Central Relaxin-3 administration causes hyperphagia in male Wistar rats

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Abstract

Relaxin-3 (INSL-7) is a recently discovered member of the insulin superfamily. Relaxin-3 mRNA is expressed in the nucleus incertus of the brainstem which has projections to the hypothalamus. Relaxin-3 binds with high affinity to the LGR7 receptor and to the previously orphan G-protein-coupled receptor GPCR135. GPCR135 mRNA is expressed predominantly in the CNS, particularly in the paraventricular nucleus (PVN). The presence of relaxin-3 and its receptors in the PVN led us to investigate its effect on appetite by examining the effect of central administration of relaxin-3 on food intake in male Wistar rats, and to investigate which receptor may be involved in mediating these effects. Intracerebroventricular (ICV) injections of human relaxin-3 (H3) in satiated rats significantly increased food intake 1h post-administration [0.96 ± 0.16g (vehicle) vs 1.81 ± 0.21g (180pmol H3), $p< 0.05$] and in the early dark phase [2.95 ± 0.45g (vehicle) vs 4.39 ± 0.39g (180pmol H3), $p< 0.05$]. IntraPVN administration of relaxin-3 significantly increased 1h food intake in satiated rats [0.34 ± 0.16g (vehicle) vs 1.23 ± 0.30g (18pmol H3), $p< 0.05$] and in the early dark phase [4.43 ± 0.32g (vehicle) vs 6.57 ± 0.42g (18pmol H3), $p< 0.05$]. Feeding behavior was increased after iPVN relaxin-3 with no other altered behaviors. Equimolar doses of human relaxin-2, which binds the LGR7 receptor but not GPCR135, did not increase feeding. There was no acute change in NPY, POMC or AgRP mRNA expression 4h following ICV relaxin-3. These results suggest a novel role for relaxin-3 in appetite regulation.

Introduction

The relaxin peptides belong to the insulin superfamily, a group of structurally related hormones typified by the presence of an A and B chain linked by disulphide bridges and an intra-chain disulphide bond, similar to insulin (1). Until recently, a single relaxin gene had been described in most mammalian species, M1 and R1 in mice and rats respectively (2;3) and H2 in humans (4). The gene product is secreted by the corpus luteum in early pregnancy and is primarily associated with female reproductive physiology, as well as having a dipsogenic effect when administered both peripherally and centrally (5;6). However, a further relaxin gene, relaxin-3, has now been identified in humans (H3) (7), mice (M3) (7) and most recently in rats (R3) (8). The gene products of H3, M3 and R3 retain their insulin-like peptide structure and are highly homologous. Whilst R1 and M1 mRNA are expressed in many tissues, R3 and M3 mRNA expression is localised to the nucleus incertus (NI) of the brainstem (8) which has extensive projections to the hypothalamus (9). These include areas such as the lateral mammillary nucleus, the supramammillary nucleus, the posterior hypothalamic nucleus, the lateral hypothalamic zone, and relatively weaker inputs from the NI to the medial and periventricular zones (9). Relaxin–like immunoreactivity has been described in the hypothalamic arcuate (ARC) and paraventricular (PVN) nuclei (10).

Unlike insulin, relaxin peptides signal via G-protein coupled receptors to modulate intracellular cAMP. The gene products of R1 and M1 act via two leucine-rich repeat-containing receptors, LGR7 and LGR8 (11). More recent studies suggest that relaxin-1 may be the endogenous ligand for LGR7 and another insulin-like peptide, INSL3, may be the physiological ligand for LGR8 (12). LGR7, expressed predominantly in

reproductive tissues but also in the CNS (11), binds relaxin-3 with high affinity (13). However, relaxin-3 is the cognate ligand for two previously orphan G-protein-coupled receptors, GPCR135 and GPCR142 (14;15). Whilst GPCR142 expression is absent in the rat, GPCR135 mRNA is highly expressed in the rat brain, particularly the PVN and the supraoptic nucleus (SON) (14;16). The distribution of relaxin-3 and its receptors suggest this system could play a role in the regulation of appetite. The aims of these studies are to investigate the effects of relaxin-3 on food intake and to examine which receptor may mediate this effect.

