nM]**	9.28 $\pm$ 0.15	[0.52 nM]###
H3 B-chain	5.75 $\pm$ 0.24	[1778.28 nM]	6.02 $\pm$ 0.24	[955.10 nM]
14s18	6.42 $\pm$ 0.13	[380.19 nM]	7.77 $\pm$ 0.13	[16.98 nM] [#]
18s22	6.02 $\pm$ 0.23	[955.10 nM]	N.d.	
Δ 14s18	6.32 $\pm$ 0.24	[478.63 nM]	7.37 $\pm$ 0.14	[42.65 nM]
Δ 13s17	6.82 $\pm$ 0.25	[151.36 nM]	8.24 $\pm$ 0.15	[5.75 nM]##
14s18-Lactam	5.89 $\pm$ 0.27	[1288.25 nM]	5.97 $\pm$ 0.71	[1071.52 nM]
14s18-Disulfide	5.80 $\pm$ 0.40	[1580.90 nM]	4.78 $\pm$ 1.5	[16.5 μ M]
14s21	7.18 $\pm$ 0.20	[66.07 nM]**	8.51 $\pm$ 0.07	[3 nM]###
14s21-Ser	7.05 $\pm$ 0.26	[89.12 nM]*	8.28 $\pm$ 0.16	[5.24 nM]##
11s18	6.48 $\pm$ 0.14	[331.13 nM]	7.78 $\pm$ 0.14	[16.65 nM] [#]

* $p < 0.05$, ** $p < 0.01$, vs H3 B-chain; [#] $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ vs H3 B-chain

The activity of a 14s18 hydrocarbon staple was compared to its corresponding 14s18-Lactam and 14s18-Disulfide peptide. The 14s18-Lactam with a salt bridge at 14s18 positions, demonstrated similar binding affinity and similar potency to H3 B-chain at RXFP3 (Figs. 2B, 2E and Table 3). Thus 14s18-Lactam was like the linear unmodified H3 B-chain peptide. The 14s18-Disulfide with a cysteine linkage demonstrated very poor binding affinity at RXFP3 and significantly lower activity than the H3 B-chain (Fig. 2E, Table 3). However, while the 14s18-Lactam showed a similar activity to H3 B-chain, the 14s18-Disulfide was not able to display the basal activity at RXFP3 receptor. The data clearly demonstrated that the lactam and cysteine linkages at 14s18 position were not able to mimic the improved activity seen with the hydrocarbon staple, which was also consistent with the studies previously published with minimized staple analogs of RXFP3 [Hojo et al., *J Med Chem* 59: 7445-7456 (2016)].

Of the *i*, *i*+7 staples (14s21 and 11s18), 14s21 retained the most potent competitive binding with significant enhancement over H3 B-chain ($pK_i = 66$ nM, $p < 0.01$ vs H3 B-chain) (Fig. 2C, Table 3). The significant increase in binding affinity was consistent with the change seen in the cAMP activity assays ($pEC_{50} = 3$ nM, $p < 0.001$ vs H3 B-chain) (Fig. 2F, Table 3). A Cys to Ser substitution in 14s21 peptide (14s21-Ser) did not have any significant difference over their binding affinity towards RXFP3 receptor. Interestingly, it was not as potent as Cys containing 14s21 peptide. Competition binding assay revealed that despite a significant enhancement in helicity profile of 11s18 peptide, it merely displayed the binding affinity of 331 nM (Fig. 2C). Consistent with binding affinity, 11s18 also displayed weak cAMP inhibitory activity with an EC_{50} value of 17 nM. However, the cAMP activity of 11s18 peptide was significantly higher than unmodified H3 B-chain peptide ($p < 0.05$) (Fig. 2F, Table 3).

Consistent with the superior binding affinity and enhanced cAMP activity the 14s21 peptide displayed over other H3 relaxin stapled analogs, we further determined the ability of 14s21 peptide to activate ERK1/2 kinase phosphorylation activity in HEK-RXFP3 cells and compared it to H3 relaxin. The 14s21 peptide stimulated ERK1/2 kinase phosphorylation in a dose-dependent fashion similar to H3 relaxin and there was no significant difference in the potency of 14s21 peptide in comparison to H3 relaxin [H3

relaxin, $pEC_{50} = 10.04 \pm 0.12$ ($n=3$) and 14s21 peptide, $pEC_{50} = 9.74 \pm 0.09$ ($n = 3$)] (Fig. 2G).

To examine the effect of stapling on conformational preference and structural stability, we tested 14s18 (*i, i+4*) and 14s21 (*i, i+7*) stapled peptides for resistance to thermal denaturation. In thermal unfolding experiments, both the *i, i+4* and *i, i+7* stapled peptides underwent a cooperative melting transition with an approximate melting temperature (T_m) range of 50-55°C (Fig. 2H). A linear dependency of the ellipticity values as a function of temperature at 222 nm was observed for both heating and cooling cycles. At higher temperature, the increase in CD intensity in the 215-230 nm range indicated the unfolded 14s21 stapled peptide becomes partially folded. The linear dependency also indicated the less energy difference between the two states. Thus, VT-CD (variable temperature-circular dichroism) results indicated that there was the lower assembly in the transition of unfolding, indicating the high peptide stability. The H3 B-chain did not have a cooperative melting transition point in this temperature range, and therefore its melting temperature was indeterminable by this method.

The stability against proteolytic degradation is an important feature of biologically active peptides. To measure the comparative protease susceptibility of unmodified H3 B-chain peptide, *i, i+4* stapled 14s18 and *i, i+7* stapled 14s21 peptide, they were subjected to trypsin digestion. Peptide digestion followed by LC-ESI-MS/MS (liquid chromatography-electrospray ionization tandem mass spectrometry) analysis on the individual peptides revealed the protein identity after the tryptic digestion over the time period of 25 hours. Stapling at *i, i+4* conferred up to a 1.6-fold enhancement in peptide half-life compared with the unmodified peptide, whereas *i, i+7* stapled analogs manifested a 4.5-fold improvement in proteolytic resistance (Fig. 2I). In this case, the *i, i+7* stapled peptides displayed superior advantage towards the proteolytic resistance of the corresponding *i, i+4*-stapled peptides. From a mechanistic standpoint, the longer *i, i+7* staple not only slowed the kinetics of proteolytic digestion but completely eliminated cleavage of two arginine sites that either localized within the protective umbrella of the staple or was immediately adjacent to it (Fig. 8).

Taken together, these data demonstrated that *i*, *i*+7 stapling optimizes the α -helicity and protease resistance, yielding a structurally stable, high-affinity and most potent agonist ligand of the RXFP3 receptor.

EXAMPLE 4

5 *Effect of central administration of H3 relaxin and 14s21 peptide on food, water intake and related behavior in rats*

To determine whether the *in vitro* activity of 14s21 peptide can be translated to an *in vivo* context, the hypothesis that H3 relaxin is involved in regulation of food intake after central administration of H3 relaxin was tested. Relaxin-3 (H3) which is known to
10 be an orexigenic peptide produces dose-dependent effects in the rats. SD rats received an acute intracerebroventricular injection (ICV) of either vehicle or H3 relaxin (1 nmol) in the early light phase. Doses used were based on previously reported effects of H3 relaxin on food and water intake studies [Otsubo et al., *Peptides* 31: 1124-1130 (2010)]. Relaxin-3 (1 nmol) was also compared to the most potent stapled peptide in the present invention,
15 14s21 (1 nmol). A single ICV injection of a high dose of relaxin-3 (H3) to satiated male SD rats significantly increased food intake in the first hour post-administration [0-1 h food intake: 4.64 ± 0.23 g (1 nmol H3); $p < 0.001$ vs vehicle, 2.17 ± 0.17 g (1 nmol 14s21 peptide); $p < 0.01$ vs vehicle, N = 6 rats/group]. There was a significant difference in the interval food intake between vehicle and treated groups which was also seen at later
20 time points (Figs. 9A-9C). The effect of H3 relaxin (1 nmol) and 14s21 peptide (1 nmol) on drinking behavior paradigm was compared. ICV relaxin-3 (1 nmol) increased the drinking behavior immediately after the drug administration [0-1 h water intake: 3.54 ± 0.36 g (1 nmol H3); $p < 0.001$, 1.83 ± 0.31 g (1 nmol) 14s21 peptide); $p < 0.001$, N = 6 rats/group]. Other parameters like grooming, locomotion and total distance travelled,
25 were analyzed, and were found to be significantly changed after the peptide treatment (Figs. 9D-9H). Further, feeding behavior equivalent to their optimal pEC_{50} values was compared. The optimal pEC_{50} values reported for 14s21 stapled peptide (8.51 ± 0.07) was approx. 10-fold lesser than relaxin-3 pEC_{50} values (9.28 ± 0.15) so the equivalent dose of relaxin-3 (100 pmol = 0.1 nmol) was used to compare the equivalent activity
30 reported by 14s21 stapled peptide (1 nmol). Single ICV administration of 14s21 peptide to satiated rats in early light phase significantly increased the food intake within the first

hour after injection ($p < 0.001$ vs Vehicle) (Fig. 3C). The amount consumed was not statistically different to that following the same molar concentration of H3 relaxin [0-1 h food intake: 0.84 ± 0.07 g (vehicle), 3.74 ± 0.1 g (H3 relaxin- 0.1 nmol); $p < 0.001$ vs vehicle and 3.41 ± 0.15 g (14s21 peptide- 1 nmol); $p < 0.001$ vs vehicle, $N = 6$ rats/group].

5 Cumulative food intake was significantly increased at 2, 3, 4 h post ICV administration of H3 and 14s21 peptide (Fig. 3C). Water intake was significantly increased 15-120 min after icv injection of H3 relaxin (0.1 nmol) and 25-80 min post icv injection of 14s21 peptide (1 nmol), the effect exerted by both the peptides was found to be statistically significant compared with vehicle. Cumulative water intake was significantly increased

10 2, 3, and 4 h post ICV injection of H3 relaxin and 14s21 peptide (Fig. 3C). Apart from feeding and drinking behavior analyzed, two-way ANOVA analysis with 10 min time bins up to 4 h in LABORAS home-cage environment revealed that there was significant difference observed (versus vehicle) in time spent in eating behavior ($F_{23, 360} = 27.78$, $p < 0.001$), time spent in drinking behavior ($F_{23, 360} = 26.81$, $p < 0.001$), time spent in grooming ($F_{23, 360} = 3.807$, $p < 0.001$), time spent in rearing ($F_{23, 360} = 17.09$, $p < 0.001$),

15 time spent in locomotion ($F_{23, 360} = 31.58$, $p < 0.001$) and total distance travelled ($F_{23, 360} = 18.34$, $p < 0.001$) (Figs. 3F-3K). One-way ANOVA, followed by Tukey's post-hoc conducted on the first 80 min data indicated that the peptide treatment has significant effect in time spent in eating (H3 relaxin; $p < 0.001$ and 14s21 peptide; $p < 0.001$), time

20 spent in drinking (H3 relaxin; $p < 0.001$ and 14s21 peptide; $p < 0.001$), time spent in grooming (H3 relaxin; $p < 0.001$, and 14s21 peptide; $p < 0.01$), time spent in rearing behavior (H3 relaxin; $p < 0.001$ and 14s21 peptide; $p < 0.001$), time spent in locomotion (H3 relaxin; $p < 0.01$, and 14s21 peptide; $p < 0.05$) and total distance travelled (H3 relaxin; $p < 0.05$) All the comparisons were made to vehicle treatments. (Inset graph, 3d-

25 i to 3d-vi). Studies revealed the causal role for the NI in locomotion and behavioral activation by employing high frequency electrical microstimulation. However, the anorectic effects of H3 relaxin may be secondary to other altered behavior such as grooming or drinking. Appetite, regulated by central and peripheral mechanisms is dysregulated in various disorders such as anxiety, depression which are associated with

30 derangements in levels of neuropeptides, neurotransmitters (Dopamine and 5-HT) including insulin, ghrelin, orexins and NPY. Intranasal delivery could be one possible alternative of bypassing the brain barrier and promising delivery route of administration

of drug compounds based on the recent studies with insulin, oxytocin/vasopressin and NPS.

EXAMPLE 5

Evaluation and optimization of intranasal drug delivery of H3 relaxin and 14s21 peptide in rats

The inventors optimized the experimental factors, such as head position, volume and method of administration which can influence the drug deposition within the nasal passage and pathways involving the CNS [Dhuria et al., *J Pharm Sci* 98: 2501-2515 (2009)].

In preliminary experiments, several concentrations of H3 relaxin (1, 10, 100 and 1000 nmol) were intranasally administered. Dose-response studies revealed, that there was significant effect of treatment (Feeding behavior: Significant effect of the H3 treatment, One-way ANOVA, $F_{4, 25} = 29.77$, $p < 0.001$, Drinking behavior: Significant effect of H3 treatment, One-way ANOVA, $F_{4, 25} = 11.98$, $p < 0.001$, Fig. 4B). Post-hoc Tukey's test revealed that only 100 nmol ($p < 0.001$ vs vehicle) and 1000 nmol ($p < 0.001$ vs vehicle) was found to be significantly effective in stimulating the feeding and drinking behavior, whereas 10 nmol of relaxin-3 ($p < 0.05$ vs vehicle) was also able to elicit the drinking behavior within first 80 min post drug administration. An optimal 100 nmol dose of relaxin-3 was chosen to analyze the effect on related behavior paradigms. Relaxin-3 (100 nmol = 0.1 μ mol) was delivered intranasally to rats and their behavior in LABORAS was analyzed. First maximal effect of activity in feeding behavior was produced in the 10 min of post drug administration ($p < 0.001$ vs vehicle) (Fig. 4D). The effect on eating duration elicited by relaxin-3 (100 nmol) was highest at the 40th min after drug administration ($p < 0.0001$ vs vehicle). After 40th min, drug effects seem to start declining and comes down to the basal level as vehicle animals. The total effect produced in 4-hour duration on feeding was analyzed in LABORAS and was statistically significant compared to that to vehicle animals (Significant effect of interaction of Time X Treatment, $F_{46, 230} = 13.16$, $p < 0.001$, Fig. 4D). After optimization of behavior parameters with relaxin-3 in LABORAS, similar dose-response relationship studies were done for the 14s21 peptide (0.1, 1, 10 and 100 μ mol). It was found that there is a significant effect of

treatment (Significant effect of treatment, $F_{4,25} = 33.8$, $p < 0.001$, Fig. 4C), post-hoc Tukey's test revealed that there is a significant increase in feeding behavior was observed with 1 μmol ($p < 0.05$ vs vehicle), 10 μmol ($p < 0.001$ vs vehicle) and 100 μmol ($p < 0.001$ vs vehicle). However, 14s21 peptide was also able to elicit the drinking behavior but only at 10 μmol concentration and was not as potent as H3 relaxin (Fig. 4C). Unlike H3 relaxin, onset of feeding behavior by 14s21 peptide was produced 20 min after the drug administration and it reached to its peak level at 50th min. Interestingly, the effect of 14s21 peptide lasted longer than H3 relaxin analyzed in the home-cage behavior paradigm. After successful dose optimization, detailed behavior analysis of 100 nmol (0.1 μmol) of H3 relaxin and 10 μmol of 14s21 peptide in LABORAS home-cage environment was analyzed. The activity of H3 relaxin and 14s21 peptide was analyzed over 4 h in LABORAS paradigm (Figs. 4D-4I) and total mean values over 80 min after IN drug administration was recorded (insets in Figs. 4D-4I). Two-way ANOVA on the data across time showed significant changes in feeding behavior ($F_{23, 360} = 70.86$, $p < 0.001$), drinking behavior ($F_{23, 360} = 33.1$, $p < 0.001$), rearing duration ($F_{23, 360} = 14.56$, $p < 0.001$) and locomotion ($F_{23, 360} = 25.31$, $p < 0.001$). There was an overall significant difference in grooming behavior ($F_{23, 360} = 31.47$, $p < 0.001$) and total distance travelled ($F_{23, 360} = 33.76$, $p < 0.001$) was observed across the entire 4 h paradigm. The peak activity analyzed over first 80 min duration showed that both H3 relaxin and 14s21 peptide could elicit the feeding behavior significantly within first 80 min post drug administration. One-way ANOVA on the data across first 80 min time showed significant difference between the treatments compared to vehicle, time spent in feeding ($F_{2, 15} = 83.58$, $p < 0.001$), Tukey's post-hoc multiple comparison analysis revealed, both H3 relaxin and 14s21 peptide elicited the feeding behavior significantly ($p < 0.001$) compared to vehicle animals. Drinking behavior was found to be stimulated more potently by H3 relaxin ($p < 0.001$) rather than 14s21 peptide ($p < 0.05$) compared to the vehicle animals, and there was a significant difference between the treatments (One-way ANOVA, $F_{2, 15} = 11.19$, $P = 0.0011$). There was a significant overall effect of the treatment of H3 relaxin and 14s21 peptide on time spent in rearing ($F_{2, 15} = 5.111$; $p < 0.05$) and total distance travelled ($F_{2, 15} = 9.494$, $p = 0.01$) was found to be significantly affected with H3 relaxin and 14s21 peptide treatment. Other parameters, time spent in grooming ($F_{2, 15} = 2.778$, $P = 0.0941$) and time spent in locomotion ($F_{2, 15} = 1.072$, $P = 0.3673$) were not found to be significantly affected by either peptide treatment. When the behavior was analyzed for both the

peptides over a 4 h time period, it was shown that the effect of the 14s21 peptide lasted longer than the H3 relaxin peptide in the feeding paradigm.

Previous studies have demonstrated the sex specific effects on food intake by H3 relaxin when given intracerebroventricularly; the significantly higher food intake and more body weight gain in response to higher dose of neuropeptide in female rats compared to male rats (Calvez et al., 2016 *Br J Pharmacol*, doi: 10.1111/bph.13530). The effect of intranasal administration of H3 relaxin (0.1 μ mol) and 14s21 peptide (10 μ mol) in feeding and drinking behavior was analyzed and compared in male and female SD rats in a LABORAS home-cage over 2 h time duration. There was no sex specific difference observed between the male and female SD rats on feeding behavior after the intranasal administration of H3 relaxin and 14s21 peptide, significant effect of interaction between sexes ($F_{2, 30} = 0.1383$, $P = 0.8714$), however drinking behavior was elicited slightly more by H3 relaxin than by 14s21 peptide in all the experiments, but the effect was not found to be statistically different between the sexes, significant effect of interaction between sexes ($F_{2, 30} = 0.3876$, $P = 0.6821$) (Fig. 4J).

After the successful optimization and establishment of intranasal administration of H3 relaxin and 14s21 peptide and with the required evidence pertinent to metabolism/eating disorders, those behavior models which are more relevant and potentially translatable to the aetiology and treatment of major depression and anxiety disorders were optimized.

EXAMPLE 6

Intranasal treatment of H3 relaxin and 14s21 peptide in anxiety-related behavior paradigms

Animals were intranasally infused with either H3 relaxin (0.1 μ mol) and 14s21 peptide (10 μ mol) or vehicle. The optimal response time window of H3 relaxin and 14s21 peptide when the maximal activity was recorded in LABORAS was chosen. Thus 30 min after the H3 relaxin and 14s21 peptide administration, the behavior of rats on elevated zero maze, light-dark box, open field test and novelty induced suppression of feeding test was recorded. One-way ANOVA followed by Tukey's post-hoc test was conducted on all the following data unless otherwise specified. All the comparisons were made

compared to vehicle. H3 relaxin infused intranasally significantly affected the percentage time spent in the open arms ($p < 0.05$, Fig. 5B) and the number of entries made in the open arms ($p < 0.05$, Fig. 5B-ii) and total number of transitions made from closed to open arms ($p < 0.05$, Fig. 5B-iii) compared to vehicle animals. However, the effect of 14s21 peptide was found to be highly significant ($p < 0.01$ vs Vehicle) in all the above parameters analyzed in EZM (Fig. 5B i-iii). The number of head dips made during the exposure to EZM was counted. The number of head dips, which is another index of anxiety was found to be significantly increased by both the peptide treatments. H3 relaxin significantly increased the head dips ($p < 0.05$), whereas the effect of 14s21 peptide was highly significant ($p < 0.001$) compared to the vehicle animals (Fig. 5B-iv). Additionally, a heat-map plot of rat position and time spent in the open and closed arms also provided the high-throughput characterization of behavior through Ethovision software detection algorithms. The heat-map indicates the cumulative time spent in different parts of the maze and thus provides a useful variable to inspect rat preferences between open and closed arms after the treatment with H3 relaxin, 14s21 peptide and vehicle during the test (Fig. 5A). In light-dark box, H3 relaxin and 14s21 peptide displayed the significant time spent in the light zone ($p < 0.05$, Fig. 5D-i) and the number of entries made in the light zone was highly significant by 14s21 peptide- ($p < 0.01$) and H3 relaxin- ($p < 0.05$, Fig. 5D-ii) treated rats. Using the automated tracking of the rat centroid, the amount of time spent and the distance travelled in the different light and dark areas of the light-dark box after the treatment of H3 relaxin and 14s21 peptide were computed (Fig. 5D-iii). In the open field exploration paradigm, H3 relaxin showed the significant time spent in center ($p < 0.05$, Fig. 5F-i) and latency to reach to center ($p < 0.01$, Figure 5F-ii), whereas 14s21 peptides also showed the significant time spent in the center ($p < 0.01$, Fig. 5F-i), latency to reach to center ($p < 0.05$, Fig. 5F-ii), velocity ($p < 0.01$, Fig. 5F-iii) and total distance travelled was found to be significantly increased ($p < 0.01$, Fig. 5F-iv) compared to the vehicle animals. However, there was no significant difference in velocity and total distance travelled by H3 treatment was observed in the animals. Heat-map represents the cumulative time spent in the arena after vehicle, H3 relaxin and 14s21 peptide treatment in open field test paradigm (Fig. 5E). In novelty induced suppression of feeding test, both H3 relaxin and 14s21 peptide displayed the significant time spent in the center (H3 relaxin; $p < 0.05$ and 14s21 peptide; $p < 0.01$, Fig. 5H-i). Both H3 relaxin and 14s21 peptide significantly increased the latency to reach food in

the center ($p < 0.05$, Fig. 5H-ii). 14s21 peptide also displayed the significant increase in velocity analyzed in this behavior paradigm ($p < 0.05$, Fig. 5H-iii). However, there was no significant difference in total distance travelled between the peptides. Corresponding heat maps represent the animal behavior and cumulative time spent in the experimental arena after the treatment with vehicle, H3 relaxin and 14s21 peptide. Thus, both H3 relaxin and 14s21 peptide showed a significant anxiolytic effect in the behavior paradigms, however the effect of 14s21 peptide performed slightly better than the H3 relaxin.

EXAMPLE 7

10 *Antidepressant behavior of H3 relaxin and 14s21 peptide in repeat rat FST*

The antidepressant behavior analysis of H3 relaxin and 14s21 peptide in the test, retest 1 and retest 2 of the repeat rat Forced Swim Test (FST) was studied. The inventors used a modified protocol of the FST using within subject design. SD rats were submitted to 15 min of training (Day1: pretest) followed by three subsequent 5 min-swimming tests one week apart (Day 2: test, Day 7: retest 1, Day 14: retest 2) (Fig. 6A). To validate the methods, daily intranasal administration of H3 relaxin (0.1 μ mol) and 14s21 peptide (10 μ mol) or vehicle was given over a two-week period. Tests and retests were further scored for the duration of immobility, swimming and climbing behaviors. On the first day of training, H3 relaxin and 14s21 peptide treated animals showed the slight decrease in immobility (One-way ANOVA, $F_{2,15} = 2.224$, $P = 0.1426$) and swimming behavior (One-way ANOVA, $F_{2,15} = 2.682$, $P = 0.1010$) and slight increase in the climbing behavior (One-way ANOVA, $F_{2,15} = 3.109$, $P = 0.0742$) but the effect did not reach a statistically significant level (Fig. 6B). After 24 hours of training, rats were subjected to a 5 min test procedure in FST [Test – Acute study]. Both H3 relaxin and 14s21 peptide showed a significant decrease in the immobility (One-way ANOVA, $F_{2,15} = 11.16$, $p < 0.01$, Tukey's HSD, H3 relaxin; $p < 0.05$, 14s21 peptide; $p < 0.01$) (Fig. 6C-i). H3 relaxin and 14s21 both induced a significant increase in the climbing behavior (One-way ANOVA, $F_{2,15} = 14.3$, $p < 0.001$, Tukey's HSD, H3 relaxin; $p < 0.001$, 14s21 peptide; $p < 0.01$) (Fig. 6C-ii). In retest 1 [Retest1- Subacute study], the duration of immobility was further decreased significantly by H3 relaxin and 14s21 peptide (One-way ANOVA, $F_{2,15} = 80.23$, $p < 0.001$, Tukey's HSD, H3 relaxin and 14s21 peptide; $p < 0.001$, Fig. 6D-i). Climbing behavior

was also found to be significantly increased (One-way ANOVA, $F_{2,15} = 14.82$, $p < 0.001$, Tukey's HSD, H3 relaxin; $p < 0.01$, 14s21 peptide, $p < 0.001$) (Fig. 6D-ii). In retest-2, chronically administered H3 relaxin did not have any significant effect in duration of immobility but the effect of 14s21 peptide was significant in reducing the immobility duration of the animals (One-way ANOVA, $F_{2,15} = 5.284$, $p < 0.05$, Tukey's HSD, 14s21 peptide; $p < 0.05$ (Fig. 6E-i). Chronically administered H3 relaxin and 14s21 peptide also increased the climbing behavior significantly (One-way ANOVA, $F_{2,15} = 14.52$, Tukey's HSD, H3 relaxin; $p < 0.01$, 14s21 peptide; $p < 0.001$ (Fig. 6E-ii). There was no significant difference in swimming behavior elicited by either H3 relaxin or 14s21 peptide observed in the entire repeat FST paradigms.

