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ORIGINAL ARTICLE

Chemical synthesis and orexigenic activity of rat/mouse relaxin-3

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Abstract The insulin-like peptide, relaxin-3 was first identified just a decade ago via a genomic database search and is now recognized to be a key neuropeptide with several roles including the regulation of arousal, stress responses and neuroendocrine homeostasis. It also has significant potential as a drug to treat stress and obesity. Its actions are mediated via its cognate G protein-coupled receptor, RXFP3, which is found in abundant numbers in the brain. However, much remains to be determined with respect to the mechanism of neurological action of this peptide. Consequently, the chemical synthesis of the rat and mouse (which share identical primary structures) two-chain, three disulfide peptide was undertaken and the resulting peptide subjected to detailed in vitro and in vivo assay. Use of efficient solid-phase synthesis methods provided the two regioselectively S-protected A- and B-chains which were readily combined via sequential disulfide bond formation. The synthetic rat/mouse relaxin-3 was obtained in high purity and good overall yield. It demonstrated potent orexigenic activity in rats in that central intracerebroventricular infusion led to significantly increased food intake and water drinking.

Keywords Solid-phase peptide synthesis · Relaxin-3 · RXFP3 · Insulin-like peptide 7 · Rat/mouse relaxin-3

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Introduction

Relaxin-3 is a highly conserved 51 amino acid neuropeptide that was discovered in 2002 (Bathgate et al. 2002, 2005; Burazin et al. 2002; Wilkinson et al. 2005). It primarily binds to and activates the G-protein-coupled receptor, Relaxin Family Peptide 3 receptor (RXFP3) in vivo (Liu et al. 2003), although it can also bind to and activate the related receptors, RXFP1 and RXFP4, both in vitro and in vivo but with lower affinity (Sudo et al. 2003; Bathgate et al. 2005, 2006b). Relaxin-3 and RXFP3 are highly expressed in the brain and studies that have mapped their neuroanatomical distribution within rat (Burazin et al. 2002; Tanaka et al. 2005; Ma et al. 2007) and mouse (Smith et al. 2010) brain have indicated that the relaxin-3/RXFP3 system likely modulates a number of key limbic, hypothalamic, and broadly distributed neuronal circuits that control modalities such as arousal, stress

responses, and metabolic and neuroendocrine homeostasis (Smith et al. 2011; Gundlach et al. 2013).

Indeed, since its discovery, the role of the relaxin-3/RXFP3 system in the brain has been investigated in a number of in vivo studies and examinations of physiology and behavior following central infusion of H3 relaxin or mimetic peptides have been particularly informative in indicating the endogenous functions of relaxin-3, although these studies have to date been exclusively conducted in rats. Most notably, acute intracerebroventricular (icv) infusion of human relaxin-3 (H3 relaxin) was shown to increase food consumption during the hour post-infusion (McGowan et al. 2005). Since this initial finding, several independent studies have confirmed that acute central infusion of H3 relaxin or specific RXFP3 agonist peptides are potently orexigenic in rats, while chronic infusion results in bodyweight gain and associated metabolic effects (McGowan et al. 2006, 2007; Hida et al. 2006; Sutton et al. 2009; Ganella et al. 2012a, b). Other effects of central icv or local infusion of H3 relaxin or specific RXFP3 agonists have been noted, such as stimulation of the hypothalamic–pituitary–adrenal (HPA) and hypothalamic–adrenal–gonadal (HPG) axes (McGowan et al. 2008; Watanabe et al. 2010), and modulation of hippocampal theta rhythm (Ma et al. 2009).

Human relaxin-2 (known as 'relaxin' in non-human species) is another member of the relaxin family of peptides and its cognate receptor is RXFP1 (Bathgate et al. 2006b). Relaxin is a hormone long studied for its roles in peripheral tissues, especially during pregnancy (Hisaw 1926; Sherwood 2004); but relaxin also signals within the brain, and stimulates water drinking in rats, via strong RXFP1 activation within the circumventricular organs and hypothalamic brain regions that control fluid balance (Hornsby et al. 2001; Sunn et al. 2002). H3 Relaxin infusion has been reported to increase water consumption to a level comparable to that mediated by H2 relaxin (Bathgate et al. 2006b; McGowan et al. 2005), but as H3 relaxin can bind/activate RXFP1, the dipsogenic response produced by H3 relaxin is likely to be primarily due to the activation of RXFP1 rather than RXFP3. This idea is supported by a recent study in which central infusion of H3 relaxin activated neurons within circumventricular organs and fluid balance-related regions in a pattern almost identical to that elicited by H2 relaxin (Otsubo et al. 2010). RXFP1 is also expressed by neurons within deeper brain tissues and circuits of rat brain (Ma et al. 2006) suggesting it can affect a range of complex behaviors, although few studies have investigated this possibility directly, apart from an early report on the effect of RXFP1 activation within the amygdala on aversive memory consolidation (Ma et al. 2005).