Methods

Materials

Human relaxin-3 (H3) was purchased from Phoenix Pharmaceuticals (Belmont, CA) and synthesized by the company using solid phase synthesis. Recombinant human relaxin-2 (H2) was purchased from Dr A. Parlow, National Hormone and Peptide Program (Torrance, CA). Reagents for Ribonuclease Protection Assay studies were purchased from Ambion (Austin, TX).

Animal studies

Male Wistar rats (Specific pathogen free, Charles River, UK) weighing 250-300g were maintained in individual cages for all studies. All animals were kept under controlled temperature (21-23C) and light (12h light, 12h dark, lights on at 0700h) with *ad libitum* access to food (pelleted RM1 chow diet, SDS, UK) and water. All procedures undertaken were approved by the British Home Office Animals Scientific Procedures Act 1986 (project license 70/5516).

Food and water intake studies

Male Wistar rats underwent third ventricle (ICV) or unilateral intra-paraventricular nucleus (iPVN) cannulation 7-10 days before feeding studies and were habituated to regular handling and injection, as previously described (17). Central injections (5µl (ICV) or 1µl (iPVN)) were administered over 1 minute via stainless steel injectors (27-gauge (ICV) or 31-gauge (iPVN)), placed in and projecting 1mm below the end of the cannula. Spread of a 1µl injection into the PVN is reported to be limited to 1 mm^3 (18). All compounds were dissolved in vehicle (10% acetonitrile in 0.9% saline) and studies were performed in satiated rats (n = 10-12) in the early light phase (0900 – 1000h) unless otherwise stated. Following injection, animals were returned to their home cage with pre-weighed chow. Food intake was measured at 1, 2, 4, 8, and 24 hours post-injection. NPY was administered as a positive control in food intake studies (5 nmol/animal ICV or 0.5 nmol/animal iPVN). Water intake was measured at 1 and 2 hours post-injection. Angiotensin II was administered ICV as a positive control (150 ng/rat) in water intake studies.

IntraPVN cannula position was verified histologically at the end of the study (17). Immediately following decapitation, 1 µl Indian ink was injected into the cannula. The brains were removed and fixed in 4% paraformaldehyde, dehydrated in 40% sucrose and frozen in liquid nitrogen and stored at -70C. Brains were sliced on a cryostat (Bright, Huntingdon, UK) into 15 µm coronal sections and correct PVN placement determined by microscopy according to the position of the Indian ink. ICV cannula position was verified by a positive dipsogenic response to angiotensin II

(150ng/rat). Only those animals with correct cannula placement were included in the data analysis.

Behavioral response following ICV and iPVN administration of relaxin-3

Behavioral responses were monitored following ICV administration of vehicle or 180 pmol relaxin-3 (H3) and iPVN administration of vehicle, 18 pmol or 180 pmol relaxin-3 (H3) (n = 10). Animals were immediately returned to their home cages and observed for 1h following injection by an investigator blinded to the experimental treatment. Behavior was classified into one of nine categories: feeding, drinking, grooming, burrowing, rearing, locomotion, sleep, head down, and tremor. Each rat was observed for three 3 sec periods every 6 min and the behavior in each period scored as previously described (19).

Hypothalamic neuropeptide expression following relaxin-3 administration

Hypothalamic neuropeptide mRNA expression was measured following ICV administration of vehicle or relaxin-3 (H3) (180 pmol) (n = 10-12). Food was removed immediately following injection and at four hours animals were killed, hypothalami dissected and snap frozen. Hypothalamic neuropeptide Y (NPY), agouti related peptide (AgRP) and pro-opiomelanocortin (POMC) mRNA expression were determined by ribonuclease protection assay (RPA). Briefly, RNA was extracted using Tri-Reagent (Helena Biosciences, Sunderland) according to the manufacturer's protocol. Rat β-actin (Ambion Inc.) was used to correct for RNA loading. RNA was hybridized overnight at 42C with 1.3 x 10^3 Bq of ^{32}P[CTP] labelled riboprobe. Reaction mixtures were digested with RNase A/T1, the protected fragments precipitated and separated on a 4% polyacrylamide gel. The dried gel was exposed to

a phosphorimager screen overnight and bands quantified by image densitometry using ImageQuant software (Molecular Dynamics, Sunnyvale, CA) (19).