Discussion

The chemical capability to produce an optimally effective stapled peptide agonist of relaxin-3 B-chain as well as the increased efficacy of this agonism in CNS-targeted drug delivery in rodent models of anxiety and depression was studied. The inventors demonstrate, for the first time, that rapid delivery of relaxin-3 and stapled 14s21 peptide to the brain by intranasal infusion has a pronounced resilient effect and has a huge potential to be developed as a potent anxiolytic and antidepressant therapeutic drug. Hydrocarbon stapling technology to fortify lengthy peptides, has revitalized the efforts to optimize natural peptides for therapeutic application. The α -helix is the most abundant secondary structure in proteins and is a frequent participant in mediating important protein-protein interactions. Synthetic strategies to enforce the α -helical conformation in the peptides have aroused considerable interest as a means of generating potential therapeutics for exploring protein-protein interactions. The incorporation of a $i, i+4$ staple, an all-hydrocarbon cross-link flanked by α -methyl groups along one face of H3 B-chain peptide can greatly increase its helix content, binding affinity and potency to activate the RXFP3 receptor (Hojo et al., *J Med Chem* 59: 7445-7456 (2016); Jayakody et al., *Peptides* 84: 44-57 (2016)]. The inventors have shown herein that simply making a peptide helical may not guarantee optimal affinity and potency for the receptor and both the peptide sequence and stapling methodology are critical. From structural activity relationships and molecular modelling, the inventors found that 11s18 and 14s21 can be two $i, i+7$ stapling strategies in H3 B-chain to optimize for increased α -helicity and *in vitro* efficacy. Surprisingly, an $i, i+7$ stapled 14s21 peptide dramatically stabilized the α -helical

conformation of a H3 B-chain sequence, leading to a high-affinity ligand with approximately 300-fold increase in the potency of activating the RXFP3 receptor. A Cys to Ser substitution in 14s21 peptide revealed no significant change in the binding affinity and activity to RXFP3 receptor, however an increased binding and activity profile seen with the Cys containing 14s21 peptide may further underscore the importance of this amino acid in the structure-activity relationship of relaxin-3 peptide. Interestingly, conformational analysis revealed that the 11s18 peptide has similar α -helical content as 14s21 peptide but it was not found to be as potent as 14s21 peptide in *in vitro* assays of binding and activation, suggesting that the edge of N-terminus helix, may not be able to provide a significant interaction with RXFP3 receptor. The incorporation of lactam and disulfide (14s18-lactam, 14s18-disulfide) did not provide any improved means over stapled peptides, and they were as similar as relaxin-3 B-chain random coils consistently showing that these substituted versions were not able to mimic the improved binding affinity and activity seen with the hydrocarbon staple. The data presented here, taken together with data reported previously in the literature, help to formulate a consensus view on the helix stabilization and *in vitro* activity by a panel of hydrocarbon-stapled peptides. The extent of helix stabilization by both *i, i+4* and *i, i+7* staples varies from peptide to peptide, and from position to position within a given peptide sequence. Computational modelling simulations of stapled peptides have indicated that existence of quasi-stable “dummy” states in the unfolded peptide diminishes helix stability, as these states are not trivial to predict, synthesis and *in vitro* screening of panels of staple-permuted peptides appears to be the most efficient route to identify a candidate having optimal receptor affinity and efficacy.

The stability against the proteolytic degradation is an important feature of hydrocarbon-stapled peptides. Since proteases bind peptides in an extended conformation rather than in helical conformation, the peptides cross-linked by longer *i, i+7*, at positions 14 and 21 in relaxin B-chain exhibited enhanced resistance to proteolysis compared to the *i, i+4* 14s18 stapled peptide. The effect of stapling on the structural stability and conformational preference in terms of thermal denaturation was also studied. VT-CD (variable temperature-circular dichroism) results indicated that there was the lower assembly in the transition of unfolding, indicating the high peptide stability.

The linear dependency further also indicates the less energy difference between the folded and unfolded states.

To further assess whether the optimal profile of 14s21 achieved with in-vitro analysis can be translated into in vivo assays, the initial screening of in vivo tests with different concentrations of relaxin-3 in well characterized rat feeding model was developed and optimized. The in vivo activity of 14s21 stapled peptide was determined in order to learn whether it will be able to mimic the effect of relaxin-3 in feeding and drinking behavior. ICV administration of 14s21 lead to a significant increase in food intake and drinking behavior post 1 h administration of drug infusion and the effect was not statistically different from relaxin-3 (0.1 nmol). It is possible that the expression of other hypothalamic neuropeptides might change after administration of relaxin-3 at different doses. There are several lines of evidence to suggest that the cross reactivity or relaxin-3 towards RXFP1 may be the reason for decreased selectivity towards the feeding behavior and the circumventricular organs may mediate the central actions of relaxin on drinking behavior as reported in previous studies. 14s21 peptide demonstrated similar properties to relaxin-3 in vitro and in vivo showing evidence that 14s21 is a full agonist for rat RXFP3.

Grooming and rearing behavior, typified as complex patterned behavior, are mediated by multiple brain regions (especially the basal ganglia and hypothalamus), as well as by various endogenous agents (neuromediators), hormones, and psychotropic drugs [Enginar et al., *Pharmacol Biochem Behav* 89: 450-455 (2008)]. Given the robust nature of grooming and rearing behavior in animal phenotypes, it is logical to assume the alternations in this domain will be seen in experimental rodent models of stress, anxiety and depression. Grooming and rearing behavior elicited by acute ICV injection of relaxin-3 may also suggest a role for the 5-HT2A receptor in the modulation of D1 receptor function.

There are serious limitations with such pharmacological studies, including the difficulty in synthesizing these complex peptides, their likely rapid metabolism- short half-life in the circulation and possibly also in the brain and the requirement of surgical implantation of cannula into the brain, making it unlikely to effectively deliver across the blood-brain barrier. To overcome at least some of the limitations stated above, the

inventors investigated the possibility of using intranasal delivery as one possible alternative of bypassing the brain barrier.

In the intranasal route of drug administration, aerosolized insulin in humans and insulin solutions in animal models has proven to use an identical route to deliver the hormone into rostral brain structures without reaching the blood serum that would elicit the systemic side effects. Due to close structural similarity between relaxin family peptides and insulin, we further attempted to employ the intranasal delivery route for the relaxin-3 and 14s21 stapled peptide.

Our findings reported the optimal dose of intranasal relaxin-3 to be administered and confirmed that centrally as well as intranasally administered relaxin-3 has an orexigenic effect. Relaxin-3 at 100 nmol (0.1 μ mol) dose significantly increased the food intake post 1 h after the drug administration, which lasted till 40 – 60 min after the drug administration and then returned to the basal activity level. Of significance has been the observation that the presence of a staple can also promote cellular uptake by the intranasal pathway that is remarkably permissive towards diversity in sequence, length and the staple location. Unlike relaxin-3, the activity of 14s21 peptide to influence behavior was not quick and although the optimal activity was seen only post 25-30 minutes after the drug administration the behavioral effect lasted longer compared to the relaxin-3. Behavior analyzed within the first 80 min post 14s21 peptide drug administration showed a significant increase in feeding, drinking and locomotion and rearing behavior. The mechanism by which stapled peptides trigger their own vesicular transport is poorly understood at present, as are the structural features in these peptides that are responsible for uptake. Relaxin-3 as well as 14s21 peptide both offered their unique advantages in ability to deliver the peptide across the brain. On the one hand, the relaxin-3 peptide onset of activity was quicker than the 14s21 peptide, but the optimal effect was only found to last for a maximum 60-80 min, after which the drug activity fell to its basal level. On the other hand, the onset of 14s21 peptide effects appeared 25 min after the drug administration but lasted for a maximum of 80-120 min before falling to basal level. To the best of our knowledge, we combined peptide stapling and nasal delivery technology for the first time in the relaxin-3/RXFP3 system, to maximize the brain exposure via a non-invasive route.

An orexigenic action exerted by H3 relaxin and 14s21 peptide is consistent with the evidence that hypothalamic structures mediate the effects of neuropeptides on food intake and thus presents a paradigm in which to explore effects of these agonist peptides in management of hyperphagia associated with depression and related anxiety disorders. We further tested the potential of H3 relaxin and 14s21 peptide in association with anxiety and depression related behavioral paradigms. After intranasal drug administration, we allowed the drug to reach the CNS for an adequate period and ran the behavior analysis at the time when the optimal activity of the peptides could be seen. The behavioral activity observed in LABORAS was a clear indication to choose the optimal time duration for H3 relaxin and 14s21 peptide, i.e. 30 min after the drug administration.

In the elevated zero maze test, intranasal administration of H3 relaxin and 14s21 peptide produced a clear anxiolytic effect in the EZM, including an increase in time spent in the open arms, increased percentage of open arms entries and transitions made from closed to open arms, which are considered measures of anxiety-like behaviors. The number of head dips, which has also been regarded as increased anxiolytic activity in rats, was found to be significantly increased by H3 relaxin and 14s21 peptide treatment. However, the effect of 14s21 peptide was more effective than H3 relaxin in the EZM paradigm.

In the light-dark box test there was a significant increase in time spent in light zone of LDB box and the entries made in the light zone by both intranasal administered H3 relaxin and 14s21 peptide, demonstrating a clear anxiolytic activity. The effect of 14s21 peptide was significantly more effective, marked by more entries made to the light zone compared to vehicle animals. The 14s21 peptide may offer a slight advantage over H3 relaxin in certain behavior paradigms like in EZM and LDB.

In the open field test, the latency to reach the center was more significantly reduced in H3 relaxin treated animals compared to the 14s21 peptide treated ones. But the locomotion activity was found to be significantly increased by 14s21 treated animals, which may support a role of relaxin-3/RXFP3 system involvement in preparing behavioral strategies to escape from stressful situations, like the role of "nucleus incertus".

In the novelty-suppressed feeding test (NSFT), the time spent in the center was significantly increased by H3 relaxin and 14s21 peptide treatment. There was a significant increase in the velocity of 14s21 treated animals and H3 relaxin treated animals, recorded in this paradigm. The increased locomotion behavior along the
5 anxiolytic profile may be dose-dependent and provide a robust effect particularly of 14s21 peptide in novelty-suppressed feeding paradigm suggesting that it could serve as a potent anxiolytic alternative to the benzodiazepines.

These preclinical data suggested that drugs that activated RXFP3 might have potential anxiolytic as well as antidepressant action. To investigate the potential of
10 antidepressant actions of H3 relaxin and 14s21 peptide, two classical acute and chronic models of depression were used in this study.

Administration of H3 relaxin and 14s21 peptide reduced the duration of immobility of rats in the test. There was a significant increase in climbing behavior observed by the treatment with H3 relaxin and 14s21 peptide. However, no significant difference in
15 swimming behavior was observed. In retest-1, rats re-exposed to the FST had even higher significance in reduction of immobility behavior after treatment with H3 relaxin and 14s21 peptide, accompanied by a significant increase in climbing behavior, which would be consistent with observations on the effects of an antidepressant treatment. In every exposure to the FST the immobility was the most frequently scored category whereas
20 diving was seldom observed irrespective of the swimming session. After 7 days of subacute treatment of H3 relaxin and 14s21 peptide, the re-exposure to FST changed the behavioral strategy to cope with stressor more efficiently, favouring behavioral activation that could facilitate that anti-immobility effects of antidepressant drugs in the rat FST. The acute or subacute treatment with H3 relaxin and 14s21 peptide did not
25 affect the behavioral score to favour behavioral withdrawal or behavioral despair, seen with decreased duration of immobility in repeat FST. However, the chronic treatment with these drugs further increased the immobility compared to the previous exposures. The antidepressant components were those loading the variable "duration of immobility" which is sensitive to the treatment with different kinds of antidepressants. The first
30 component is classified as "putative serotonin-noradrenaline reuptake inhibitor (SNRI)-like" because it also includes the variable "duration of climbing" that is commonly affected by the treatment with SNRI. The loading of duration of "swimming" led to the classification

of the second component as putative SSRI. Although both serotonergic and noradrenergic antidepressants reduce the duration of immobility in this test, selective serotonin reuptake inhibitors (SSRIs) increase “swimming” behavior, whereas selective NE reuptake inhibitors (SNRIs) increase “climbing” behavior. The third component
5 (putative motor) comprised of variables related to the locomotor activity such as frequency of immobility and frequency of climbing [Mezadri et al., *J Neurosci Methods* 195: 200-205 (2011)]. Moreover, both H3 relaxin and 14s21 peptide, as the “putative SNRI-like” component explained most of the total variance in the analysis of the test, retest 1 and retest 2.

10 Norepinephrine produced primarily by neurons in the locus coeruleus and other nuclei with noradrenergic neurons receive afferent projections from the central amygdala, bed nucleus of stria terminalis, hypothalamus, nucleus of solitary tract and cerebral cortex. In addition, relaxin-3 immunoreactive fibers and RXFP3 mRNA are highly concentrated in brain regions involved in stress response and anxiety-like
15 behavior, including the amygdala, BNST and hypothalamic PVN, strongly implicating the interactions between serotonin (5-HT) and CRF signaling [Keck, *Amino Acids* 31: 241-250 (2006)] implicating in the aetiology of anxiety and depression. These pharmacological effects might be mediated by actions in central and medial amygdala, which is largely responsible for conferring anxiety-related symptoms that are commonly
20 experienced during depression. Furthermore, drugs that modify the monoaminergic signaling and metabolism have been widely used for many years as treatments of psychiatric disorders, such as anxiety, PTSD, schizophrenia and autism. Although there is a wealth of data showing multiple types of interactions between noradrenergic and serotonergic neurons [Dale et al., *Biochem Pharmacol* 95: 81-97 (2015)], the possibility
25 that H3 relaxin and 14s21 peptide may activate systems other than serotonergic and noradrenergic cannot be denied. Drugs that increase DA release have also been reported to increase climbing behaviors in the FST. Since both H3 relaxin and 14s21 peptide have been shown to display superior anxiolytic effects and also modulate noradrenergic-like mechanisms of antidepressant action, that these molecules might
30 play a role in antidepressant-like effects is consistent with current theories linking anxiety and depression.

In summary, this study showed that acute, subacute and chronic intranasal administration of both H3 relaxin and 14s21 peptide have antidepressant-like effects. Either serotonin or norepinephrine, or both, are involved in its anxiolytic-like effect in the anxiety-related behavioral paradigms, whereas norepinephrine seems to be more necessary for its antidepressant-like effect in the FST. That relaxin-3 and 14s21 can activate both neurotransmitter systems distinguish it from drugs activating primarily a single system (i.e., SSRIs or selective NRIs) and makes it somewhat more similar to high doses of venlafaxine or monoamine oxidase inhibitors. Such dual-acting serotonin/norepinephrine reuptake inhibitors like venlafaxine (Effexor) are reported to exhibit a faster clinical onset of action and be more effective in treating depression that is refractory to other types of antidepressants. To maximize the optimal response of the administered compound, it would be beneficial to target it to areas of the brain where it could exert the most significant effect on cognition, such as the hippocampus, and away from areas where it might exert unwanted actions.

Altogether the present findings suggest that 14s21 peptide will serve as a potentially useful tool for further probing the importance of relaxin-3/RXFP3 system in emotional processes, and represent a novel strategy for the treatment of major depressive and anxiety disorders.

References

Any listing or discussion of an apparently prior-published document in this specification should not necessarily be taken as an acknowledgement that such document is part of the state of the art or is common general knowledge.

- 5 Berge et al., Pharmaceutical Salts. *J. Pharmaceutical Sciences* 66:1-19 (1977).
- Berridge, C. W., Schmeichel, B. E., Espana, R. A., 2012. Noradrenergic modulation of wakefulness/arousal. *Sleep Med Rev* 16, 187-197.
- 10 Bird, G. H., Madani, N., Perry, A. F., Princiotta, A. M., Supko, J. G., He, X., Gavathiotis, E., Sodroski, J. G., Walensky, L. D., 2010. Hydrocarbon double-stapling remedies the proteolytic instability of a lengthy peptide therapeutic. *Proc Natl Acad Sci USA* 107, 14093-14098.
- 15 Blackwell, H. E., Sadowsky, J. D., Howard, R. J., Sampson, J. N., Chao, J. A., Steinmetz, W. E., O'Leary, D. J., Grubbs, R. H., 2001. Ring-closing metathesis of olefinic peptides: design, synthesis, and structural characterization of macrocyclic helical peptides. *J Org Chem* 66, 5291-5302.
- 20 Bryn et al., *Solid-State Chemistry of Drugs*, Second Edition, published by SSCI, Inc of West Lafayette, IN, USA, 1999, ISBN 0-967-06710-3.
- Burazin, T. C., Bathgate, R. A., Macris, M., Layfield, S., Gundlach, A. L., Tregear, G. W., 2002. Restricted, but abundant, expression of the novel rat gene-3 (R3) relaxin in the dorsal tegmental region of brain. *J Neurochem* 82, 1553-1557.
- 25 Caldji, C., Francis, D., Sharma, S., Plotsky, P. M., Meaney, M. J., 2000. The effects of early rearing environment on the development of GABAA and central benzodiazepine receptor levels and novelty-induced fearfulness in the rat. *Neuropsychopharmacology* 22, 219-229.
- 30 Calvez, J., de Avila, C., Timofeeva, E., 2016. Sex-specific effects of relaxin-3 on food intake and body weight gain. *Br J Pharmacol*, doi: 10.1111/bph.13530.
- 35 Crawley, J. N., 1981. Neuropharmacologic specificity of a simple animal model for the behavioral actions of benzodiazepines. *Pharmacol Biochem Behav* 15, 695-699.
- Dale, E., Bang-Andersen, B., Sanchez, C., 2015. Emerging mechanisms and treatments for depression beyond SSRIs and SNRIs. *Biochem Pharmacol* 95, 81-97.
- 40 Dhuria, S. V., Hanson, L. R., Frey, W. H., 2nd, 2009. Intranasal drug targeting of hypocretin-1 (orexin-A) to the central nervous system. *J Pharm Sci* 98, 2501-2515.
- Eliel, E. L. Stereochemistry of Carbon compounds (McGraw-Hill, N.Y., 1962).

- Enginar, N., Hatipoglu, I., Firtina, M., 2008. Evaluation of the acute effects of amitriptyline and fluoxetine on anxiety using grooming analysis algorithm in rats. *Pharmacol Biochem Behav* 89, 450-455.
- 5 Ganella, D. E., Ryan, P. J., Bathgate, R. A., Gundlach, A. L., 2012. Increased feeding and body weight gain in rats after acute and chronic activation of RXFP3 by relaxin-3 and receptor-selective peptides: functional and therapeutic implications. *Behav Pharmacol* 23, 516-525.
- 10 Griebel, G., Holsboer, F., 2012. Neuropeptide receptor ligands as drugs for psychiatric diseases: the end of the beginning? *Nat Rev Drug Discov* 11, 462-478.
- Hilinski, G. J., Kim, Y. W., Hong, J., Kutchukian, P. S., Crenshaw, C. M., Berkovitch, S. S., Chang, A., Ham, S., Verdine, G. L., 2014. Stitched alpha-helical peptides via bis ring-closing metathesis. *J Am Chem Soc* 136, 12314-12322.
- 15 Hojo, K., Hossain, M. A., Tailhades, J., Shabanpoor, F., Wong, L. L., Ong-Palsson, E. E., Kastman, H. E., Ma, S., Gundlach, A. L., Rosengren, K. J., Wade, J. D., Bathgate, R. A., 2016. Development of a Single-Chain Peptide Agonist of the Relaxin-3 Receptor Using Hydrocarbon Stapling. *J Med Chem* 59, 7445-7456.
- 20 Hudson, P., Haley, J., John, M., Cronk, M., Crawford, R., Haralambidis, J., Tregear, G., Shine, J., Niall, H., 1983. Structure of a genomic clone encoding biologically active human relaxin. *Nature* 301, 628-631.
- 25 Jacques et al., Enantiomers, Racemates and Resolutions (Wiley Interscience, New York, 1981).
- Jayakody, T., Marwari, S., Lakshminarayanan, R., Tan, F. C., Johannes, C. W., Dymock, B. W., Poulsen, A., Herr, D. R., Dawe, G. S., 2016. Hydrocarbon stapled B chain analogues of relaxin-3 retain biological activity. *Peptides* 84, 44-57.
- 30 Keck, M. E., 2006. Corticotropin-releasing factor, vasopressin and receptor systems in depression and anxiety. *Amino Acids* 31, 241-250.
- 35 Liu, C., Eriste, E., Sutton, S., Chen, J., Roland, B., Kuei, C., Farmer, N., Jornvall, H., Sillard, R., Lovenberg, T. W., 2003. Identification of relaxin-3/INSL7 as an endogenous ligand for the orphan G-protein-coupled receptor GPCR135. *J Biol Chem* 278, 50754-50764.
- 40 Lukas, M., Neumann, I. D., 2012. Nasal application of neuropeptide S reduces anxiety and prolongs memory in rats: social versus non-social effects. *Neuropharmacology* 62, 398-405.
- 45 Mezadri, T. J., Batista, G. M., Portes, A. C., Marino-Neto, J., Lino-de-Oliveira, C., 2011. Repeated rat-forced swim test: reducing the number of animals to evaluate gradual effects of antidepressants. *J Neurosci Methods* 195, 200-205.

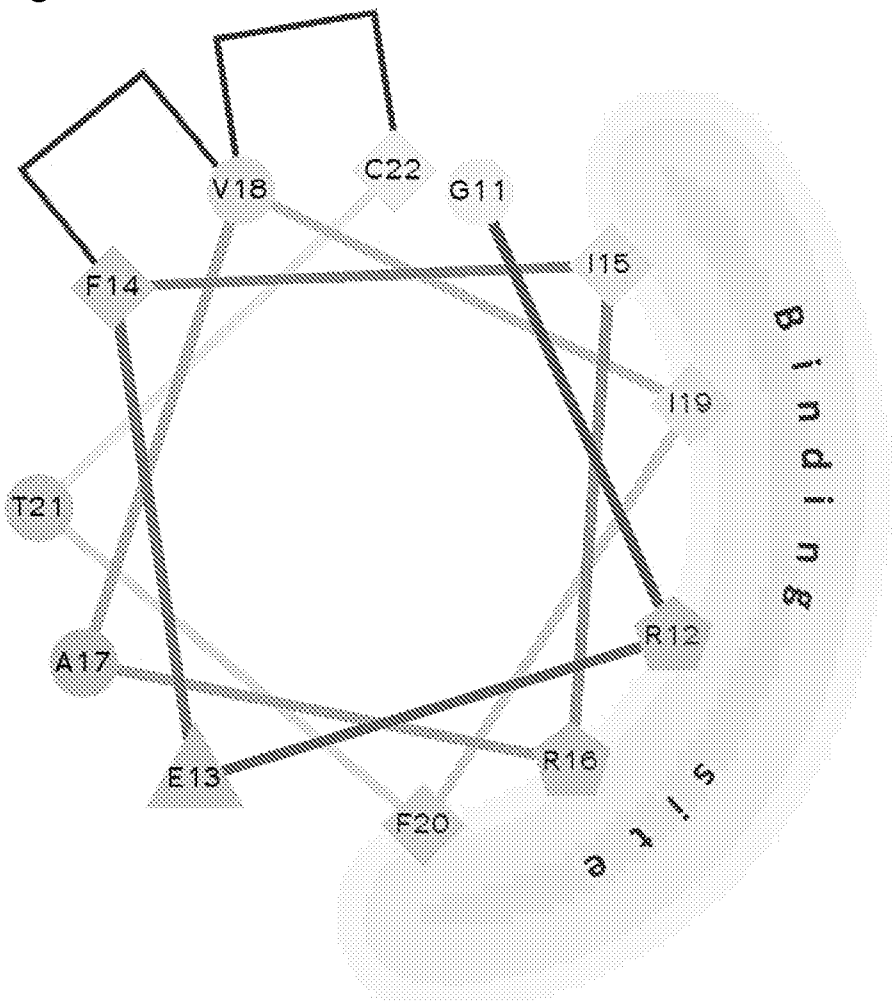
- Otsubo, H., Onaka, T., Suzuki, H., Katoh, A., Ohbuchi, T., Todoroki, M., Kobayashi, M., Fujihara, H., Yokoyama, T., Matsumoto, T., Ueta, Y., 2010. Centrally administered relaxin-3 induces Fos expression in the osmosensitive areas in rat brain and facilitates water intake. *Peptides* 31, 1124-1130.
- 5 Rajkumar, R., See, L. K., Dawe, G. S., 2013. Acute antipsychotic treatments induce distinct c-Fos expression patterns in appetite-related neuronal structures of the rat brain. *Brain Res* 1508, 34-43.
- 10 Rosengren, K. J., Lin, F., Bathgate, R. A., Tregear, G. W., Daly, N. L., Wade, J. D., Craik, D. J., 2006. Solution structure and novel insights into the determinants of the receptor specificity of human relaxin-3. *J Biol Chem* 281, 5845-5851.
- 15 Schafmeister, C. E., Po, J., Verdine, G. L., 2000. An all-hydrocarbon cross-linking system for enhancing the helicity and metabolic stability of peptides. *J Am Chem Soc* 122, 5891-5892.
- Shaul, Y., Seger, R., 2006. The detection of MAPK signaling. *Curr Protoc Mol Biol* Chapter 18, Unit 18 12.
- 20 Thorne, R. G., Pronk, G. J., Padmanabhan, V., Frey, W. H., 2nd, 2004. Delivery of insulin-like growth factor-I to the rat brain and spinal cord along olfactory and trigeminal pathways following intranasal administration. *Neuroscience* 127, 481-496.
- 25 Verdine, G. L., Hilinski, G. J., 2012. Stapled peptides for intracellular drug targets. *Methods Enzymol* 503, 3-33.
- 30 Whiteford, H. A., Degenhardt, L., Rehm, J., Baxter, A. J., Ferrari, A. J., Erskine, H. E., Charlson, F. J., Norman, R. E., Flaxman, A. D., Johns, N., Burstein, R., Murray, C. J., Vos, T., 2013. Global burden of disease attributable to mental and substance use disorders: findings from the Global Burden of Disease Study 2010. *Lancet* 382, 1575-1586.
- Wilen S. H., et al., *Tetrahedron* 33: 2725 (1977).
- Wilen, S. H. Tables of Resolving Agents and Optical Resolutions p. 268 (E. L. Eliel, Ed., Univ of Notre Dame Press, Notre Dame, Ind. 1972).
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CLAIMS:

1. A single chain relaxin-3 polypeptide or analog, derivative, fragment or mimetic thereof having RXFP3 receptor agonist activity, comprising a relaxin B-chain polypeptide stapled at amino acid positions 11 and 18 or at positions 14 and 21 of the relaxin-3 B-chain; wherein the amino acids at positions 11 and 18 or 14 and 21 are covalently cross-linked.
2. The polypeptide of claim 1, wherein the staple is an all-hydrocarbon staple.
3. The polypeptide of claim 2, wherein the staple is an 11-carbon cross-link.
4. The polypeptide of any one of claims 1 to 3, wherein the B-chain polypeptide is stapled at positions 14 and 21.
5. The polypeptide of any one of claims 1 to 4, wherein the B-chain has a polypeptide sequence of Formula 1:
R A A P Y G V R L C G R E **X1** I R A V I F **X2** C G G S R W; (SEQ ID NO: 12),
wherein **X1** and **X2** are cross-linked amino acids.
6. The polypeptide of any one of claims 1 to 3, wherein the B-chain polypeptide is stapled at positions 11 and 18, wherein the B-chain has a polypeptide sequence of Formula 2:
R A A P Y G V R L C **X1** R E F I R A **X2** I F T C G G S R W; (SEQ ID NO: 13),
wherein **X1** and **X2** are cross-linked amino acids.
7. The polypeptide of any one of claims 1 to 6, wherein the stapled amino acids are α , α -di-substituted amino acids.
8. The polypeptide of any one of claims 1 to 7, wherein the B-chain polypeptide sequence is from human relaxin-3.
9. The polypeptide of any one of claims 1 to 8, wherein the B-chain polypeptide sequence comprises the amino acid sequence set forth in SEQ ID NO: 1.
10. The polypeptide of any one of claims 1 to 9, wherein the B-chain sequence is from human relaxin-3 and the cysteine residues at positions 10 and 22 of the native human relaxin-3 sequence are replaced by serine residues.