With the aim of facilitating future pharmacological studies in rodents to further probe the function of relaxin-3, here we describe the novel synthesis of rat/mouse relaxin-3 (i.e., the sequence of relaxin-3 is identical in both species) (Wilkinson et al. 2005; Bathgate et al. 2006a). Although rat/mouse relaxin-3 only differs from H3 relaxin by four amino acids, the injection into rats or mice of 'native' peptide, rather than a foreign peptide species, is favored where possible to eliminate the potential of an immune response and to minimize 'non-endogenous' cross-reactive receptor binding. Therefore, the present studies describe the efficient chemical synthesis, structural analysis and detailed in vitro and in vivo biological activity of rat/mouse relaxin-3.

Materials and methods

Materials

9-Fluorenylmethoxycarbonyl (Fmoc) protected L-α-amino acids and 1-[bis(dimethylamino)methylene]-1*H*-benzotriazolium hexafluorophosphate 3-oxide (HBTU) were purchased from GL Biochem (Shanghai, China). Piperidine (PPD) and trifluoroacetic acid (TFA) were purchased from Auspep (Melbourne, Australia). Fmoc-PAL-PEG-PS resins with substitution of 0.18 mmol/g were purchased from Applied Biosystems Inc. (Melbourne, Australia). Dimethylformamide (DMF), methanol, diethyl ether, and dichloromethane (DCM) were purchased from Merck (Melbourne, Australia). 3,6-Dioxa-1,8-octanedithiol (DODT), triisopropylsilane (TIPS), diisopropylethylamine (DIPEA) and trifluoromethanesulfonic acid (TFMSA) were purchased from Sigma-Aldrich (Sydney, Australia). 2,2-Dipyridyl disulfide (DPDS) was purchased from Fluka (Switzerland). Acetonitrile was purchased from BDH Laboratory Supplies, (Poole, UK). All other reagents were from Sigma-Aldrich (Sydney, Australia).

Methods

Solid-phase peptide synthesis

For rat/mouse and human relaxin-3, regioselectively S-protected A- and B-chains were separately synthesized by the continuous flow Fmoc solid-phase method using an automatic PerSeptive Biosystems Pioneer peptide synthesizer (Framingham, MA, USA) or using microwave-assisted synthesis on a Liberty system (CEM Corporation, Charlotte, NC, USA) (Wade et al. 2012). The synthetic A- and B-chains were then subjected to sequential disulfide bond formation as previously described (Hossain et al. 2008).

Peptide characterization

The purity of the synthetic peptides was assessed by analytical RP-HPLC on a Phenomenex C18 column (pore size

300 Å, particle size 5 μm, 4.6 × 250 mm) using a gradient of acetonitrile in 0.1 % aqueous trifluoroacetic acid. The product was confirmed by MALDI-TOF mass spectrometry using a Bruker Autoflex II instrument (Bremen, Germany) in the linear mode at 19.5 kV. The peptides were quantified by amino acid analysis of a 24 h acid hydrolysate using a Shimadzu microbore RP-HPLC system.

Circular dichroism spectra

Circular dichroism (CD) spectra were recorded between 200 and 250 nm on JASCO (J-185, Tokyo, Japan) at 25 °C using 1-mm path length cell. The peptide was dissolved in 10 mM phosphate buffer with 137 mM NaCl (pH 7.4) at a concentration of 0.1 mg/ml.

Whole cell RXFP3 binding assay

CHO-K1 cells stably expressing RXFP3 were plated into a 96 well plate (Viewplate; opaque white wall and clear bottom, PerkinElmer, Glen Waverly, Australia) at a density of 5×10^4 cells/well and grown over night to reach ~90 % confluence before experimentation. Binding assays were conducted as described (Shabanpoor et al. 2011). Briefly, the competition binding assay was conducted using a single concentration of Eu-labeled INSL5A/H3 relaxin B (Eu-R3/I5) (0.5 nM) in the presence of increasing concentrations of rat/mouse relaxin-3. The binding affinity of rat/mouse relaxin-3 was compared with synthetic H3 relaxin. Each concentration point was performed in triplicate and the data expressed as the mean ± SEM of three independent experiments.