Statistical analysis

Results are shown as mean ± S.E.M. Data from feeding and water intake studies were compared by ANOVA with *post-hoc* LSD test (Systat, Evanston, IL). Neuropeptide expression data were compared by unpaired Student's t-test between control and treated groups. Behavioral data were non-parametric and are expressed as median number of occurrences of behavior (interquartile ranges are expressed in square brackets). Comparison between groups was made by Mann-Whitney U test. In all cases, $p < 0.05$ was considered to be statistically significant.

Results

Feeding studies

To investigate the hypothesis that relaxin-3 is involved in regulation of appetite, food intake was determined following central relaxin-3 administration.

Study 1: Effect of ICV relaxin-3 on food intake in rats in early light phase and early dark phase

Animals received an ICV injection of either vehicle or relaxin-3 (18, 54 or 180 pmol H3) in the early light phase. Doses used were based on previously reported effects of porcine relaxin-1 on water intake (6). ICV relaxin-3 significantly increased food intake in the first hour at both 54 pmol and 180 pmol [0.96 ± 0.16 g (vehicle) vs 1.80 ± 0.27 g (54 pmol H3) and 1.81 ± 0.21 g (180 pmol H3), $p< 0.05$], (Fig 1A). There was no significant difference in interval food intake between control and treated

groups at later time points. However, cumulative food intake was significantly increased at all doses of relaxin-3 at 2h and 4h following ICV administration (Fig 1B).

Rats received an ICV injection of either vehicle or relaxin-3 (180 pmol H3) at the beginning of the dark phase. Nocturnal food intake was significantly increased in the first hour following relaxin-3 administration [2.95 ± 0.45 g (vehicle) vs 4.39 ± 0.39 g (180 pmol H3), $p < 0.05$], (Fig 1C). There was no significant effect on interval food intake at later time points or in cumulative food intake.

Study 2: Effect of ICV relaxin-2 on food intake in satiated rats in early light phase

To differentiate the receptor mediating the effects of relaxin-3 on food intake, the feeding response to relaxin-3, which binds both LGR7 and GPCR135 receptors, was compared to that following administration of relaxin-2 (H2), which binds LGR7 but not GPCR135. Satiated rats received an ICV injection of either 180 pmol H3 or 180 pmol H2 in the early light phase. Following ICV administration of equimolar doses, relaxin-3 stimulated one-hour food intake as previously shown [0.21 ± 0.09 g (vehicle) vs 1.50 ± 0.40 g (180 pmol H3), $p < 0.05$] (Fig 2), as well as cumulative food intake up to 4h [0.75 ± 0.27 g (vehicle) vs 2.18 ± 0.46 g (180 pmol H3), $p < 0.05$]. In contrast, relaxin-2 had no significant effect on food intake at any time point following administration [0.21 ± 0.09 g (vehicle) vs 0.43 ± 0.13 g (180 pmol H2) at 1h], (Fig 2).

Study 3: Effect of ICV relaxin-2 and relaxin-3 on water intake in satiated rats in early light phase

To confirm the bioactivity of relaxin-2 (H2), the water intake response was assessed in water replete, satiated male Wistar rats in the early light phase. Administration of ICV relaxin-2 (180 pmol) significantly increased water intake in the first hour [0.43 ± 0.26 ml (vehicle) vs 2.50 ± 0.81 ml (180 pmol H2), $p< 0.05$] (Fig 3), and at 2h [0.63 ± 0.26 ml (vehicle) vs 2.87 ± 0.79 ml (180 pmol H2), $p< 0.05$]. ICV relaxin-3 (180 pmol H3) increased water intake in the first hour but this did not reach statistical significance [0.43 ± 0.26 ml (vehicle) vs 2.11 ± 0.67 ml (180 pmol H3)], (Fig 3).