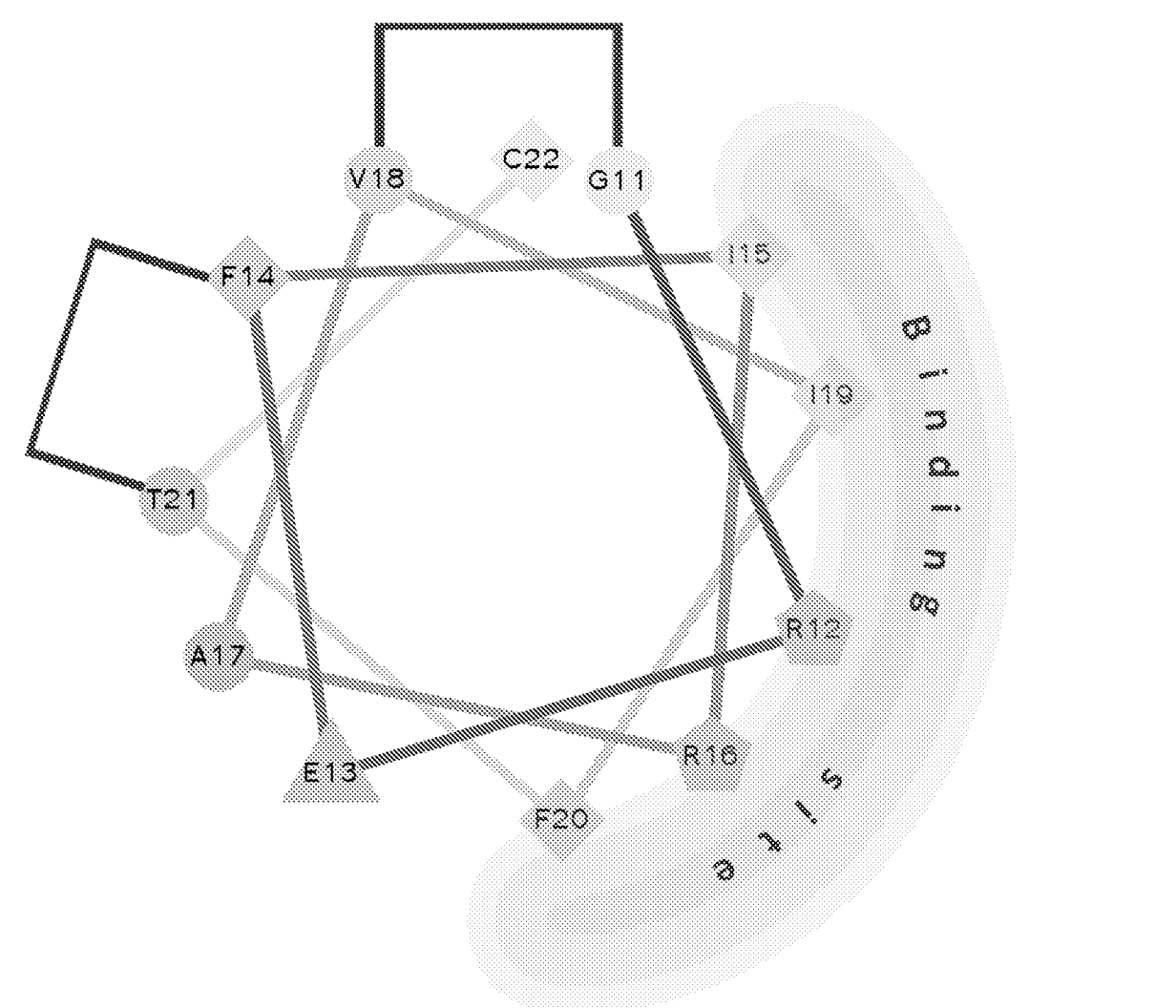
11. The polypeptide of any one of claims 1 to 5 and 7 to 10, wherein the alpha carbon of the residue at position 14 has (R) stereochemistry and the alpha carbon of the residue at position 21 has (S) stereochemistry.
- 5 12. The polypeptide of claim 11, wherein the B-chain polypeptide sequence is from human relaxin-3 and the amino acids at positions 14 and 21 are replaced by (R)-2-(4'-pentenyl) alanine and (S)-2-(7'-octenyl) alanine, respectively, as set forth in SEQ ID NO: 9 or SEQ ID NO: 10.
13. The polypeptide of any one of the preceding claims, wherein the polypeptide has helical secondary structure, preferably alpha-helical secondary structure.
- 10 14. The polypeptide of any one of the preceding claims, in a pharmaceutically acceptable form.
15. The polypeptide of claim 14, wherein the pharmaceutically acceptable form is a free base, salt or solvate form thereof.
- 15 16. The polypeptide of any one of claims 1 to 15, wherein the polypeptide decreases stress-related and/or anxiety-related and/or depressive-like behavior and/or increases feeding activity.
17. A pharmaceutical composition comprising a polypeptide according to any one of claims 1 to 16, optionally together with one or more pharmaceutically acceptable carriers, excipients or diluents for the treatment of an anxiety, stress-related, eating or depressive disorder.
- 20 18. A method for the treatment of an anxiety, stress-related neuropsychiatric, feeding or depressive disorder, the method comprising administering to a subject in need thereof an effective amount of a polypeptide according to any one of claims 1 to 16, or a pharmaceutical composition according to claim 17.
- 25 19. The method of claim 18, wherein the administration is by intranasal or intravenous route.
- 30 20. Use of a polypeptide according to any one of claims 1 to 16, or a pharmaceutical composition according to claim 17 for the manufacture of a medicament for the treatment of an anxiety, any stress-related neuropsychiatric, eating or depressive disorder.

Fig. 1A



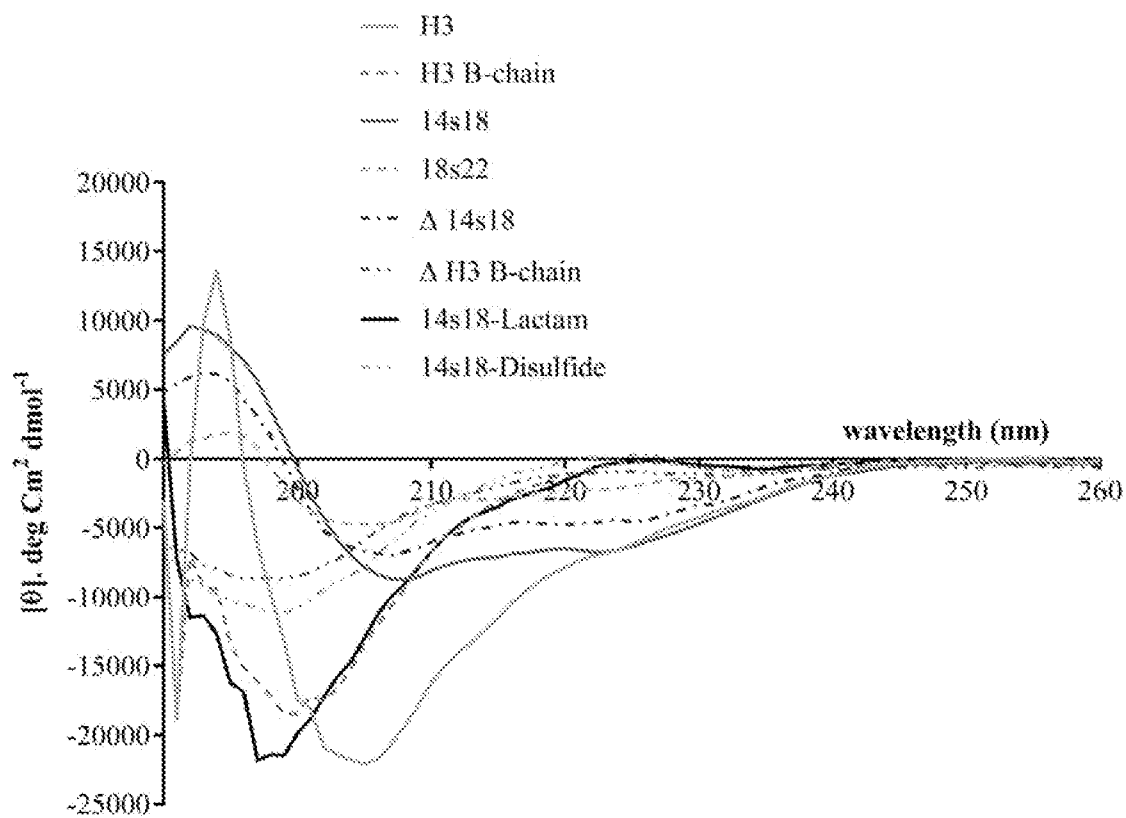
H3 ligands	Peptide Sequence	SEQ ID NO:
H3 B-chain	RAAPYGVRLSGREFIRAVIFTSGGSRW	1
14s18	RAAPYGVRLSGRE S5 IRA S5 IFTSGGSRW	2
18s22	RAAPYGVRLSGREFIRA S5 IFT S5 GGSRW	3
Δ 14s18	RLSGRE S5 IRA S5 IFTSGGSRW	4
Δ H3 B-chain	RLSGREFIRAVIFTSGGSRW	6
14s18-Lactam	RAAPYGVRLSGRE K IRA D IFTSGGSRW	7
14s18-Disulfide	RAAPYGVRLSGRE C IRA C IFTSGGSRW	8

Fig. 1B



H3 ligands	Peptide Sequence	SEQ ID NO:
H3 B-chain	RAAPYGVRLSGREFIRAVIFTSGGSRW	1
14s18	RAAPYGVRLSGRE S5 IRA S5 IFTSGGSRW	2
18s22	RAAPYGVRLSGREFIRA S5 IFT S5 GGSRW	3
14s21	RAAPYGVRLCGRE R5 IRAVIF S8 CGGSRW	9
11s18	RAAPYGVRLC R5 REFIRA S8 IFTCTGGSRW	11

Fig. 1C



Ligands	Θ_{222} nm	% Helicity
H3 B-chain	-650.60	1.81
14s18	-6720.10	18.52
18s22	-2240.02	6.17
Δ 14s18	-4647.92	12.80
Δ H3 B-chain	-1030.60	2.84
14s18-Lactam	-693.68	1.91
14s18-Disulfide	-104.82	-0.29

Fig. 1D

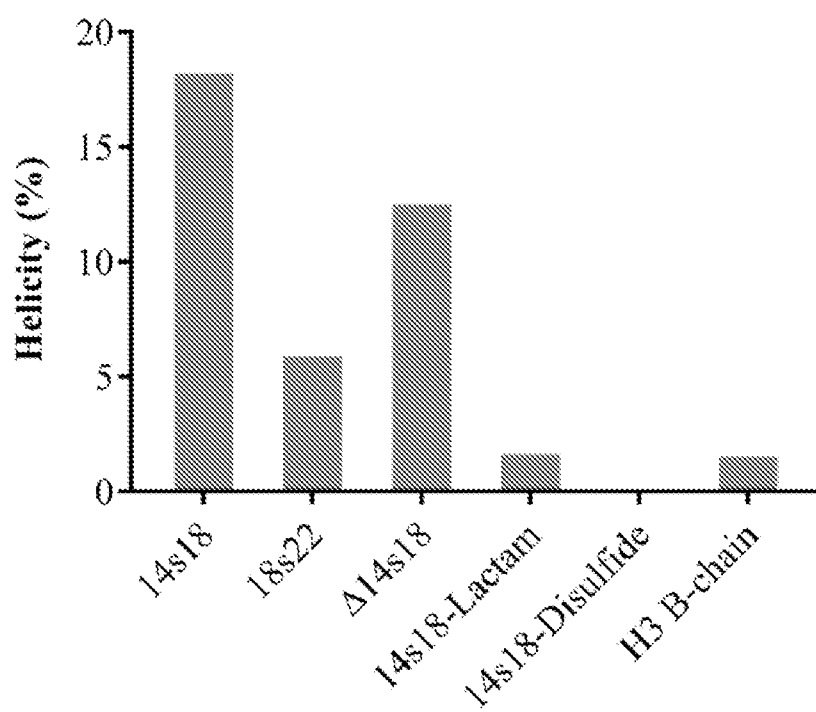
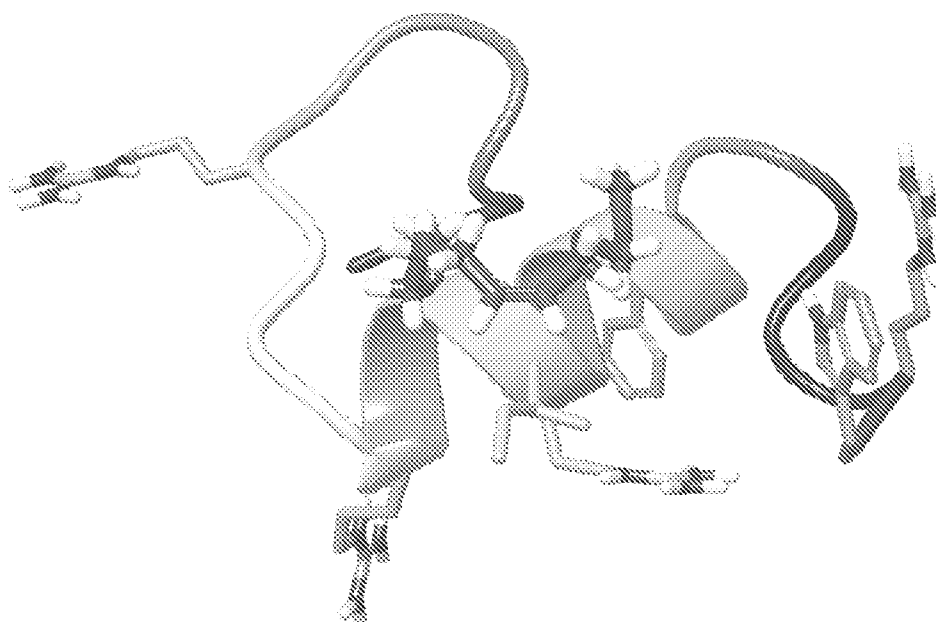
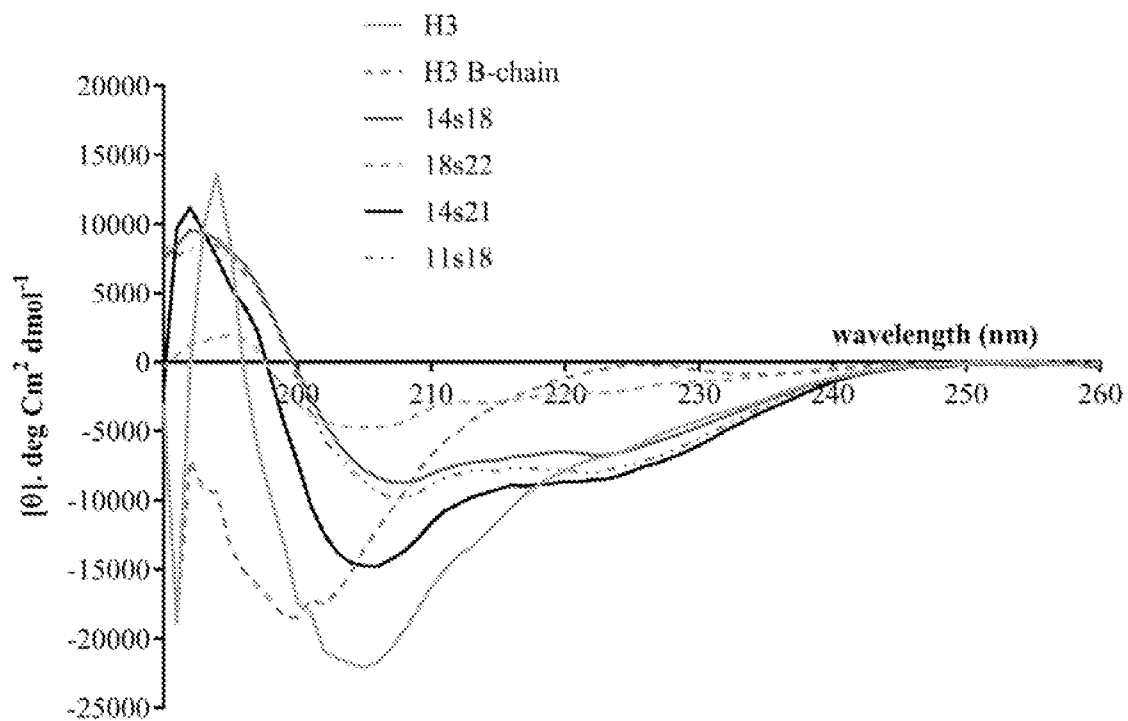


Fig. 1E



Ligands	Θ_{222} nm	% Helicity
H3 B-chain	-650.60	1.81
14s18	-6720.10	18.52
18s22	-2240.02	6.17
14s21	-8535.70	23.51
11s18	-7958.82	21.92

Fig. 1F

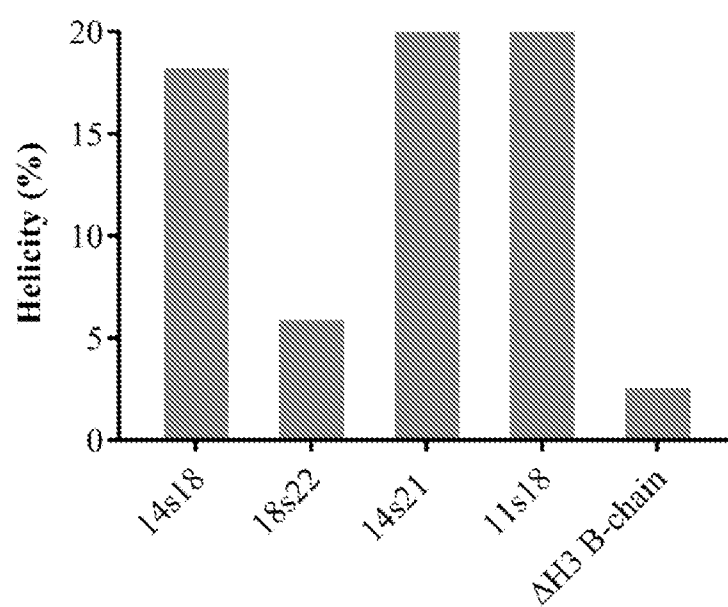
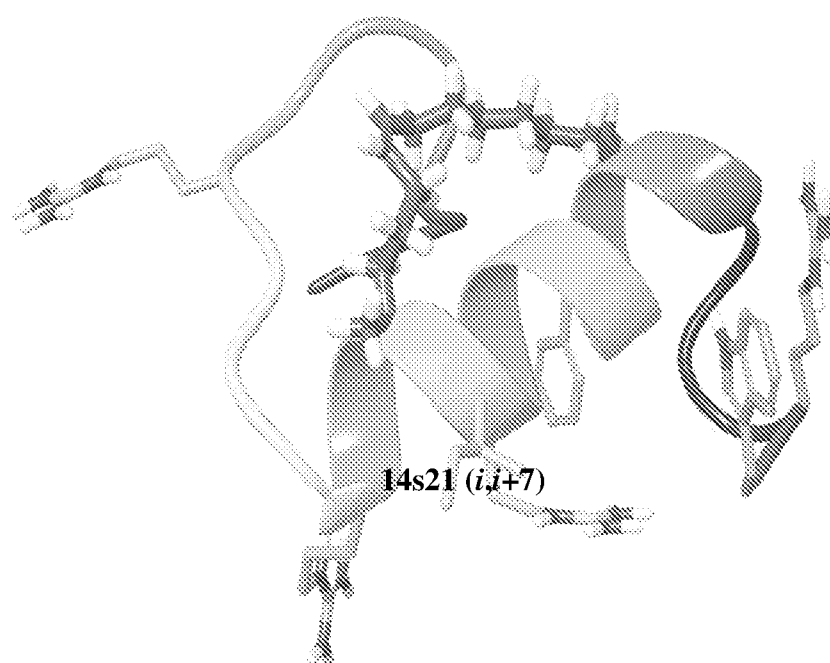