Inhibition of forskolin induced cAMP accumulation

The potency of the synthetic rat/mouse relaxin-3 and H3 relaxin was assessed by measuring their influence on forskolin-induced cAMP signalling in CHO-K1 cells stably expressing RXFP3 as previously reported (Shabanpoor et al. 2012).

Animals

Male Sprague–Dawley rats ($n = 15$; 250–300 g), supplied by the Animal Resources Centre (Perth, Australia) were housed under ambient conditions (21 °C) and maintained on a 12-h light–dark cycle (lights on 0700–1900 hours) with access to laboratory chow and water ad libitum. Experiments were conducted with the approval of the Florey Institute Animal Welfare Committee and according to the ethical guidelines issued by the National Health and Medical Research Council of Australia.

Stereotaxic implantation of cannula into lateral ventricle

Rats were deeply anesthetized with 4 % isoflurane in oxygen, 2 l/min (Delvet, Seven Hills, Australia), and maintained with 2–3 % isoflurane in oxygen, 0.2 L/min. Each rat was positioned in a stereotaxic frame (David Kopf Instruments, Tujunga, USA) and a stainless-steel guide cannula (22 gauge, cut 5 mm below pedestal; Plastics One, Roanoke, USA) was implanted with the cannula tip inserted into the lateral ventricle (coordinates: anteroposterior, −0.8 mm; mediolateral, −1.5 mm; dorsoventral −3.5 mm) (Paxinos and Watson 2007) and affixed to the skull using surgical screws and dental cement. Rats were then placed under a heat lamp in a clean cage until regaining consciousness. Meloxicam (3 mg/kg, i.p.; Troy Laboratories, Smithfield, Australia) and 0.5 mg/ml paracetamol in 5 % sucrose/water (for 3 days) were administered to provide acute and ongoing post-operative analgesia, respectively. Baytril 50 (enrofloxacin) (2 mg/kg, i.p; Bayer Australia Ltd, Pymble, Australia) was administered post-operatively as a prophylactic antibiotic.

Rats were single-housed and allowed to recover for 7 days, during when, they were handled and weighed daily to habituate them to the experimenter. A stylet of stainless steel wire (30-gauge) was inserted into each cannula that extended no further than the base of the cannula to maintain patency.

Infusion procedure and verification of cannulation using angiotensin II

Lateral ventricle infusions were made using 29-gauge hypodermic tubing (Small Parts Inc., Miramar, USA) connected to a 10 μl Hamilton microsyringe (Hamilton Instruments, Reno, USA) by polyethylene tubing (0.80-mm outer and 0.40-mm internal diameters; Microtube Extrusions, North Rocks, Australia). Correct positioning of the cannula was verified in each rat by injecting 5 μl of a 4 ng/μl solution of human angiotensin II (Auspep, Parkville, Australia) in artificial cerebrospinal fluid (aCSF; 147 mM NaCl; 4 mM KCl; 0.85 mM $MgCl_2$; 2.3 mM $CaCl_2$), and observing whether this produced a positive dipsogenic response, defined as repeated drinking episodes of ≥5 s that commenced within 1 min of angiotensin II administration. Injectors that extended 1.5 or 2 mm below the base of the cannula were tested as required on consecutive days and the length of the effective injector was recorded for each rat and used for all subsequent experiments. Rats that failed to display a drinking response were used as controls in subsequent experiments.

Peptide treatment

Rat/mouse relaxin-3 was dissolved in aCSF vehicle at a concentration of 2 μg/μl. Rats were divided into two

groups: group 1 received a 5 µl injection of rat/mouse relaxin-3 (equivalent to 10 µg); group 2 received 5 µl of aCSF (vehicle).

Food and water intake

Rats were habituated for a minimum of 7 days to the holding room and behavioral studies were performed during the light phase, beginning at 1100 hours. Groups of rats were injected as described. Following infusion, rats were returned to their home cage where a pre-weighed amount of rat chow (10–13 g) was located in the food compartment of the wire cage lid. A pre-weighed water bottle was also placed in its usual compartment, and food and water were then weighed 1 h post-infusion.