Study 4: Effect of iPVN relaxin-3 on food intake in satiated rats in early light phase and early dark phase

Animals received an iPVN injection of either vehicle or relaxin-3 (1.8, 5.4 or 18 pmol H3) in the early light phase. Doses used were ten-fold less than those eliciting a feeding response following ICV administration (17). Intra-PVN relaxin-3 administration significantly increased food intake in the first hour at 18 pmol [0.34 ± 0.16 g (vehicle) vs 1.23 ± 0.30 g (18 pmol H3), $p< 0.05$], (Fig 4A). There was no significant difference in interval food intake but cumulative food intake was significantly increased at 2h and 4h following iPVN administration of 18 pmol relaxin-3 [0.38 ± 0.18 g (vehicle) vs 1.49 ± 0.31 g (18 pmol H3) at 2h and 0.63 ± 0.27 g (vehicle) vs 1.61 ± 0.35 g (18 pmol H3) at 4h, $p< 0.05$].

Rats received an iPVN injection of either vehicle or relaxin-3 (18 pmol H3) at the beginning of the dark phase. Nocturnal food intake was significantly increased in the first hour following relaxin-3 administration [4.43 ± 0.32 g (vehicle) vs 6.57 ± 0.42 g (18 pmol H3), $p< 0.05$] (Fig 4B). There was no significant effect on interval food intake at later time points but cumulative food intake was significantly increased in

relaxin-3-treated animals for 4h following administration in the early dark phase [9.68 ± 0.60 g (vehicle) vs 12.28 ± 0.76 g (18 pmol H3), $p < 0.05$].

Study 5: Effect of iPVN relaxin-2 on food intake in satiated rats in early light phase

To reinforce the ICV findings suggesting that relaxin-3 probably mediates its orexigenic action via the GPCR135 receptor, satiated rats received an iPVN injection of either 1.8-18 pmol H3 or 1.8-18 pmol H2. Following an iPVN administration of equimolar doses, relaxin-3 stimulated one-hour food intake as previously shown [0.27 ± 0.11 g (vehicle) vs 1.52 ± 0.51 g (18 pmol H3), $p < 0.05$]. In contrast, relaxin-2 had no significant effect on food intake at any time point following administration [0.27 ± 0.11 g (vehicle) vs 0.14 ± 0.04 g (18 pmol H2) at 1h] (Fig 5).

Study 6: Behavioral response following ICV and iPVN administration of relaxin-3

There were no significant differences in feeding or drinking behaviors following an ICV injection of relaxin-3 (180 pmol H3) to satiated rats in the early light and dark phase, and no abnormal behaviors observed. Feeding behavior was significantly increased following iPVN administration of relaxin-3 (180 pmol H3) to satiated rats in the early light phase. There were no significant differences in other behaviors and there were no abnormal behaviors following iPVN injection of relaxin-3 (Table 1).

Study 7: Hypothalamic neuropeptide mRNA expression

Following an ICV injection of 180 pmol H3, there was no difference in hypothalamic NPY, AgRP or POMC mRNA expression 4 hours post-injection compared to vehicle treated animals [NPY: 26.8 ± 1.26 (vehicle) vs 27.8 ± 2.90 (180 pmol H3). AgRP: 13.1 ± 1.35 (vehicle) vs 13.0 ± 0.78 (180 pmol H3). POMC: 1.90 ± 0.17 (vehicle) vs 1.85 ± 0.24 (180 pmol H3), units are arbitrary].

Discussion

The insulin superfamily comprises functionally diverse peptides with a common structure: A and B chains with interchain disulphide bridges. Relaxin-1 in mice and rats and the human homologue, relaxin-2, were among the first hormones described but it is only recently that an additional relaxin peptide, relaxin-3, and its receptors have been identified. Unlike relaxin-1, relaxin-3 mRNA is expressed in few peripheral tissues and only at low levels. There is less than 50% homology between relaxin-1 and relaxin-3 peptides (7). The dominant brainstem expression of relaxin-3, the extensive projections from the NI to several hypothalamic nuclei and the rich expression of GPCR135 receptors in the hypothalamic PVN and SON suggest that this ligand and its receptor may play an important role in the central nervous system. The PVN is crucial in the control of appetite and this led us to investigate the role of relaxin-3 in food intake.

We have shown for the first time that ICV relaxin-3 significantly increased food intake both in satiated animals in the early light phase and at the beginning of the dark phase. Similarly, relaxin-3 injection into the PVN, an area with a high level of expression of GPCR135, also stimulated food intake in the early light phase and was able to potentiate nocturnal feeding. These studies were performed using human relaxin-3. However, there is a high level of homology among the relaxin-3 peptides of different species, the mouse and rat peptides are identical and share 92% sequence identity to human relaxin-3 (16). At the present time, only human relaxin-3 is commercially available but this binds with high affinity to rat GPCR135 (16).