Fig. 2A

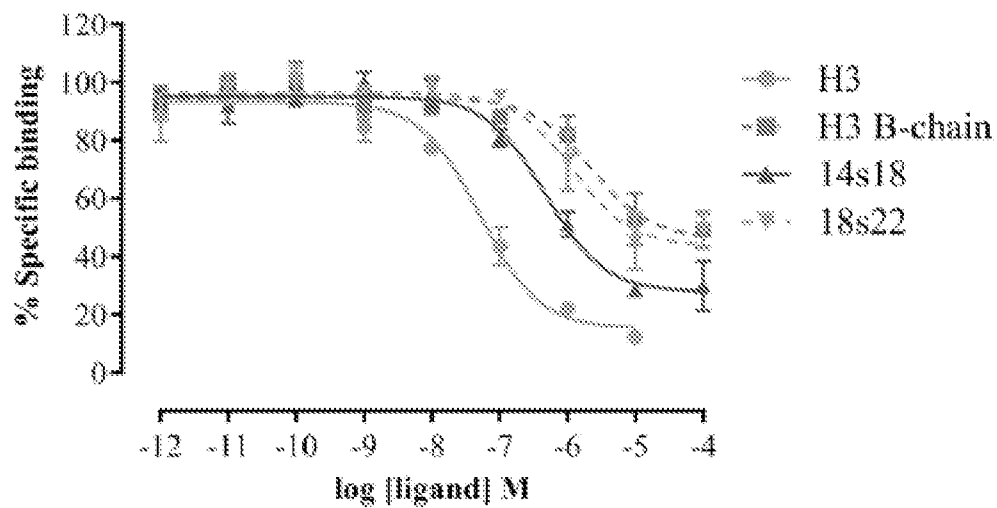


Fig. 2B

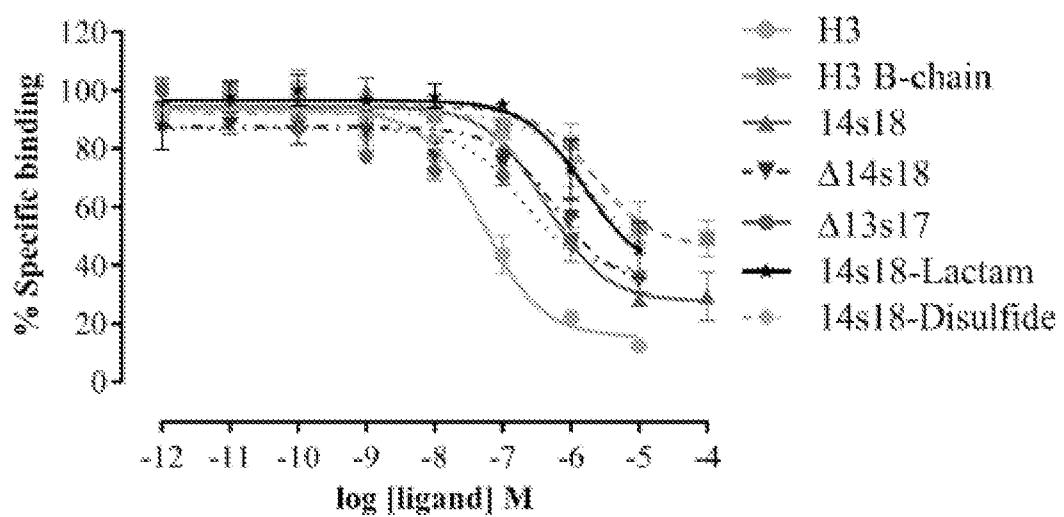


Fig. 2C

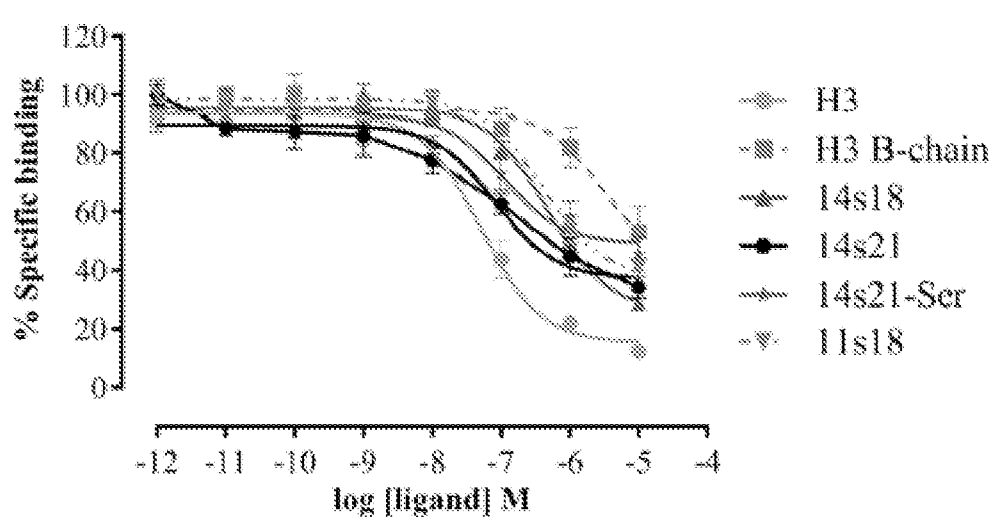


Fig. 2D

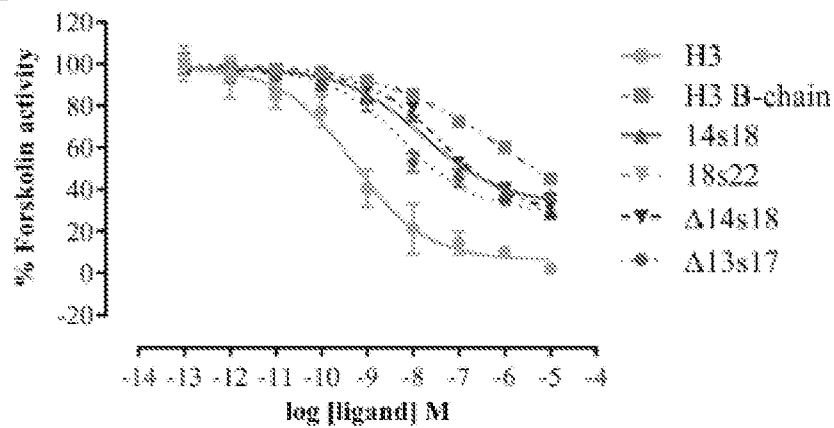


Fig. 2E

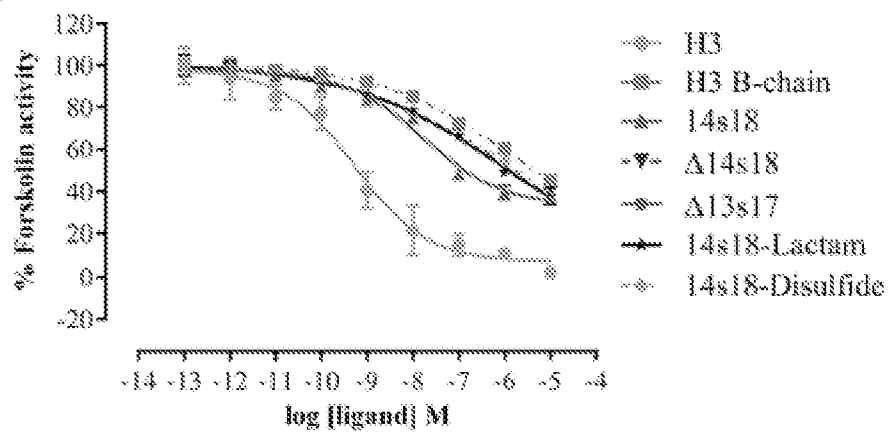


Fig. 2F

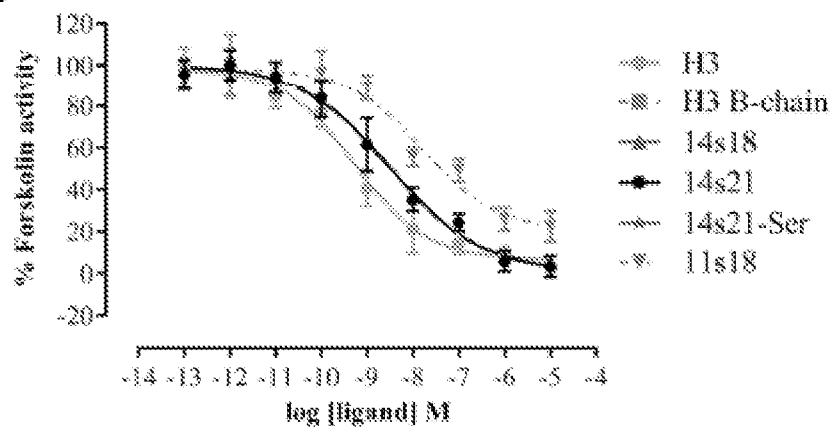


Fig. 2G

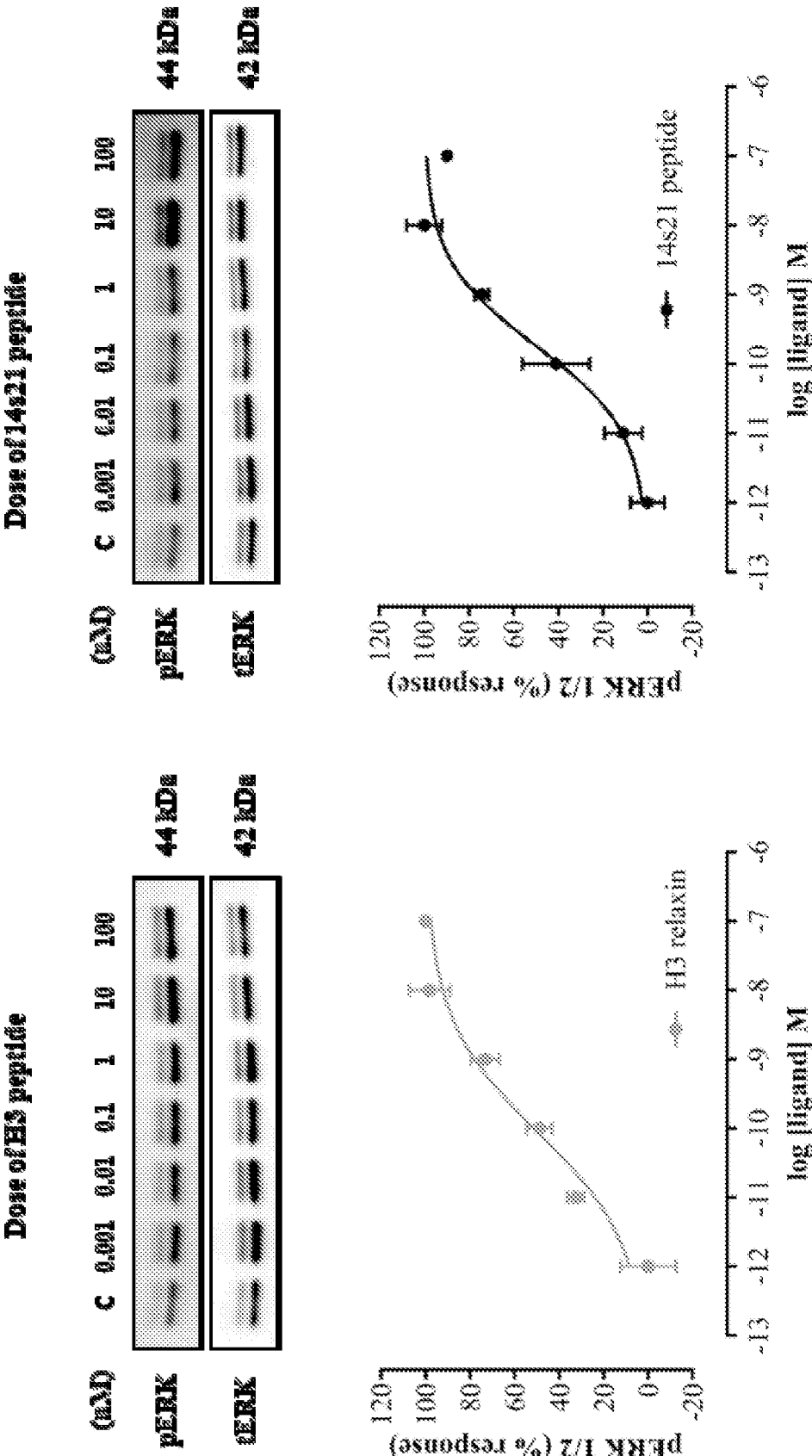


Fig. 2H

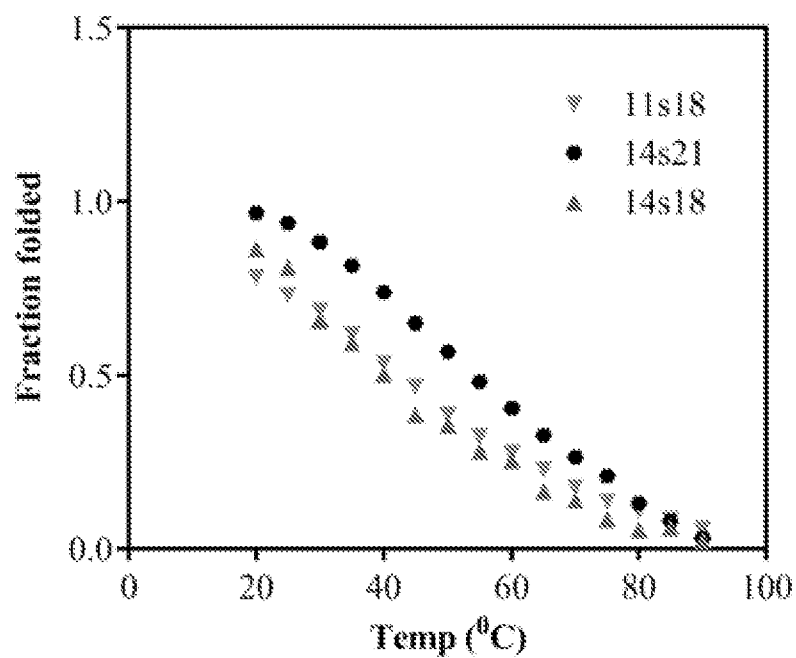
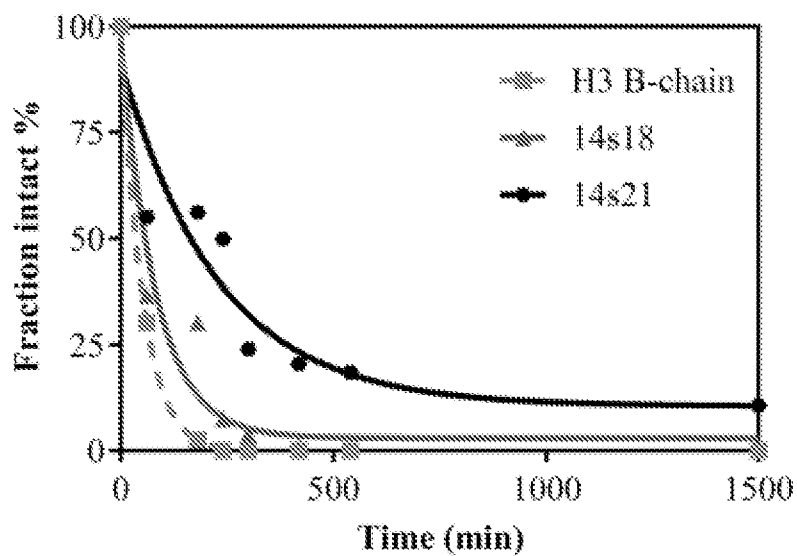


Fig. 2I



Ligands	Half-life, min.	95% CI (min)
H3 B-chain	35	34 to 36
14s18	56	18 to 138
14s21	158	38 to 449

Fig. 3A



Figure 3B

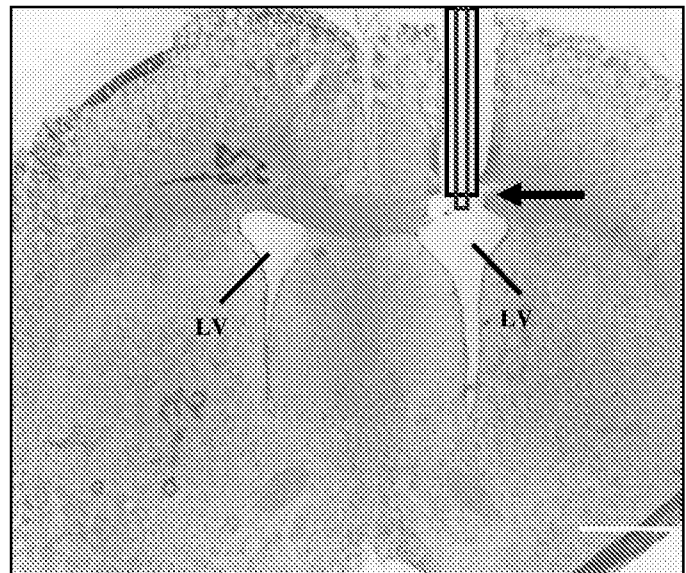


Fig. 3C

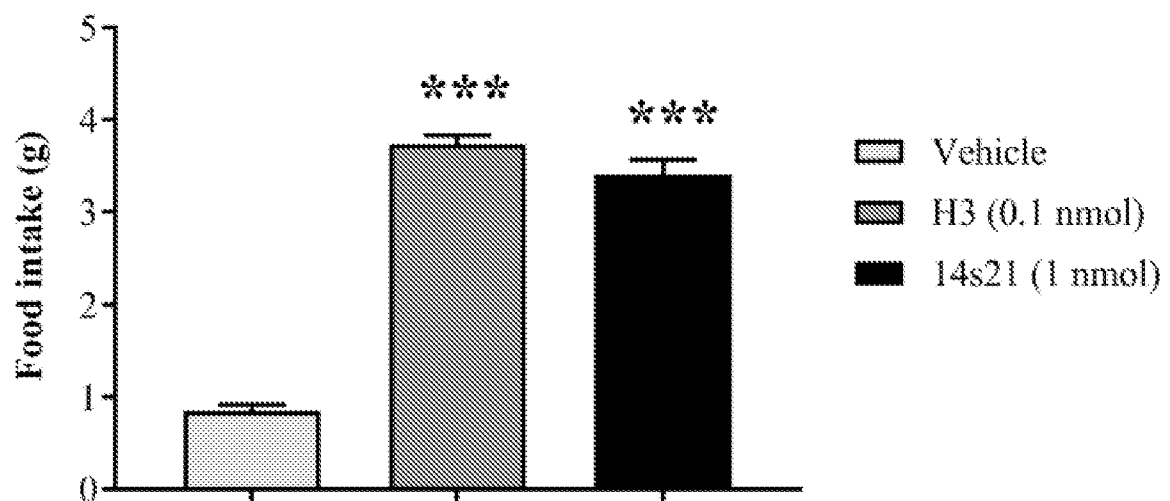


Fig. 3D

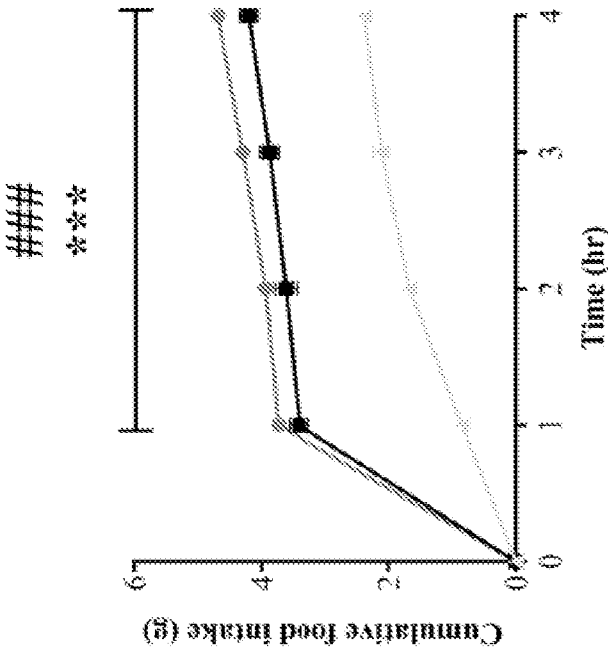


Fig. 3E

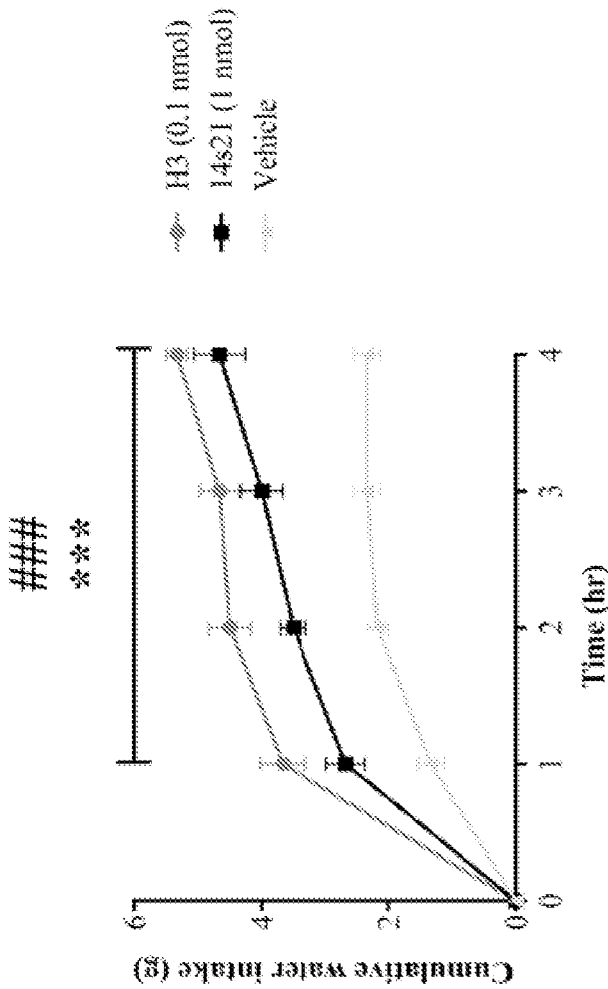


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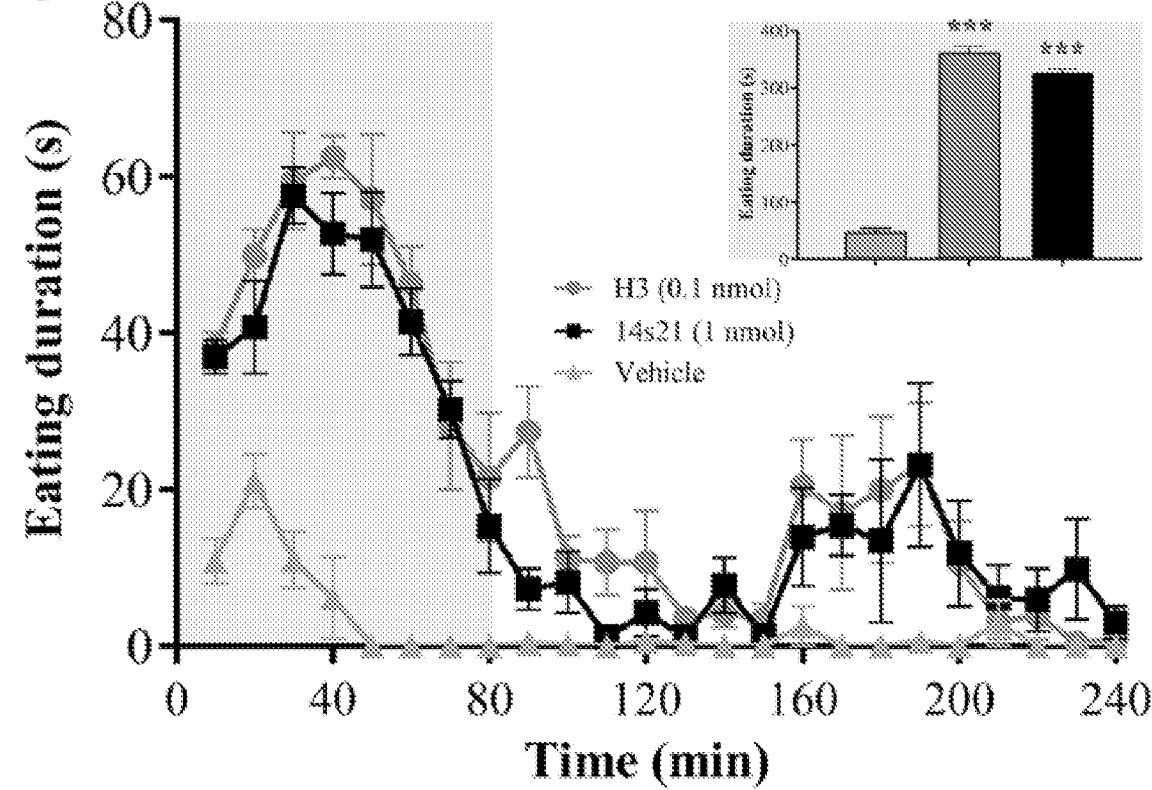