Data analysis

Data were analyzed using GraphPad Prism V5.00 for Windows (GraphPad Software, San Diego, USA). Results are expressed as mean ± SEM. Statistical significance was evaluated using a Student's *t* test with $P < 0.05$ considered significant. Food and water intake levels of rats that were 'mock injected' were combined with data from the vehicle injected group, as there was no significant difference between them.

Results and discussion

Definitive physiological roles of H3 relaxin in human are not yet known, but anatomical, neurochemical and pharmacological studies in rodents have identified the extensive brain relaxin-3/RXFP3 signalling networks as potential targets for the treatment of a range of psychiatric conditions, including anxiety, depression, sleep disorders, and associated cognitive deficits (Smith et al. 2011). Over recent years, in vivo pharmacological studies to explore the function of the relaxin-3/RXFP3 system have been greatly facilitated by the development of several synthetic RXFP3 agonist and antagonist peptides (Shabanpoor et al. 2012; Haugaard-Kedstrom et al. 2011; Kuei et al. 2007; Liu et al. 2005; Shabanpoor et al. 2011). In line with these endeavors, the present studies describe the synthesis of rat/mouse relaxin-3, which represents an important addition to the pharmacological tool box available to investigate relaxin-3/RXFP3 systems in the major experimental species of rodents.

Chemical assembly of rat/mouse relaxin-3

A regioselective disulfide synthesis approach was used to synthesize rat/mouse relaxin-3. Three differential cysteine S-protecting groups (Trt, tBu and Acm) were used that allowed the directed formation of three disulfide bonds. Solid-phase synthesis of the separate, selectively S-protected A- and B-chains followed by their purification and subsequent stepwise formation of each of the three disulfides via oxidation, thioloysis, and iodolysis (Hossain et al. 2008, 2009, 2010) led to the successful preparation of rat/mouse relaxin-3. Its analytical RP-HPLC profile (Fig. 1a) and MALDI TOF MS spectrometry (theoretical, MH+5497.51; found, MH+5497.87; Fig. 1b) revealed a highly purified synthetic product. The peptide content (81.6 %) and amino acid composition were determined by amino acid analysis. Rat/mouse relaxin-3 was made as the C-termini amide and the yield (~12 %) was comparable to H3 relaxin amide (11 %), and was twice that of H3 acid (~6 %) (Bathgate et al. 2006b). We also synthesized H3 relaxin for comparative purposes.

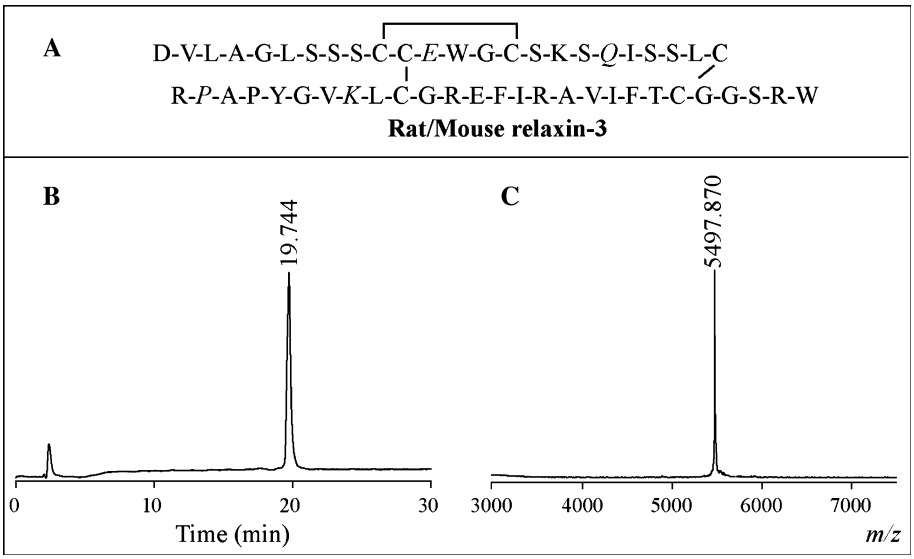

Fig. 1 **a** Primary structure. **b** RP-HPLC trace and **c** MALDI-TOF MS trace of rat relaxin-3 which is 100 % identical to mouse relaxin-3

Conformation of rat/mouse relaxin-3

The conformation of synthetic rat/mouse relaxin-3 was analyzed by CD spectroscopy (Fig. 2). H3 relaxin was used as a control due to its sequence similarity with rat/mouse relaxin-3, and because it has been well characterized (Bathgate et al. 2006b). Both human and rat/mouse relaxin-3 form a significant degree of helical conformation. The helix content of rat/mouse relaxin-3, calculated from the mean residual weight ellipticity at 222 nm, $[\theta]_{222}$ (Scholtz et al. 1991), was overlapping with H3 relaxin (32 %), indicating that rat/mouse relaxin-3 has the same or similar secondary structure to H3 relaxin, which does not appear to be affected by the four amino acid differences between the two peptides.