Some orexigenic neuropeptides, for example orexin A, have been found to alter behaviors such as increasing spontaneous physical activity and arousal following iPVN administration (20). Our behavioral studies did not show any abnormal behaviors following ICV administration of relaxin-3. There was, however, a significant increase in feeding behavior following iPVN relaxin-3 administration with no change in other behaviors that could have accounted for the significant increase in food intake.

The doses of relaxin-3 required to elicit a significant feeding response are in the picomolar range and similar to effective doses of other orexigenic peptides such as ghrelin. For example, a significant orexigenic response with iPVN ghrelin has been seen at 30 pmoles (17) compared to 18 pmoles of H3 relaxin. Similarly, the lowest dose of the potent orexigenic peptide NPY to significantly stimulate feeding in the PVN is 24 pmol (21). As with NPY and ghrelin, the effect of relaxin-3 occurs in the first hour following administration but cumulative food intake remains elevated for several hours.

Centrally administered porcine relaxin has been shown to increase water intake in male and female rats (6). We have shown that human relaxin-2 (H2), which binds to the LGR7 receptor with a similar affinity to porcine relaxin (13), significantly increases water intake following ICV administration in male Wistar rats (Figure 3). This indicates that the commercially available relaxin-2 (H2) used was biologically active. Relaxin-3 (H3) also increased water intake at one hour although this did not reach statistical significance. The effect of relaxin-3 on water intake, albeit less

potent compared to relaxin-2, is likely to occur via LGR7 receptors in the subfornical organs and related circuits (22).

In contrast to relaxin-3, equimolar doses of human relaxin-2 did not elicit any increase in feeding following ICV or iPVN administration to satiated animals in the early light phase. Whilst both relaxin-2 and relaxin-3 bind to the LGR7 receptor with high affinity, only relaxin-3 binds to GPCR135 with similarly high affinity. This suggests that the GPCR135 receptor may mediate the effects of relaxin-3 on food intake. In keeping with this, neither relaxin-1 null mice nor LGR7 null mice have any reported obesity or feeding phenotype (23).

The action of some orexigenic peptides, for example ghrelin, is mediated via NPY, AgRP and the melanocortin system (24). Central administration of ghrelin upregulates the expression of NPY and AgRP mRNA in the hypothalamus 4h after injection (25;26). In contrast, relaxin-3 (180 pmol H3) did not alter acute hypothalamic NPY, POMC or AgRP mRNA expression 4h after ICV administration. The absence of change in mRNA expression does not exclude altered expression of these important regulatory neuropeptides following administration of relaxin-3 at different doses and/or time points. Nevertheless, these studies suggest that altered NPY, POMC or AgRP mRNA expression may not be required in the orexigenic action of relaxin-3. It would be of interest to determine the effect of relaxin-3 on other hypothalamic mRNA and peptides and to determine whether it acts via an unknown mechanism or downstream of NPY, AgRP and POMC.

In summary, these results suggest that ICV and PVN administration of relaxin-3 stimulate feeding in male rats and that this effect may be mediated via the GPCR135 receptor. The mechanism for the orexigenic action of relaxin-3 remains to be established but does not appear to be via regulation of hypothalamic NPY, POMC or AgRP expression. Determining the role of relaxin-3 in appetite regulation is currently limited by the absence of specific antagonists for relaxin receptors or antisera for rat relaxin-3. Further work is required to determine if relaxin-3 plays a physiological role in regulation of appetite and body weight.

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Legends

Figure 1: Effect of ICV administration of relaxin-3 in satiated male Wistar rats. A) Effect of relaxin-3 (18-180 pmol H3) on 1h food intake * = $p < 0.05$ vs vehicle in early light phase B) Effect of relaxin-3 (18-180 pmol H3) on cumulative food intake over 4h in early light phase. & = $p < 0.05$ at 18 pmol vs vehicle**,** * = $p < 0.05$ at 54 pmol vs vehicle, # = $p < 0.05$ at 180 pmol vs vehicle C) Effect of relaxin-3 (180 pmol H3) on 1h food intake in early dark phase, * = $p < 0.05$ vs vehicle.