Fig. 3G

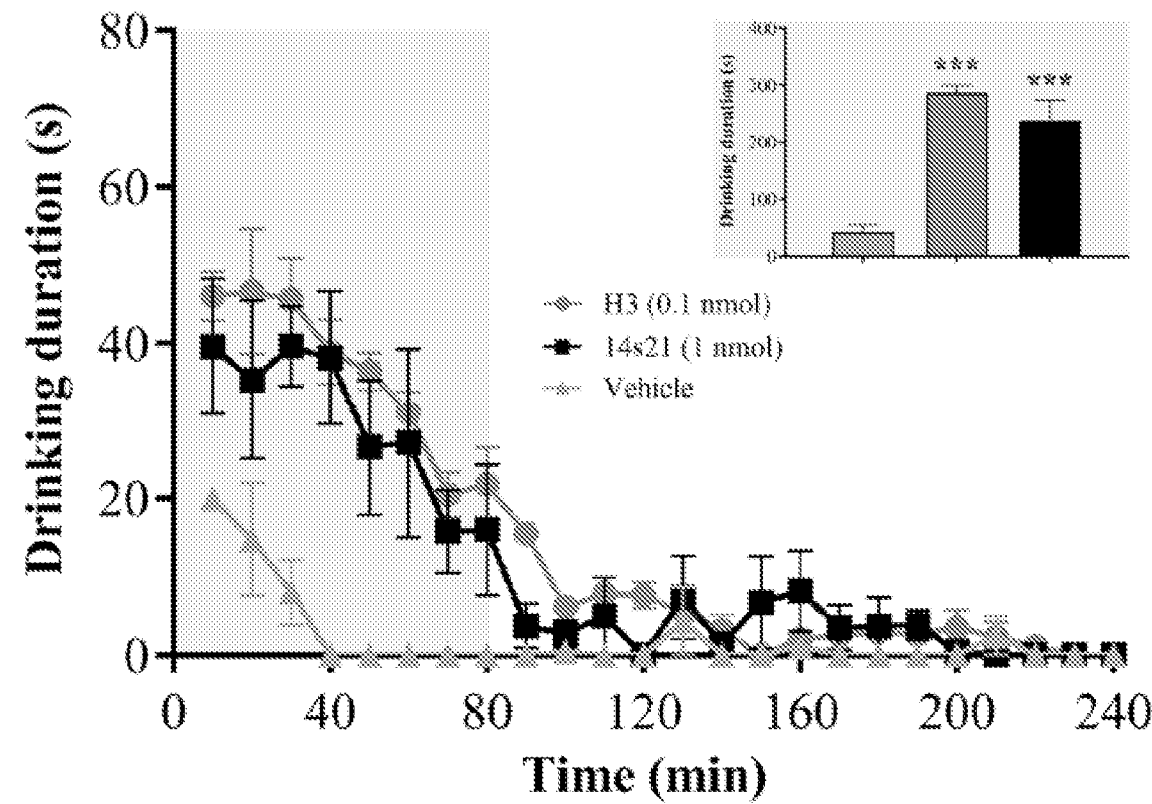


Fig. 3H

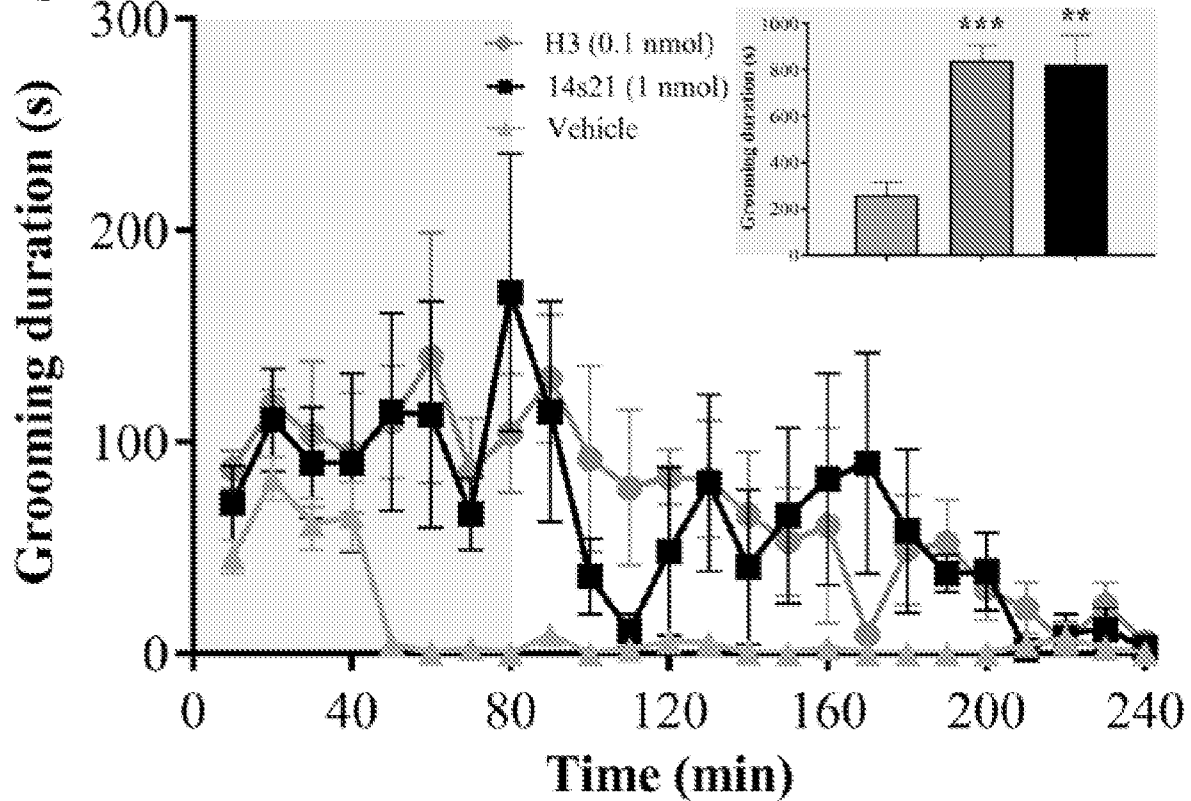


Fig. 3I

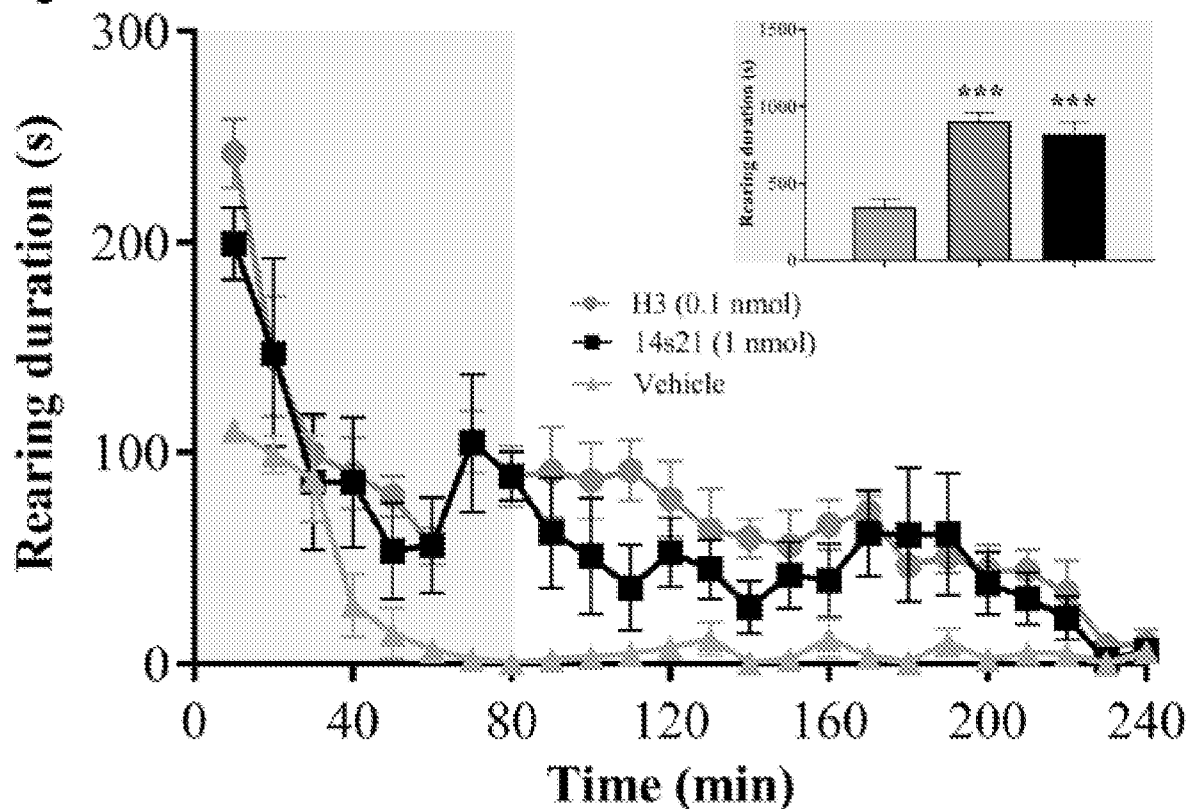


Fig. 3J

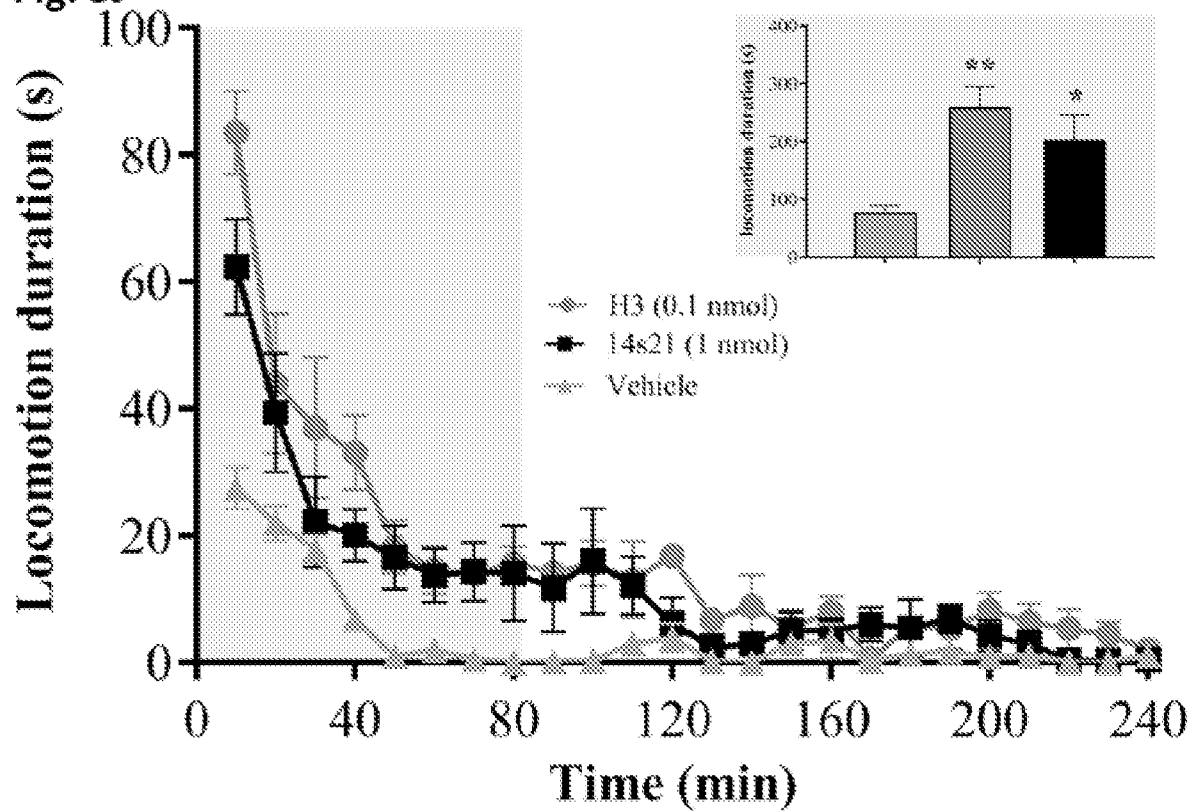


Fig. 3K

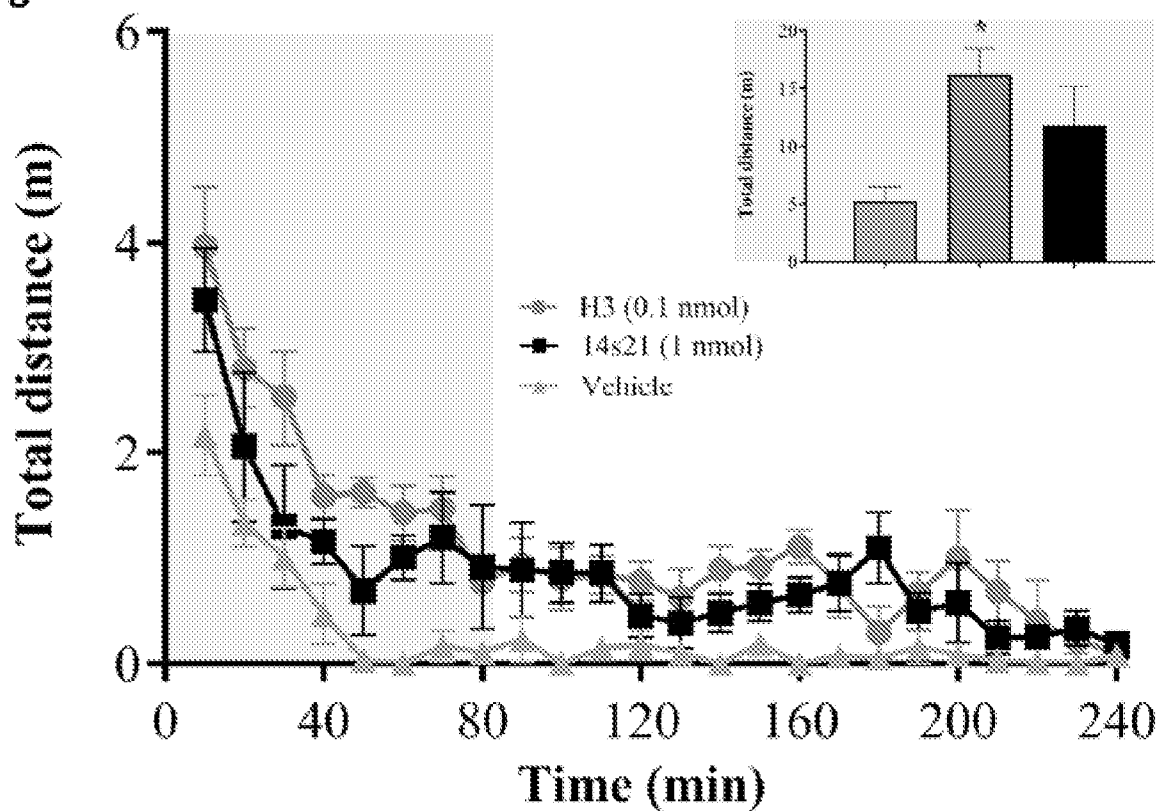


Fig. 4A

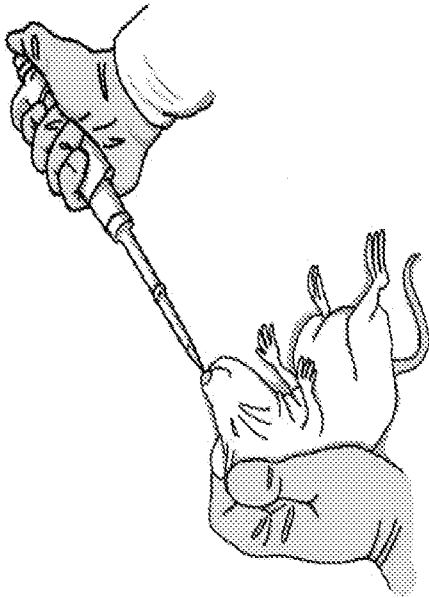


Fig. 4B

H3 relaxin dose response

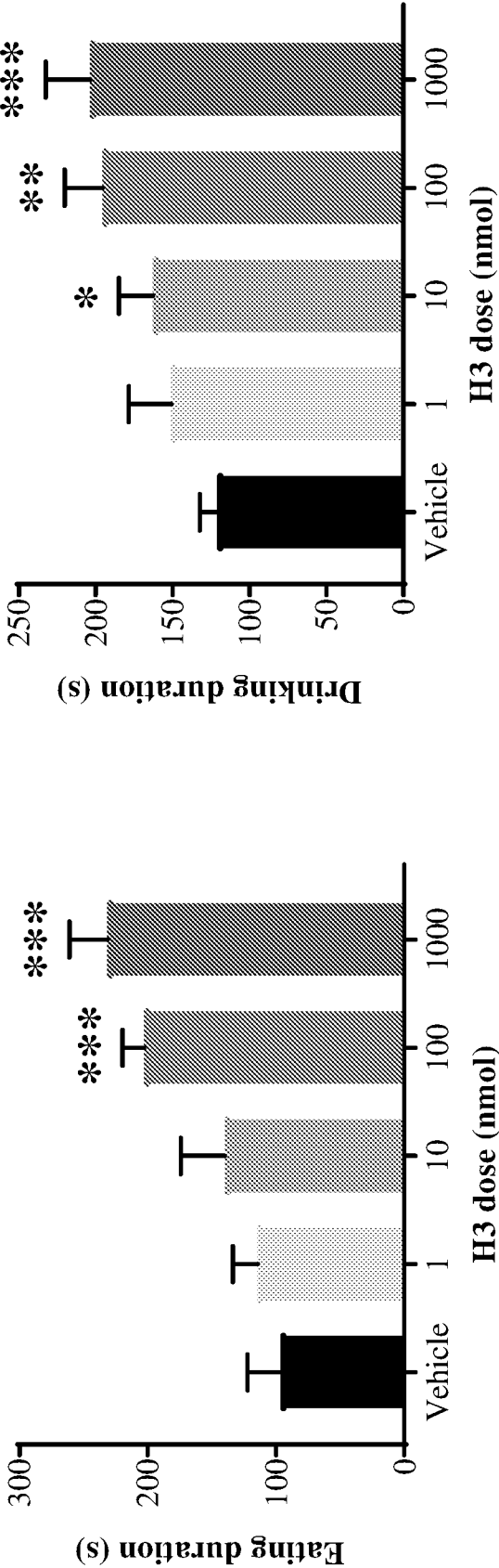


Fig. 4C

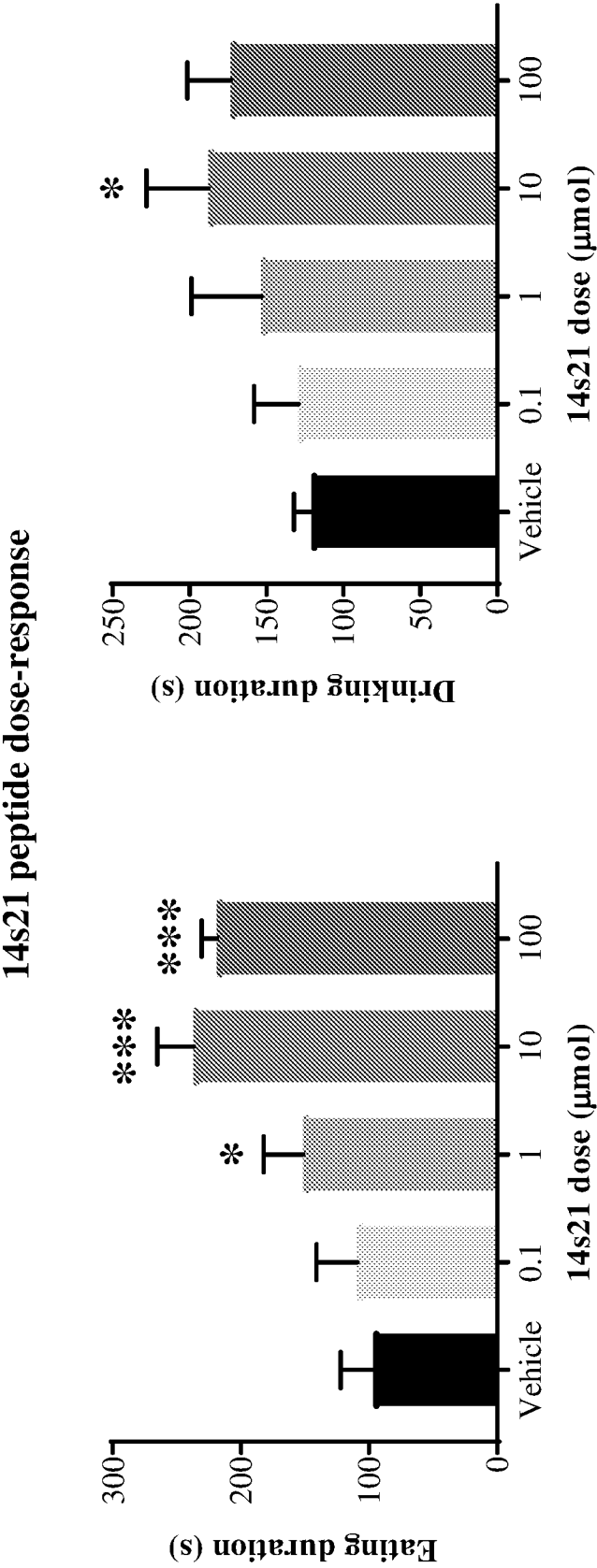


Fig. 4D

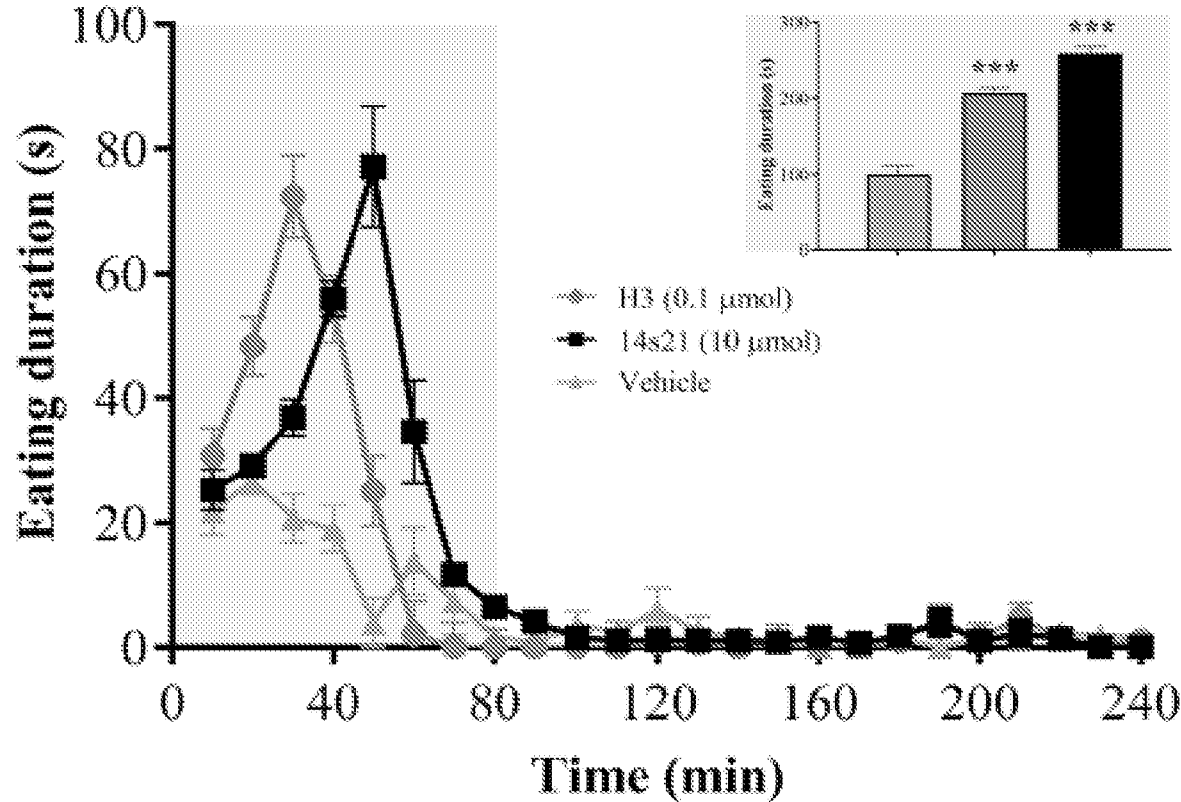


Fig. 4E

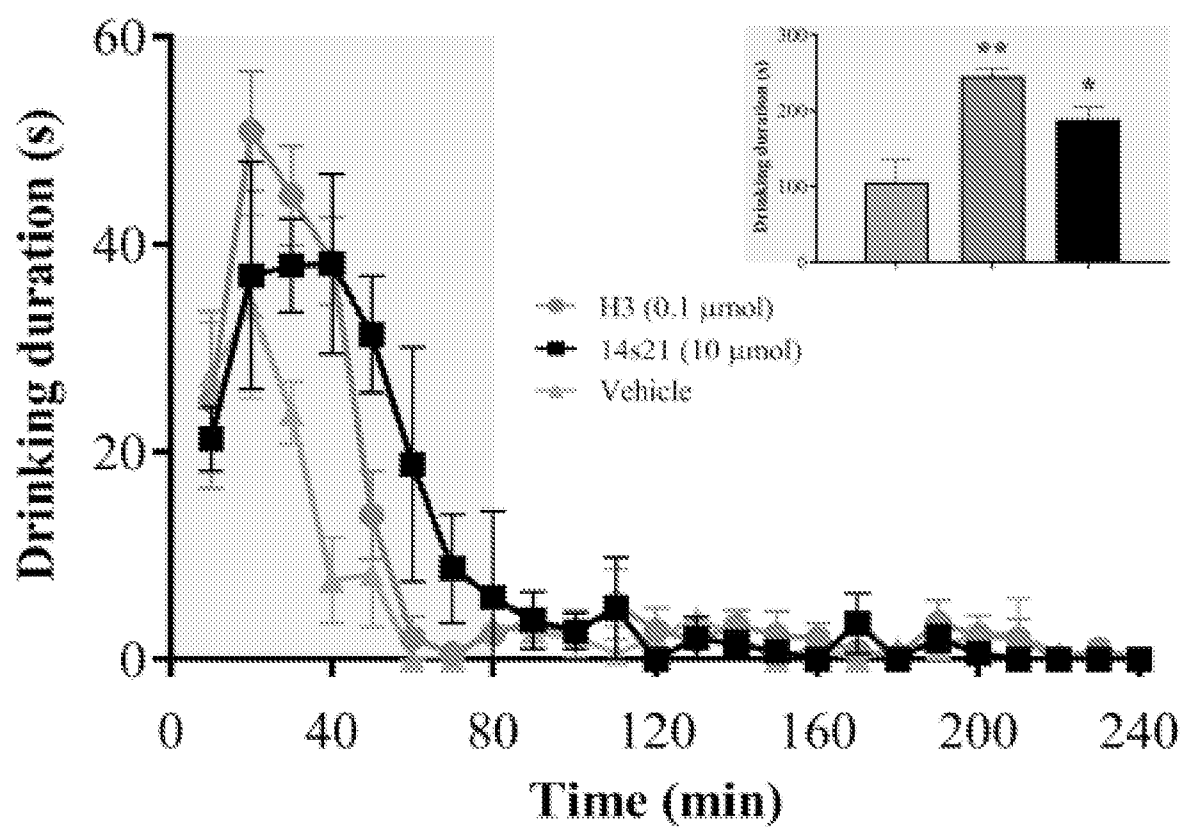