Activity of rat/mouse relaxin-3 at RXFP3

Synthetic rat/mouse relaxin-3 was first tested for its ability to bind human RXFP3 (Fig. 3a), and it demonstrated slightly higher affinity at RXFP3 than H3 relaxin, although the difference was not statistically significant. Rat/mouse relaxin-3 was then tested for its ability to inhibit forskolin-induced cAMP production in human RXFP3 transfected CHO cells, as RXFP3 is $G_{i/o}$-coupled and its activation has been shown to inhibit cAMP (Liu et al. 2003). Rat/mouse relaxin-3 was able to decrease forskolin induced cAMP activity in a dose-dependent manner and to a similar degree as H3 relaxin (Fig. 3b). These findings demonstrate that rat/mouse relaxin-3 is a high affinity, potent ligand for human RXFP3 in vitro, with properties indistinguishable from H3 relaxin.

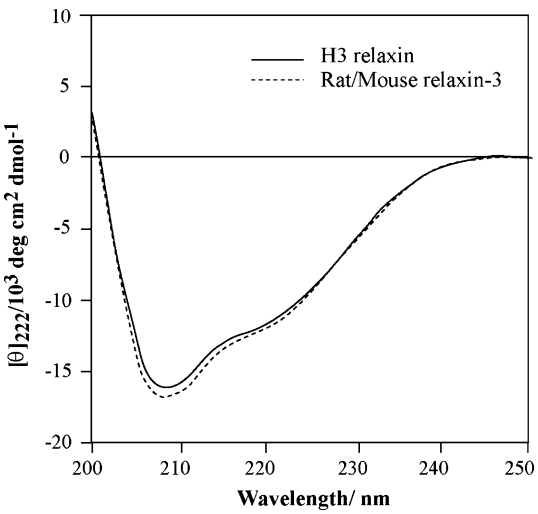

Fig. 2 Circular dichroism spectra (CD) of rat/mouse relaxin-3 compared to human relaxin-3 (H3 relaxin). CD was performed in 10 mM phosphate buffer at pH 7.5

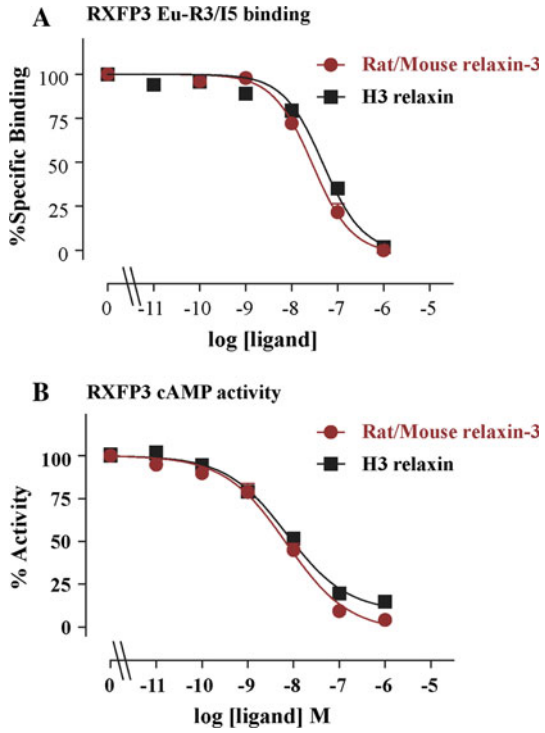

Fig. 3 Activity of rat/mouse relaxin-3 at RXFP3. **a** Competition binding of rat/mouse relaxin-3 or H3 relaxin with europium labeled R3/I5 in membranes from CHO-K1 cells stably expressing RXFP3. Data are expressed as percentage of specific binding and are pooled data from at least three experiments performed in triplicate. **b** Inhibition of forskolin-stimulated cAMP activity in RXFP3 expressing CHO-K1 cells using a pCRE-β-galactosidase reporter gene system. Data are expressed as percentage of forskolin response and are pooled data from at least three experiments performed in triplicate