Fig. 4F

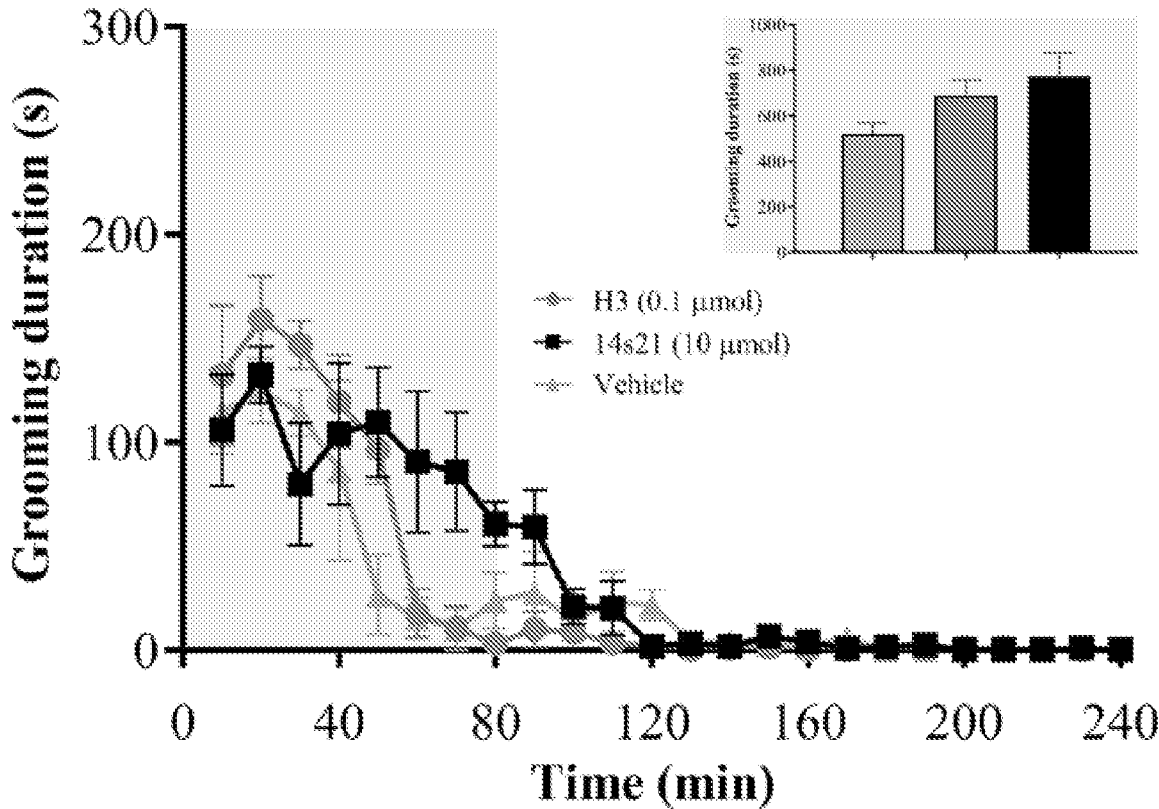


Fig. 4G

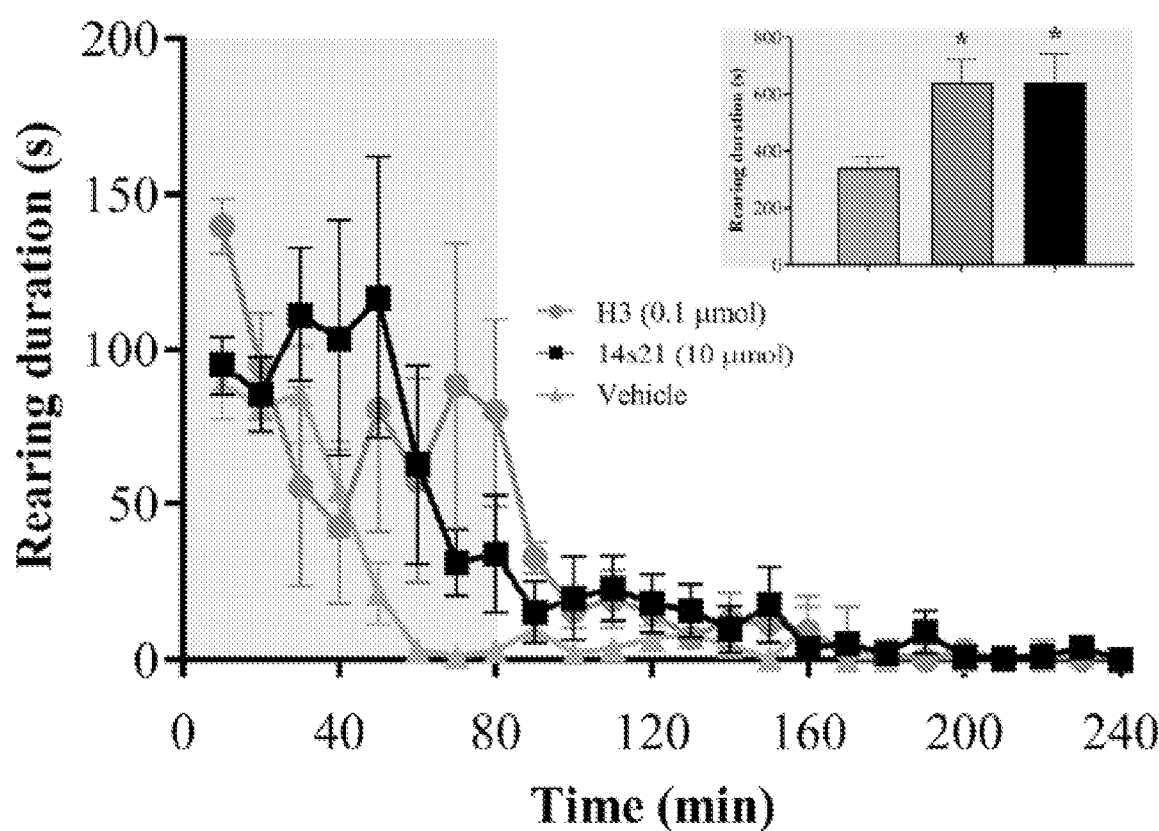


Fig. 4H

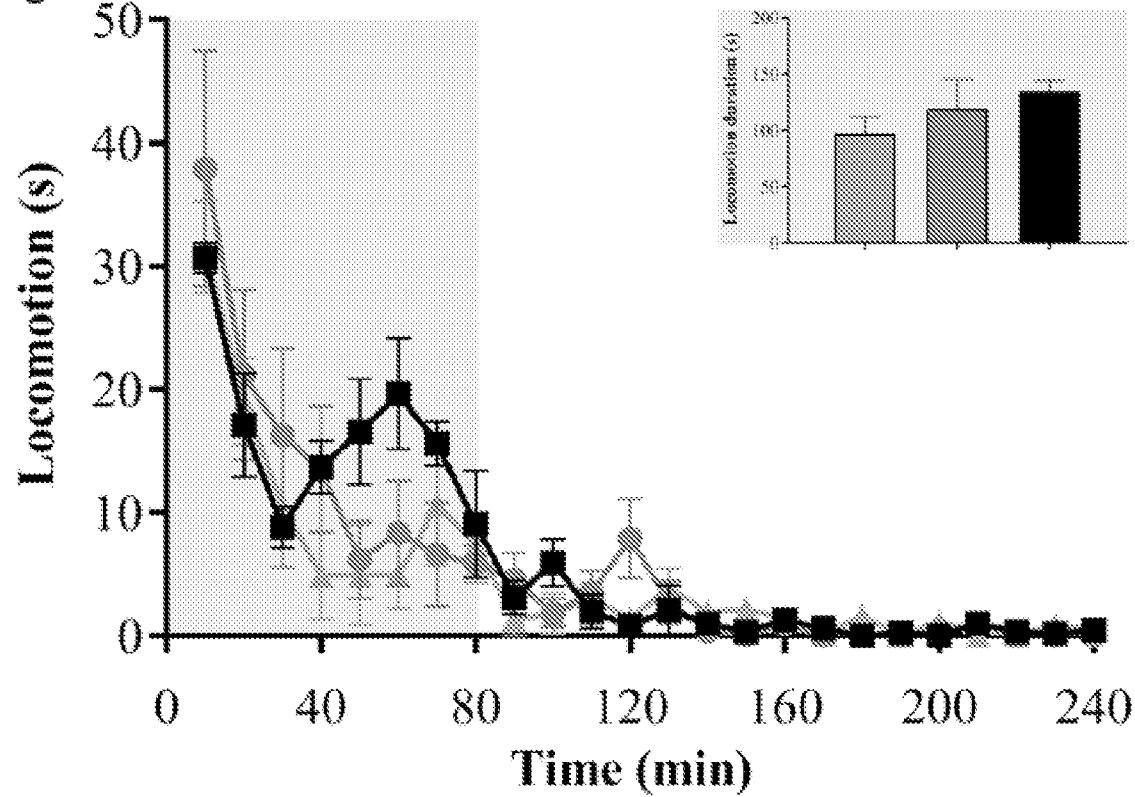


Fig. 4I

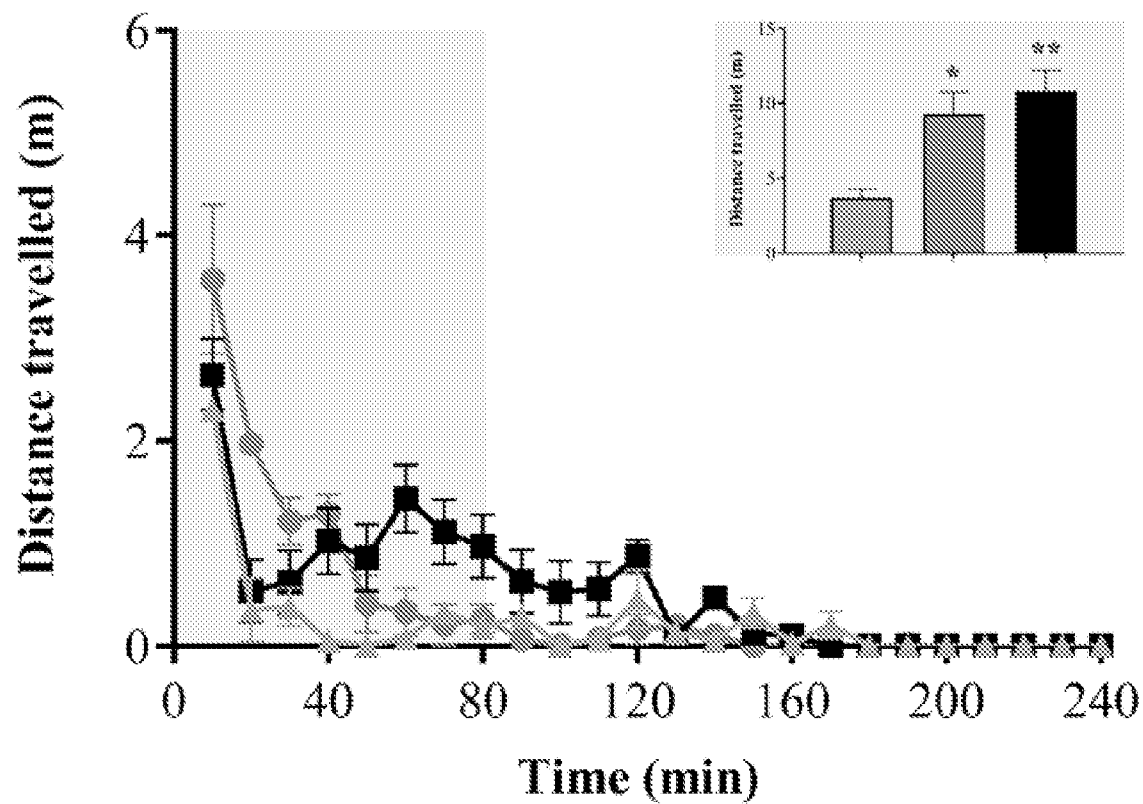


Fig. 4J

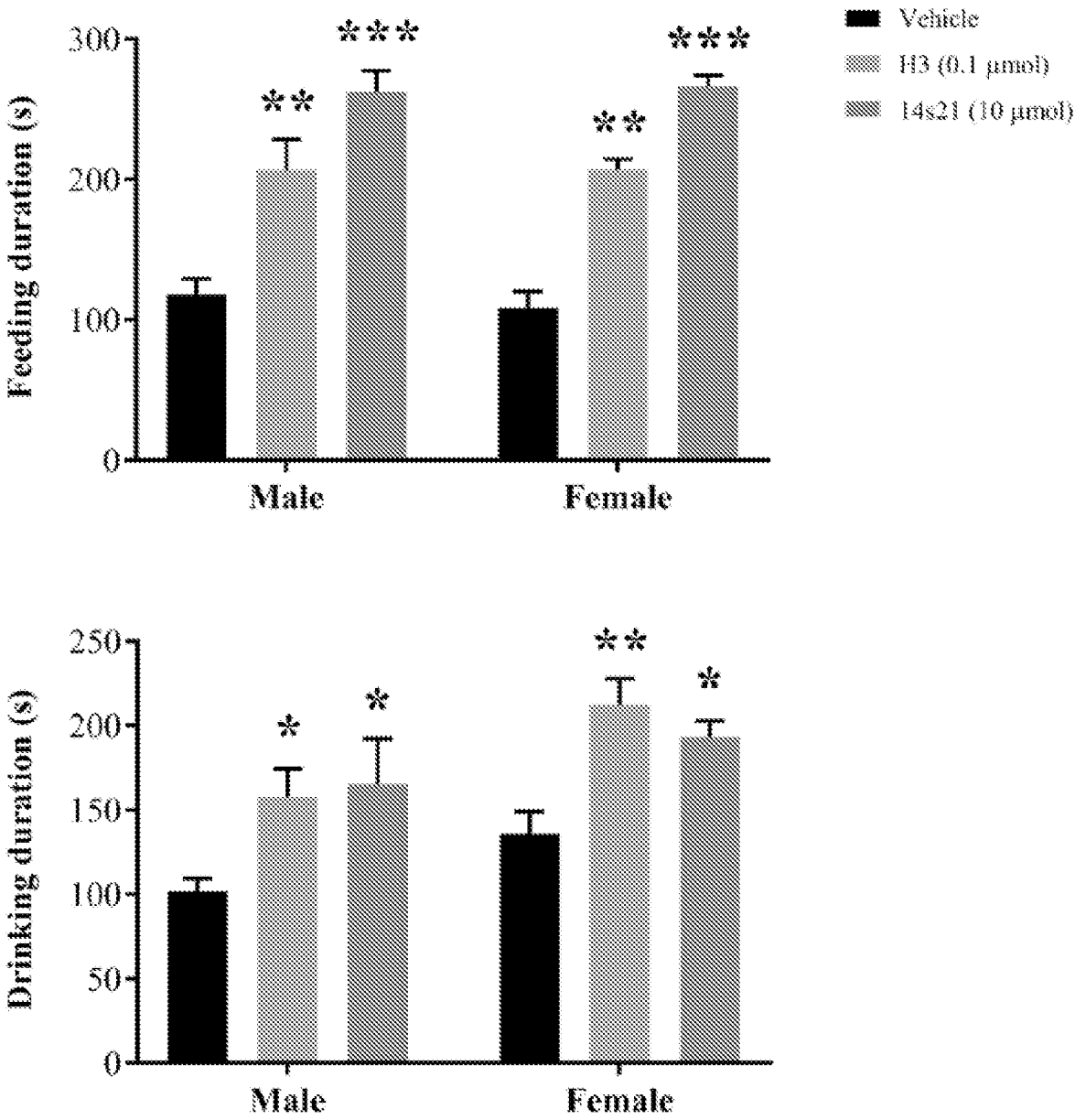


Fig 5A

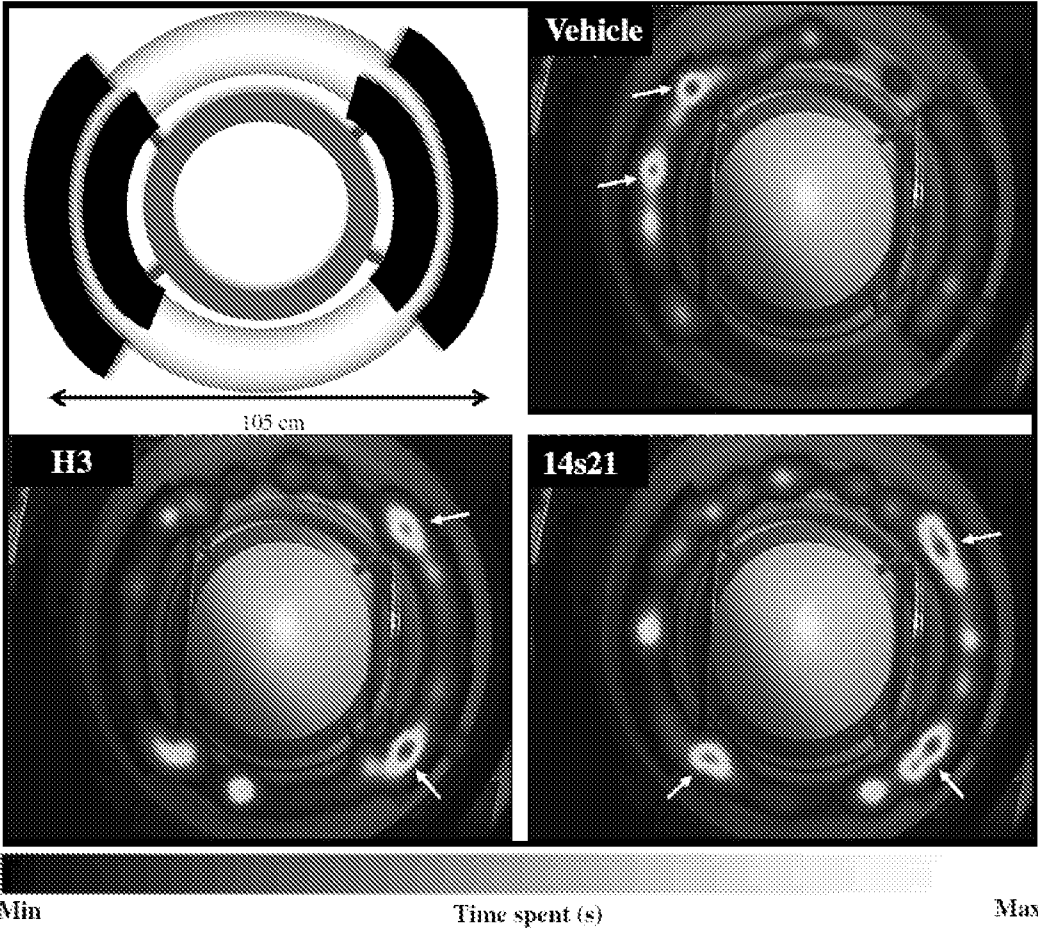


Fig. 5B

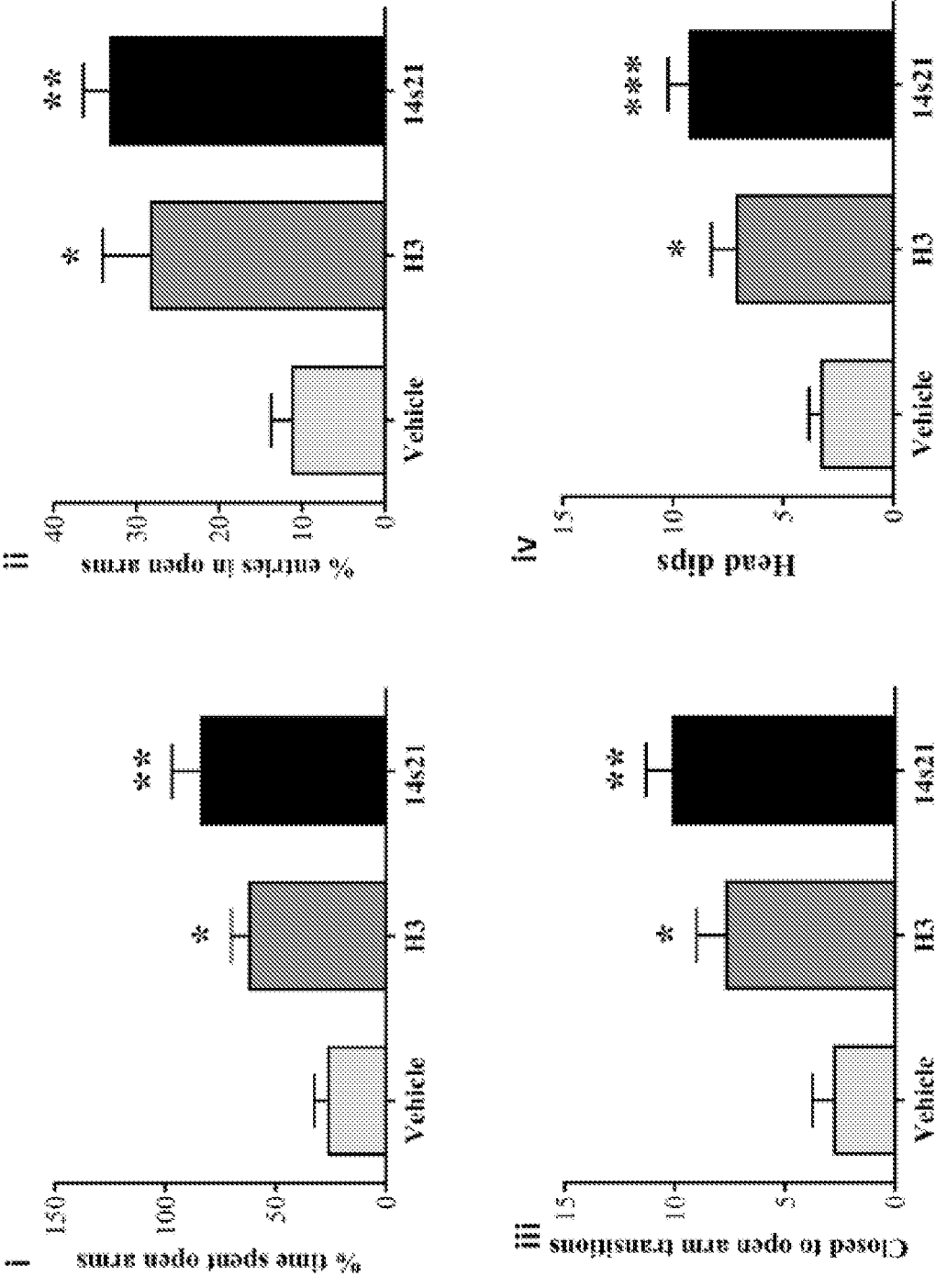


Fig. 5C

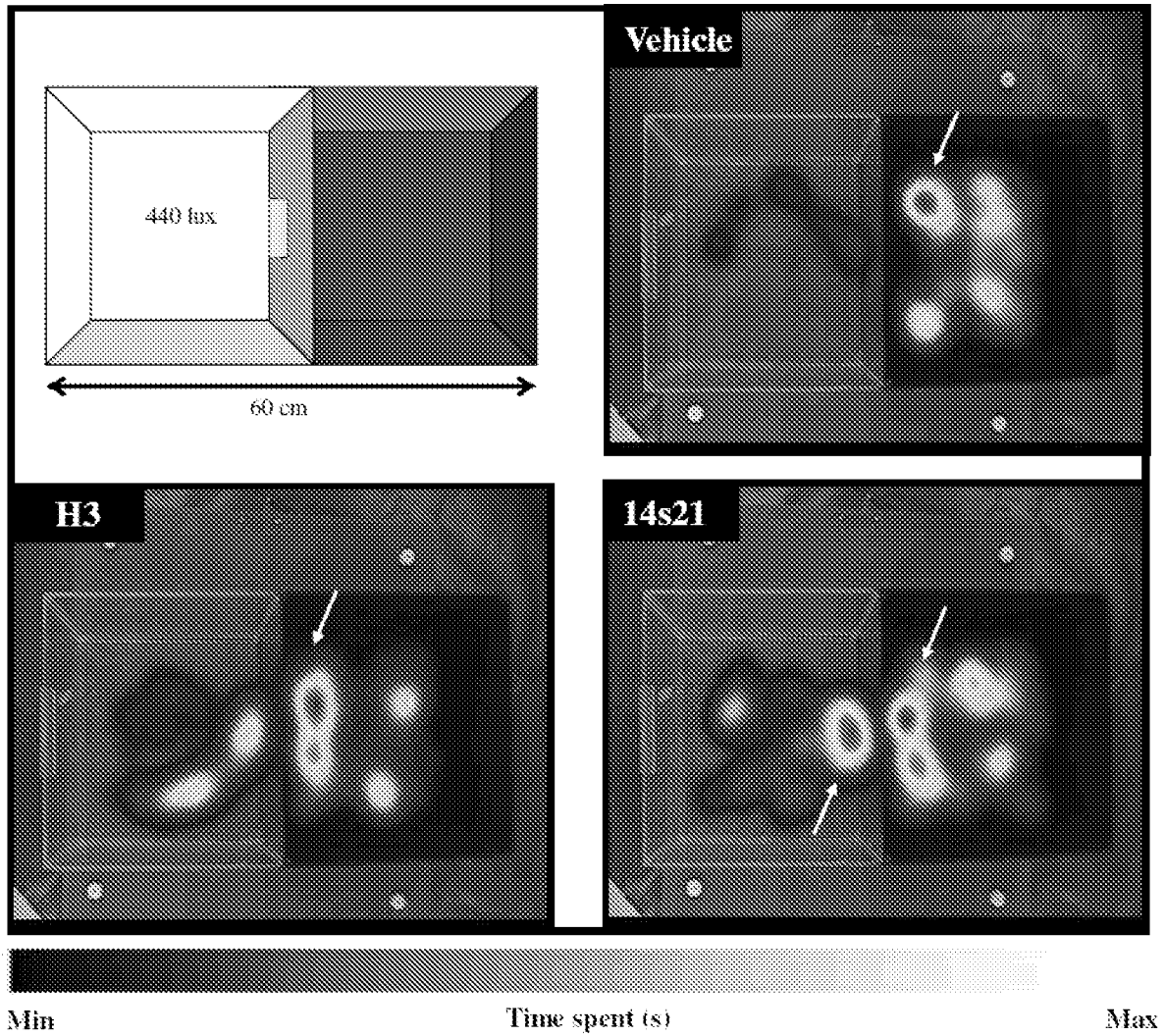


Fig. 5D

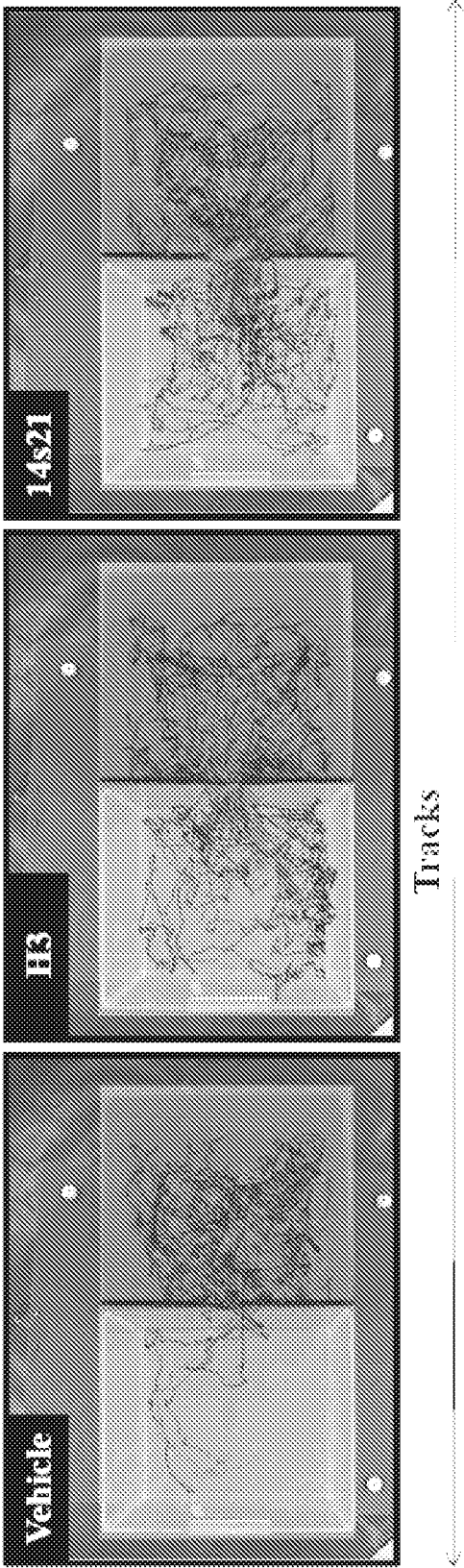
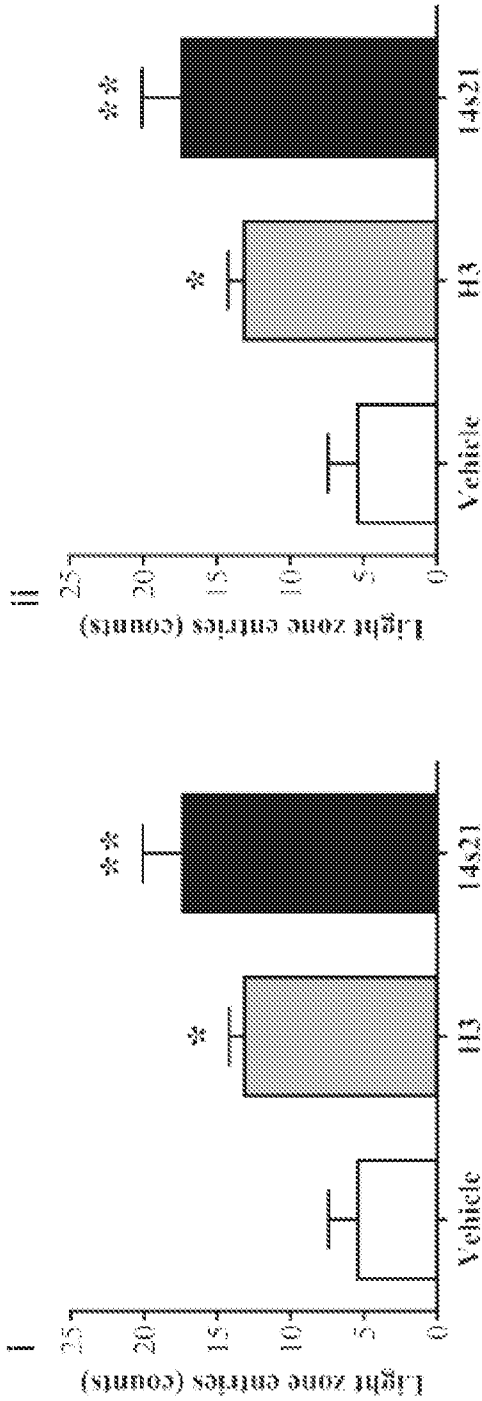


Fig. 5E

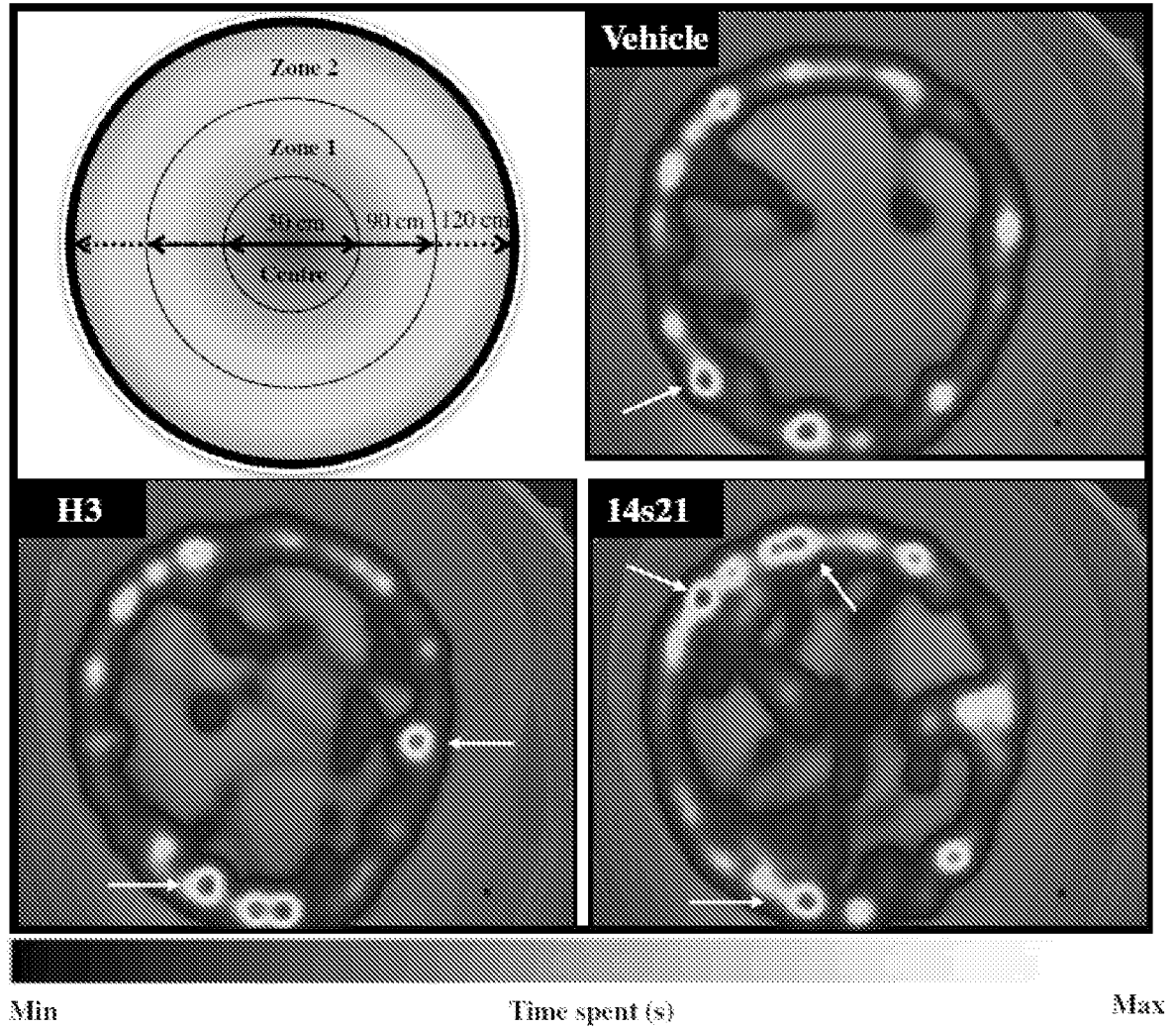


Fig. 5F

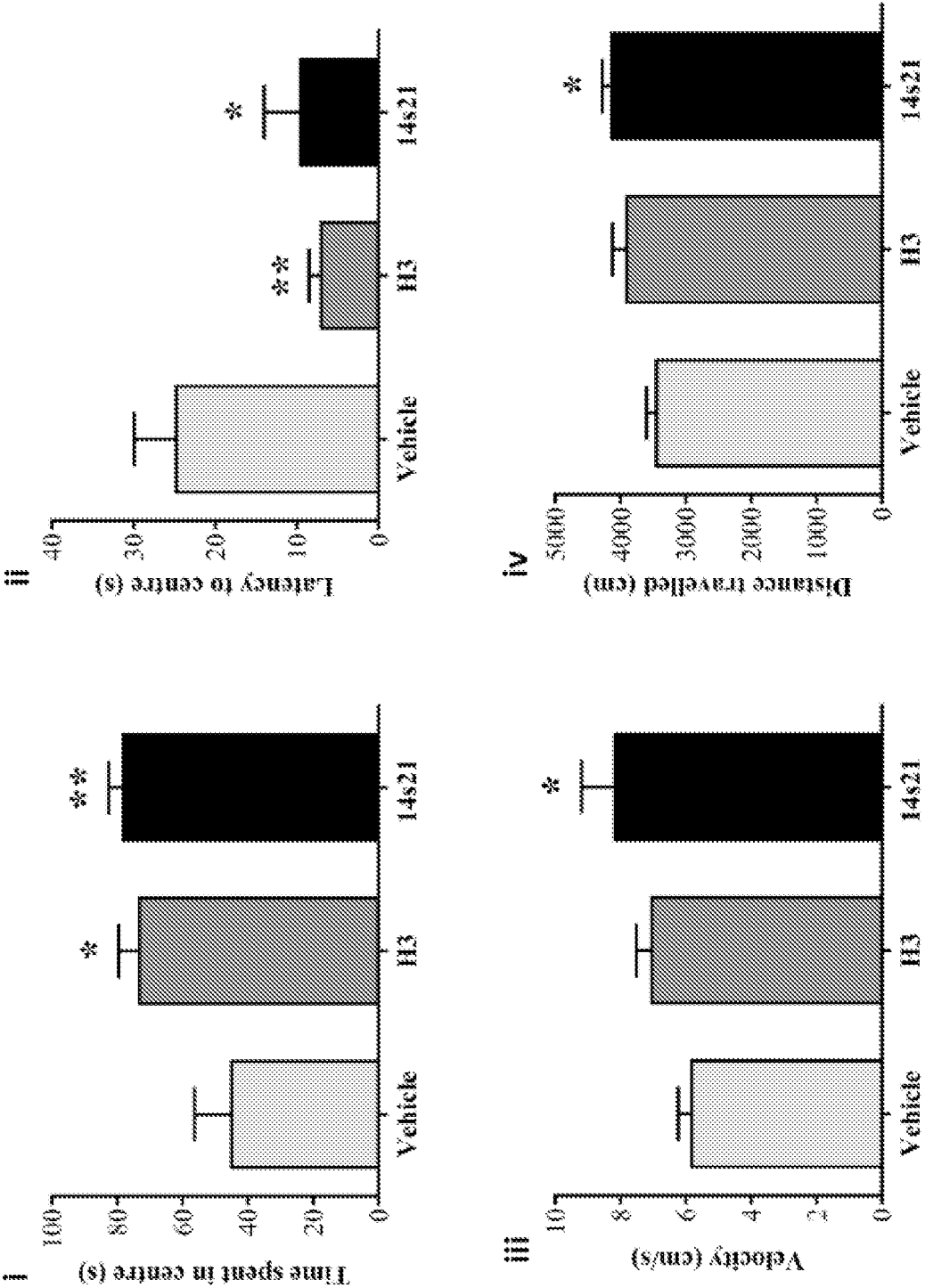


Fig 5G

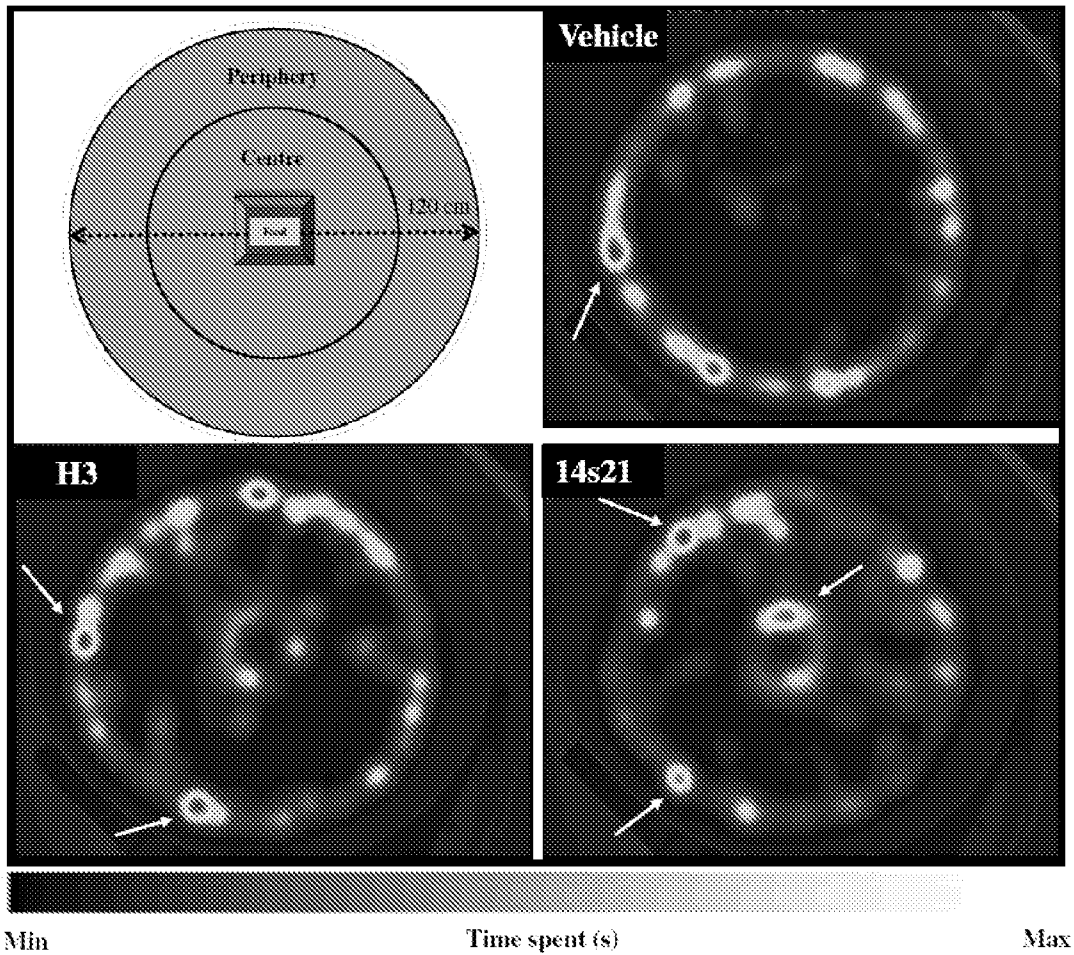


Fig. 5H

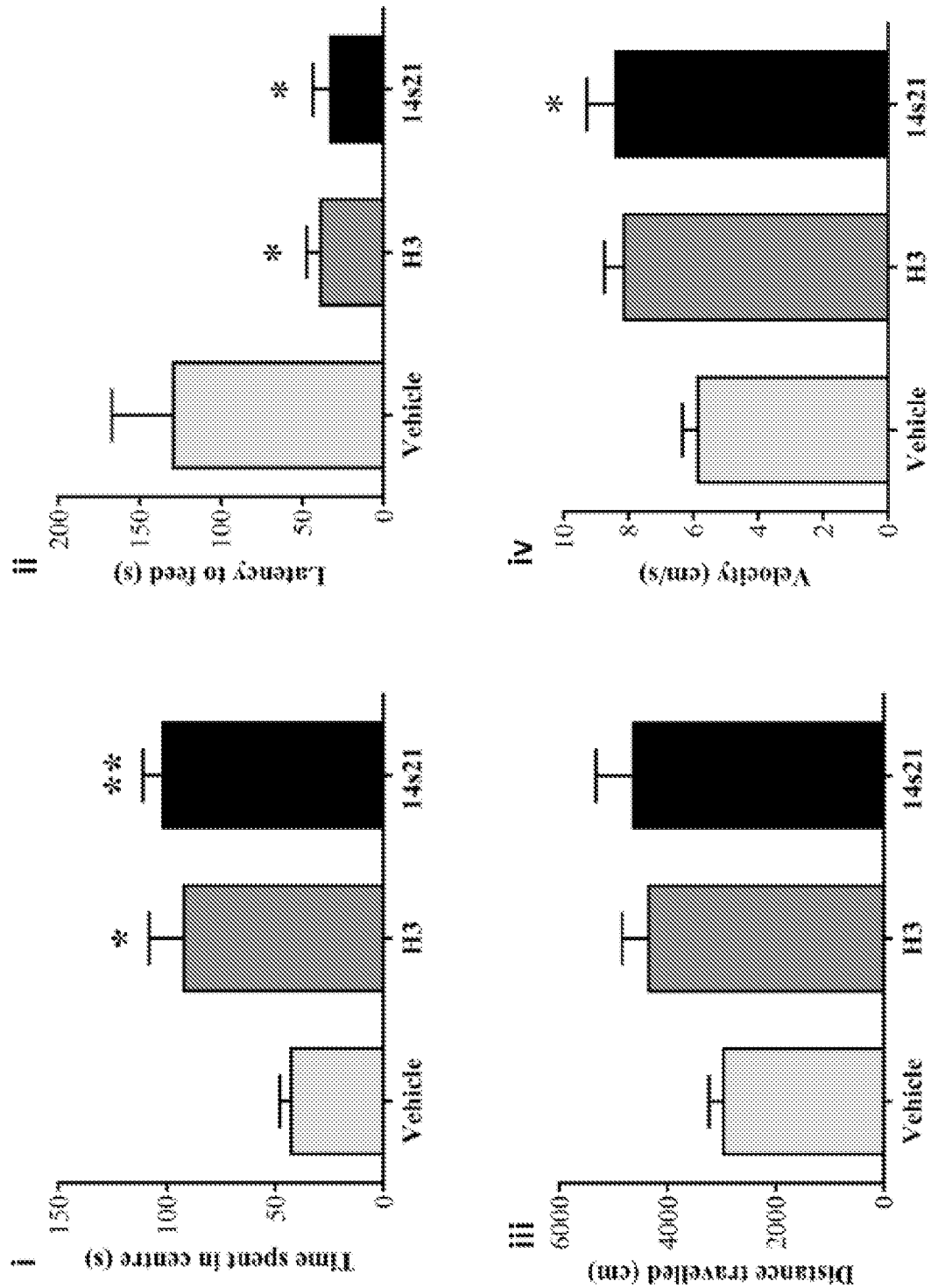


Fig. 6A

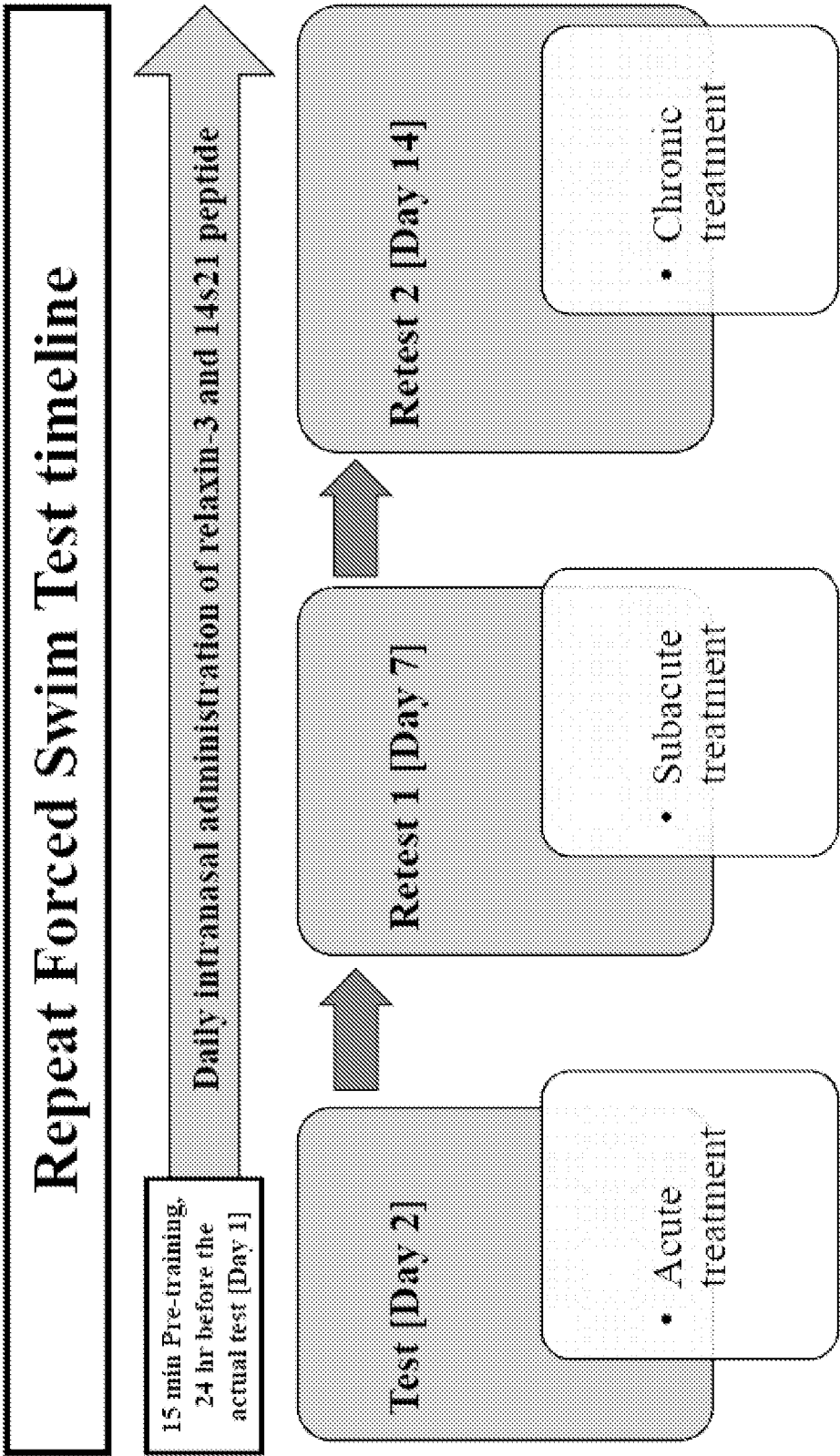


Fig. 6B

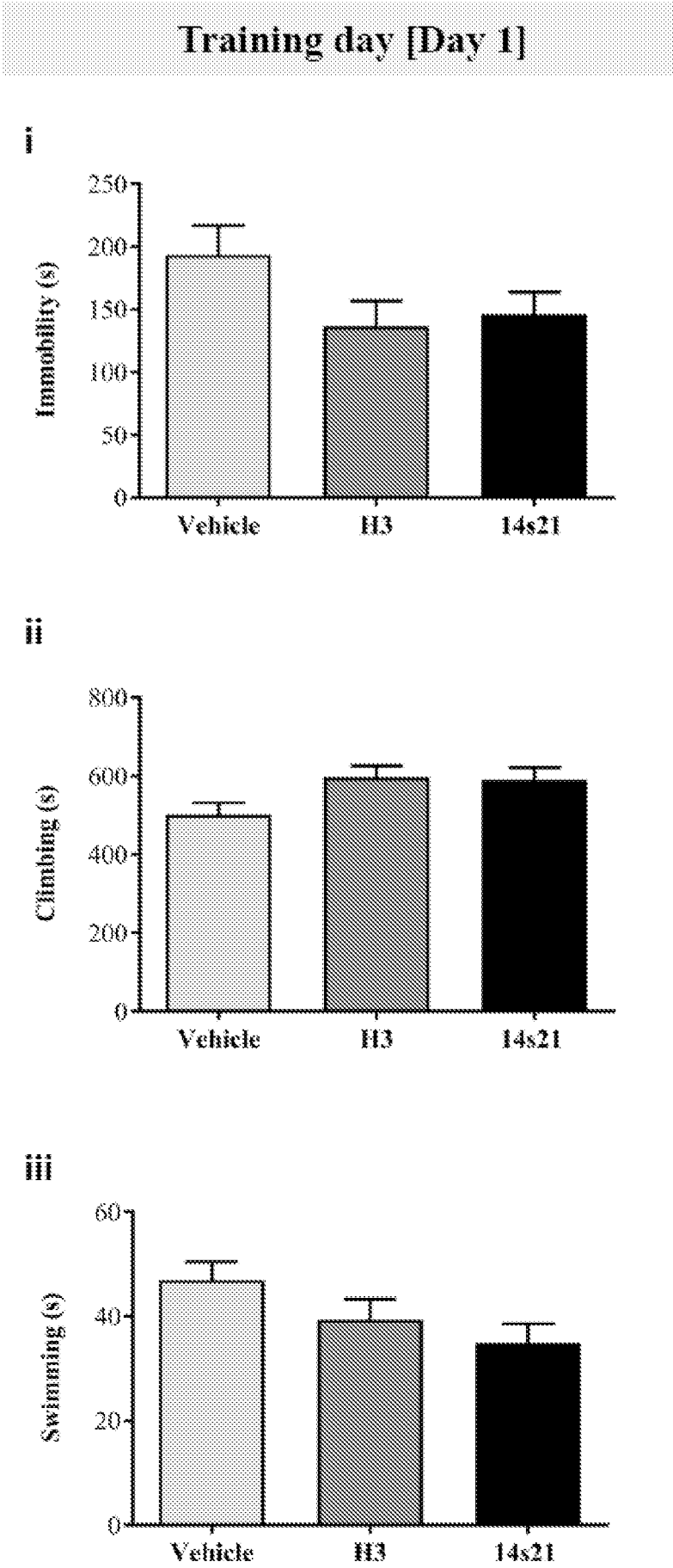


Fig. 6C

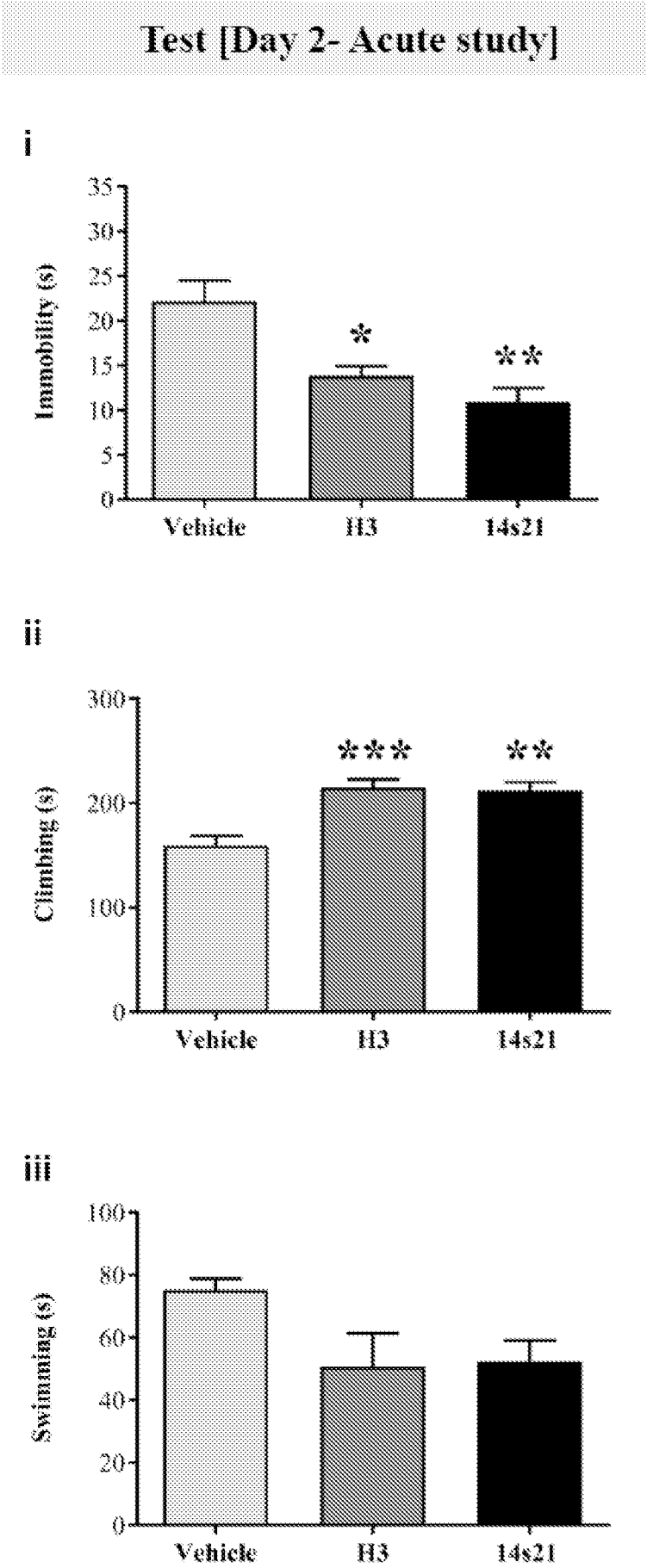


Fig. 6D

Retest 1 [Day 7- Subacute study]

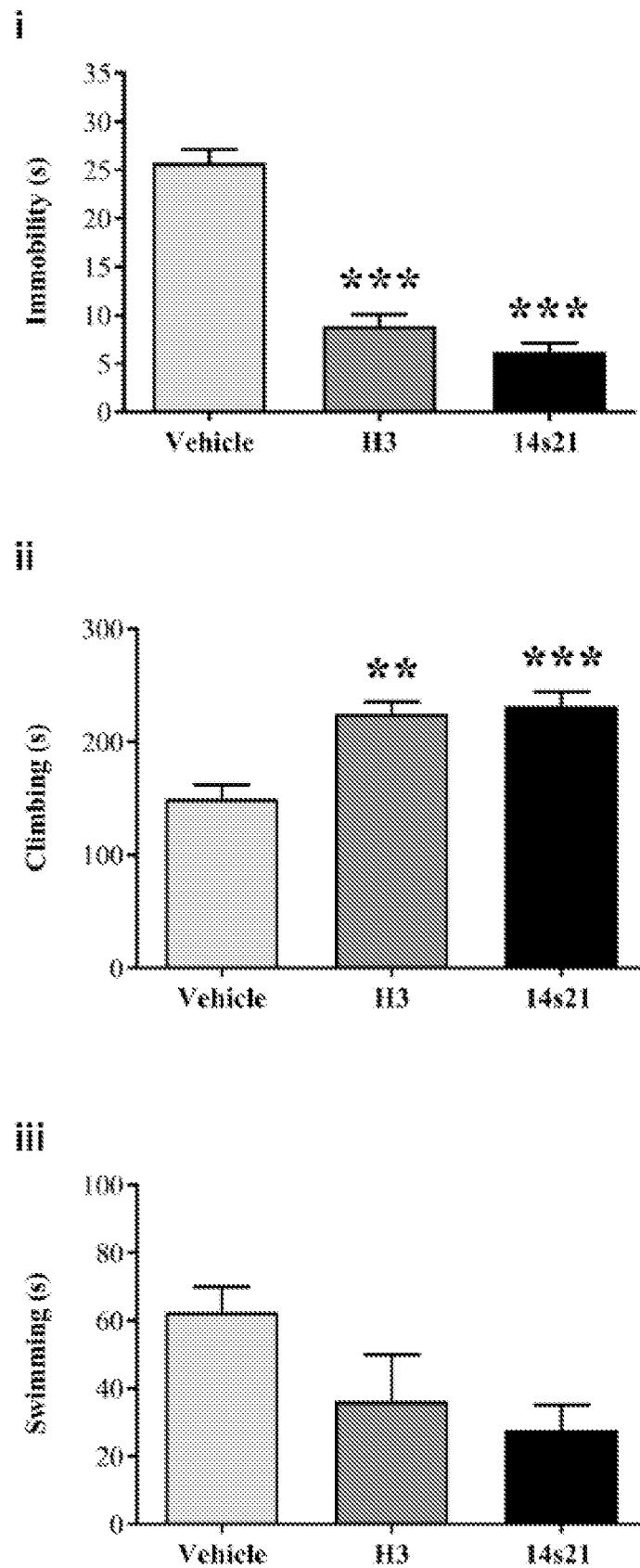


Fig. 6E Retest 2 [Day 14- Chronic study]

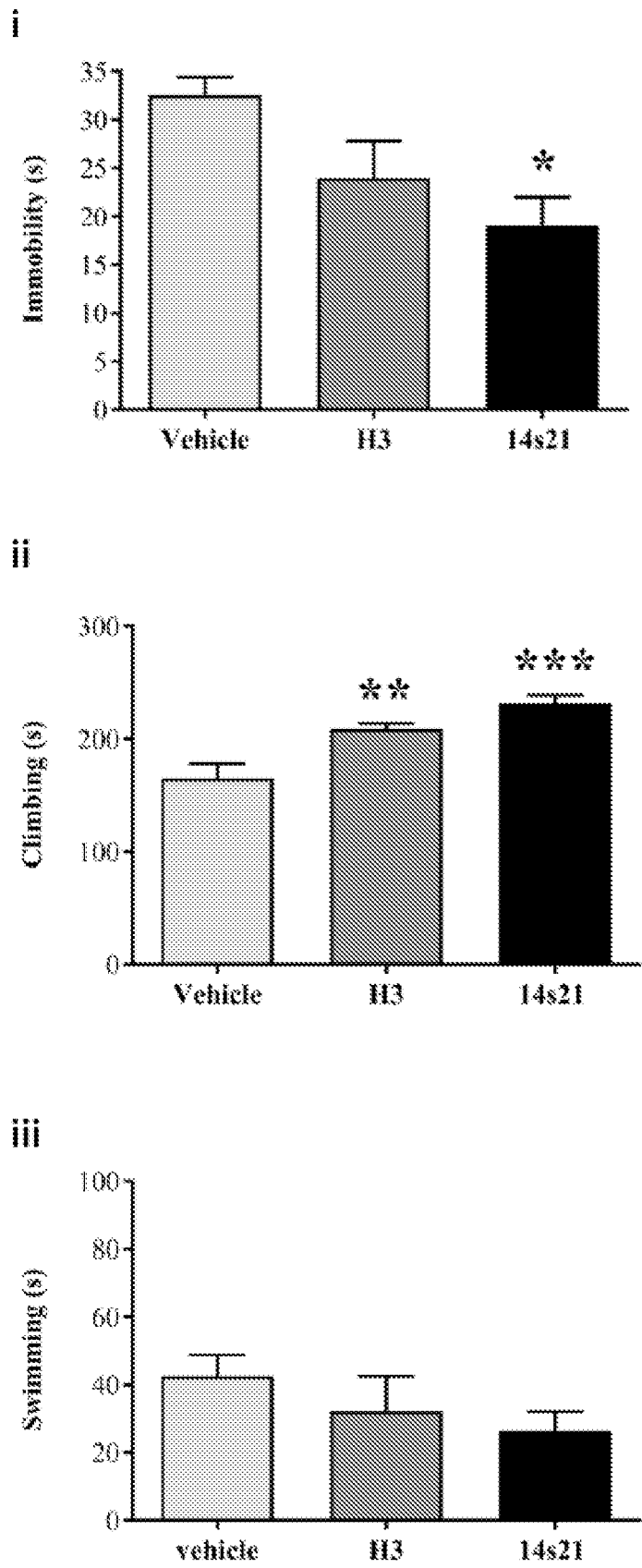


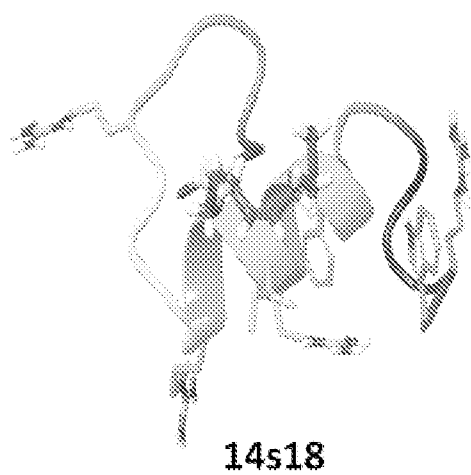
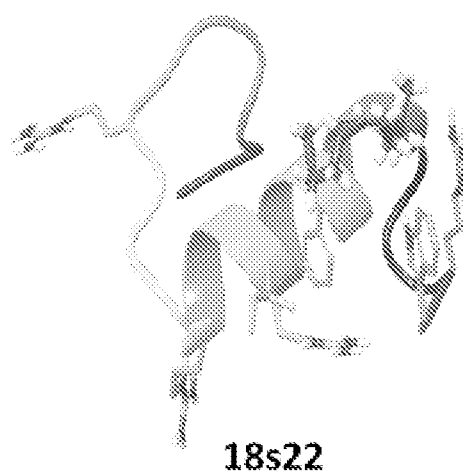
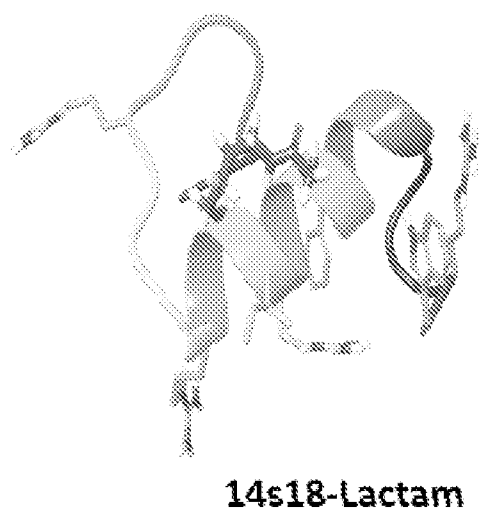
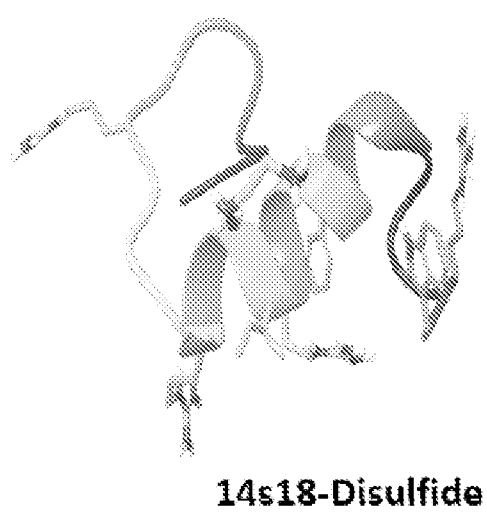
Fig. 7A**Fig. 7B****Fig. 7C****Fig. 7D**

Fig. 7E

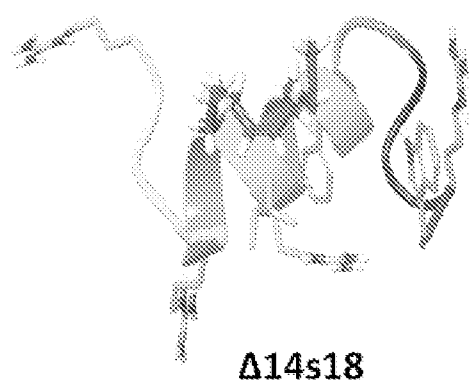


Fig. 7F

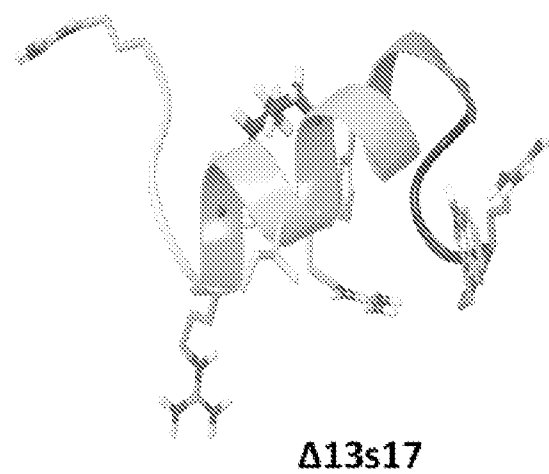


Fig. 7G

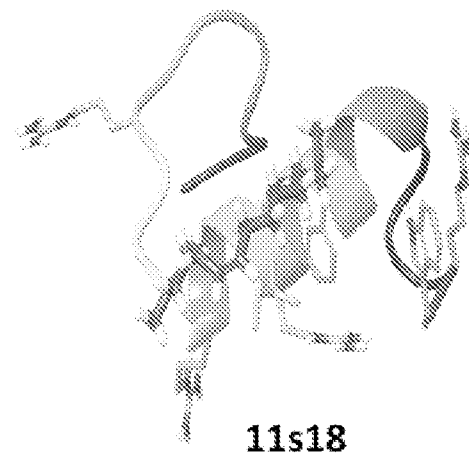


Fig. 7H

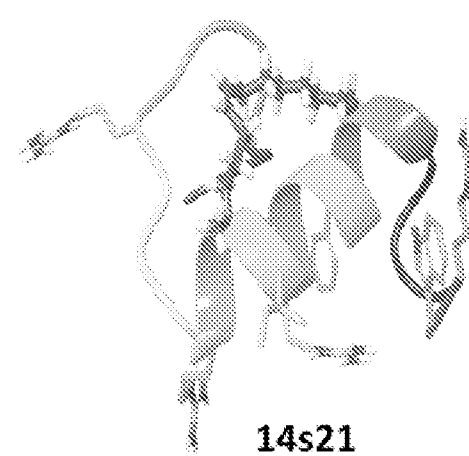
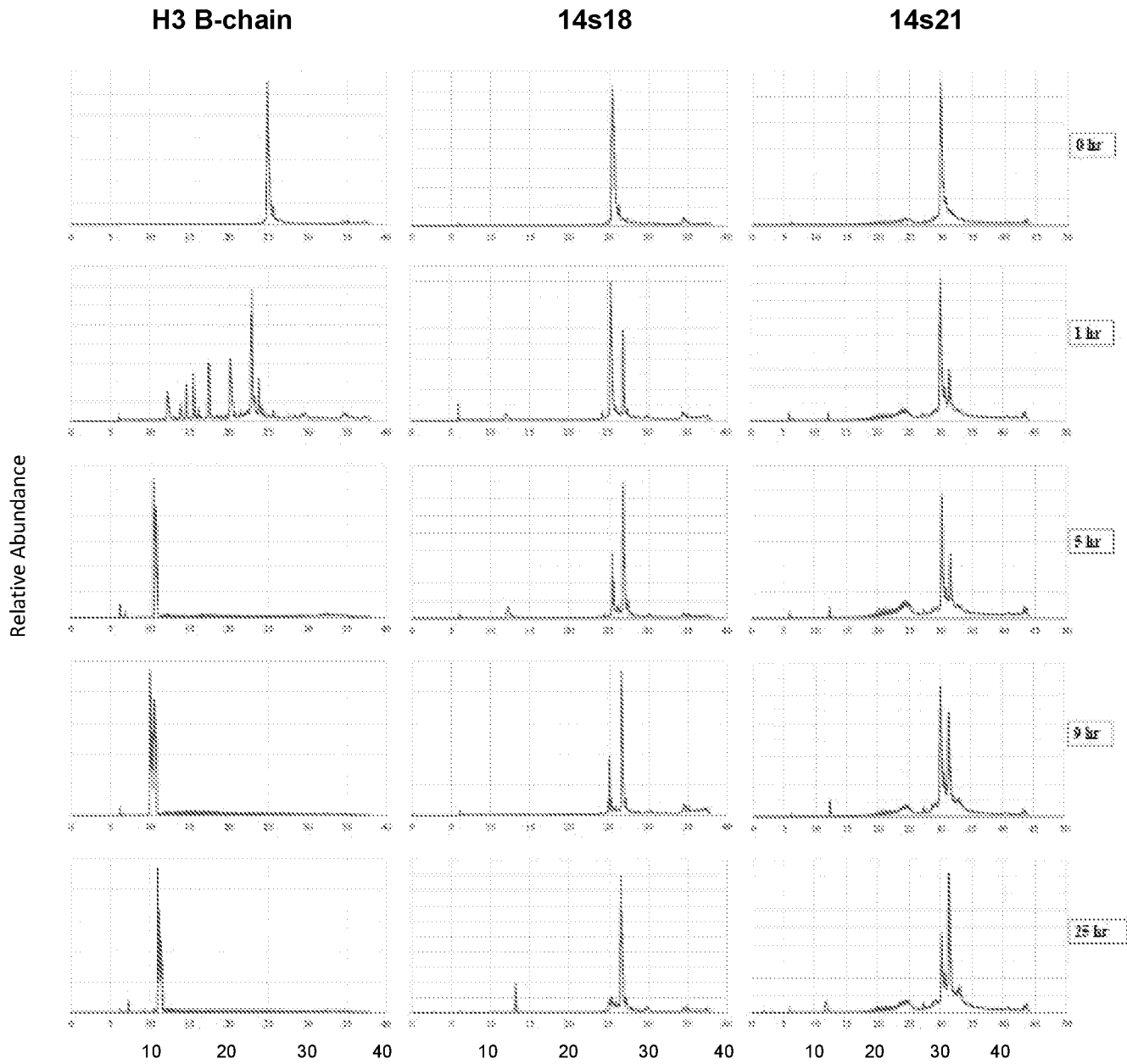


Fig. 8



INTERNATIONAL SEARCH REPORT

International application No.

PCT/SG2018/050568

A. CLASSIFICATION OF SUBJECT MATTER

See Supplemental Box

According to International Patent Classification (IPC)

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
FAMPAT/CAPLUS/BIOSIS/EMBASE/MEDLINE: relaxin-3, stapled, neuropsychiatric or eating disorders, and similar terms.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	JAYAKODY T. ET AL., Hydrocarbon stapled B chain analogues of relaxin-3 retain biological activity. <i>Peptides</i> , 3 August 2016, Vol. 84, pages 44-57 [Retrieved on 2019-01-28] <DOI: 10.1016/J.PEPTIDES.2016.08.001> Section 2.2 "Designing and modelling of the stapled peptides"; Section 2.3 "Stapled peptides"	1-20
X	HOJO K. ET AL., Development of a Single-Chain Peptide Agonist of the Relaxin-3 Receptor Using Hydrocarbon Stapling. <i>J Med Chem</i> , 27 July 2016, Vol. 59, No. 16, pages 7445-7456 [Retrieved on 2019-01-28] <DOI: 10.1021/ACS.JMEDCHEM.6B00265> Results "Design and Synthesis of Stapled Peptides"; Fig. 1b	1-20