Central infusion of rat/mouse relaxin-3 in rats

Several studies have demonstrated that central infusion of H3 relaxin or RXFP3-selective agonist peptides in rats is potently orexigenic (see "Introduction"), and that this effect is mediated via RXFP3, as pre-administration of an RXFP3 antagonist blocks the increase in food intake (Haugaard-Kedstrom et al. 2011), and H2 relaxin decreases food intake following local injection into the hypothalamus (McGowan et al. 2010). Furthermore, central infusion of H3 relaxin in rats increases water drinking via RXFP1 activation (Bathgate et al. 2006b; McGowan et al. 2005; Otsubo et al. 2010). Therefore, to test the ability of rat/mouse relaxin-3 to activate RXFP3 and RXFP1 in vivo, food and water consumption following acute icv infusion of rat/mouse relaxin-3 (10 µg, ~2 nmol) was assessed in rats.

During the hour post infusion, rats injected with rat/mouse relaxin-3 consumed significantly more food than control rats (Fig. 4a; $p < 0.05$), and also drank more water (Fig. 4b; $p < 0.05$), consistent with biological activity of rat/mouse relaxin-3 at RXFP3 and RXFP1. Rats were used rather than mice for several reasons. Firstly, the behavioral

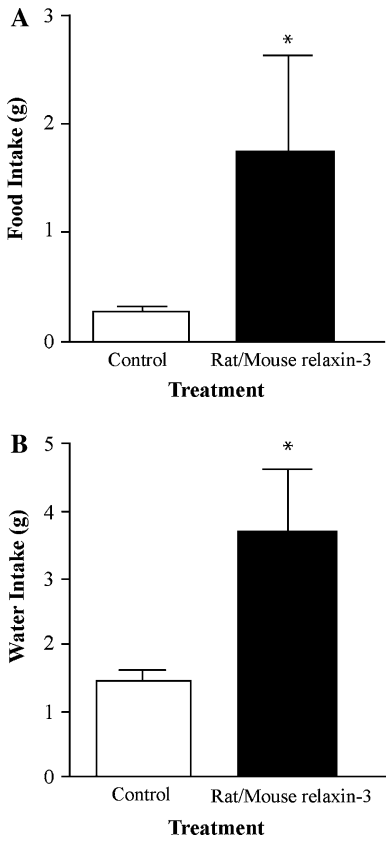

Fig. 4 Effect of central (icv) administration of rat/mouse relaxin-3 on **a** food and **b** water consumption in adult Sprague–Dawley rats during a 60-min post-injection period. Rat/mouse relaxin-3 ($n = 6$ rats); control ($n = 11$ rats)

response of rats to icv infusion of H3 relaxin or RXFP3 selective agonists is known to be robust and reproducible, whereas the equivalent response in mice is not yet well characterized, and secondly, although H3 relaxin (and rat/mouse relaxin-3) can pharmacologically bind/activate RXFP4 in vitro (Liu et al. 2005; Bathgate et al. 2013), RXFP4 is a pseudogene in rats (Chen et al. 2005), and hence, cannot contribute to any effect of relaxin-3 observed in vivo. However, as RXFP4 expression is absent or very low (undetectable) in mouse brain (Sutton et al. 2009), rat/mouse relaxin-3 could be used in future studies of native relaxin-3 effects in mouse brain, relative to the effects of RXFP3 selective peptides.

Conclusion

These studies describe the first chemical preparation of rat/mouse relaxin-3. In vitro testing of the peptide revealed correct secondary structure conformation and potent RXFP3 binding activity, while icv infusion in rats increased food intake and water drinking, consistent with predicted biological activity at both relaxin-3 and relaxin receptors, RXFP3 and RXFP1, respectively. Synthetic rat/mouse relaxin-3, thus, provides a valuable complement to H3 relaxin for future in vivo pharmacological studies in rats or mice. Such approaches are powerful for probing the endogenous role of relaxin-3 in the brain under different physiological and pathological conditions, particularly in combination with the use of relevant transgenic mouse models and complementary viral-based methods (Ganella et al. 2012a; Smith et al. 2012). Current efforts are focusing on the preparation of stable analogues of relaxin-3 (Werle and Bernkop-Schnurch 2010) with the goal of delivering it via iv or ip routes, possibly with the aid of brain targeting adducts (Li et al. 2012). Such studies are an important scientific and translational endeavor, as the relaxin-3/RXFP3 system represents a promising target for the development of treatments for a variety of metabolic, neuroendocrine and psychiatric diseases.

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