A	WALENSKY L.D. AND BIRD G.H., Hydrocarbon-Stapled Peptides: Principles, Practice, and Progress. <i>J Med Chem</i> , 19 February 2014, Vol. 57, No. 15, pages 6275-6288 [Retrieved on 2019-01-28] <DOI: 10.1021/JM4011675> Whole document	-

☒ Further documents are listed in the continuation of Box C.

☐ See patent family annex.

*Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

28/01/2019

(day/month/year)

Date of mailing of the international search report

29/01/2019

(day/month/year)

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INTERNATIONAL SEARCH REPORT

International application No.

PCT/SG2018/050568

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	KUMAR J.R. ET AL., Relaxin' the brain: a case for targeting the nucleus incertus network and relaxin-3/RXFP3 system in neuropsychiatric disorders. <i>Br J Pharmacol</i>, 6 September 2016, Vol. 174, No. 10, pages 1061-1076 [Retrieved on 2019-01-28] <DOI: 10.1111/BPH.13564> <i>Whole document</i>	-

INTERNATIONAL SEARCH REPORT

International application No.

PCT/SG2018/050568

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:
 - a. ☐ forming part of the international application as filed:
 - ☐ in the form of an Annex C/ST.25 text file.
 - ☐ on paper or in the form of an image file.
 - b. ☐ furnished together with the international application under PCT Rule 13~~ter~~.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.
 - c. ☐ furnished subsequent to the international filing date for the purposes of international search only:
 - ☐ in the form of an Annex C/ST.25 text file (Rule 13~~ter~~.1(a)).
 - ☐ on paper or in the form of an image file (Rule 13~~ter~~.1(b) and Administrative Instructions, Section 713).
2. ☐ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

Although a sequence listing has been filed or furnished, it was not used for the purposes of this search.

Since only one version or copy of a sequence listing has been filed or furnished, the statements under item 2 are not required.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/SG2018/050568

Supplemental Box (Classification of Subject Matter)

Int. Cl.

C07K 14/64 (2006.01)

C07K 1/113 (2006.01)

A61K 38/22 (2006.01)

A61P 25/22 (2006.01)

A61P 25/24 (2006.01)