Figure 2: Effect of ICV administration of relaxin-3 (180 pmol H3) and relaxin-2 (180 pmol H2) on 1h food intake in early light phase in satiated male Wistar rats, * = $p < 0.05$ vs vehicle.

Figure 3: Effect of ICV administration of relaxin-3 (180 pmol H3) and relaxin-2 (180 pmol H2) on water intake in satiated male Wistar rats, * = $p < 0.05$ vs vehicle.

Figure 4 : Effect of iPVN administration of relaxin-3 in male Wistar rats. A) Effect of relaxin-3 (1.8-18 pmol H3) on 1h food intake in early light phase B) Effect of relaxin-3 (18 pmol H3) on 1h food intake in early dark phase, * = $p < 0.05$ vs vehicle.

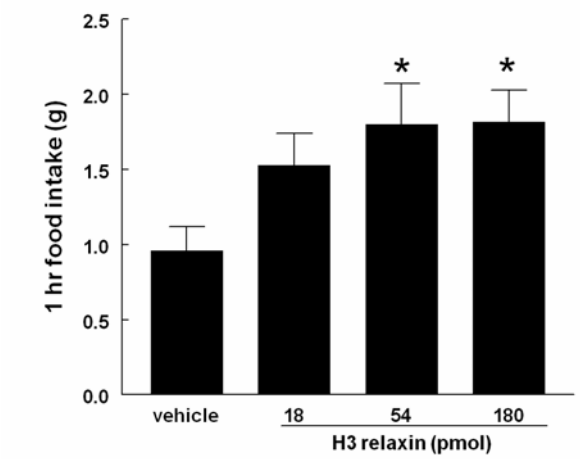
Figure 5: Effect of iPVN administration of equimolar doses of relaxin-3 (H3) and relaxin-2 (H2) on 1h food intake in satiated male Wistar rats, * = $p < 0.05$ vs vehicle.

Table 1: Effect of iPVN administration of relaxin-3 (18 pmol or 180 pmol) on behavior in the first hour following injection. Behavior was classified into one of nine categories. Each rat was observed for three 3 sec periods every 6 min and the behavior in each period scored as previously described (19). Behavioral data are expressed as median number of occurrences of behavior (interquartile ranges are expressed in square brackets), * = $p < 0.05$ vs vehicle.

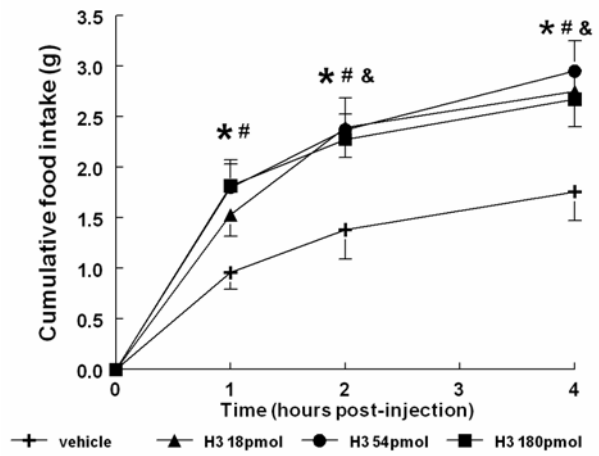
Figures

Figure 1

A



B



C

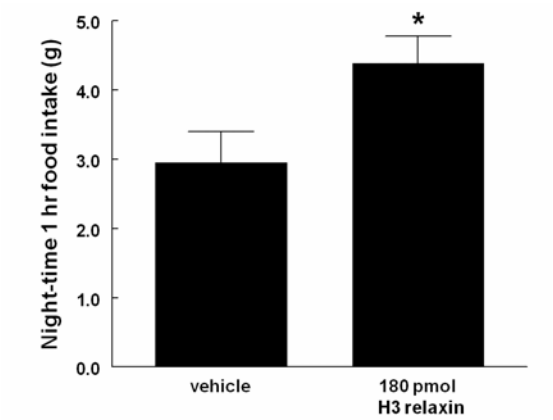


Figure 2

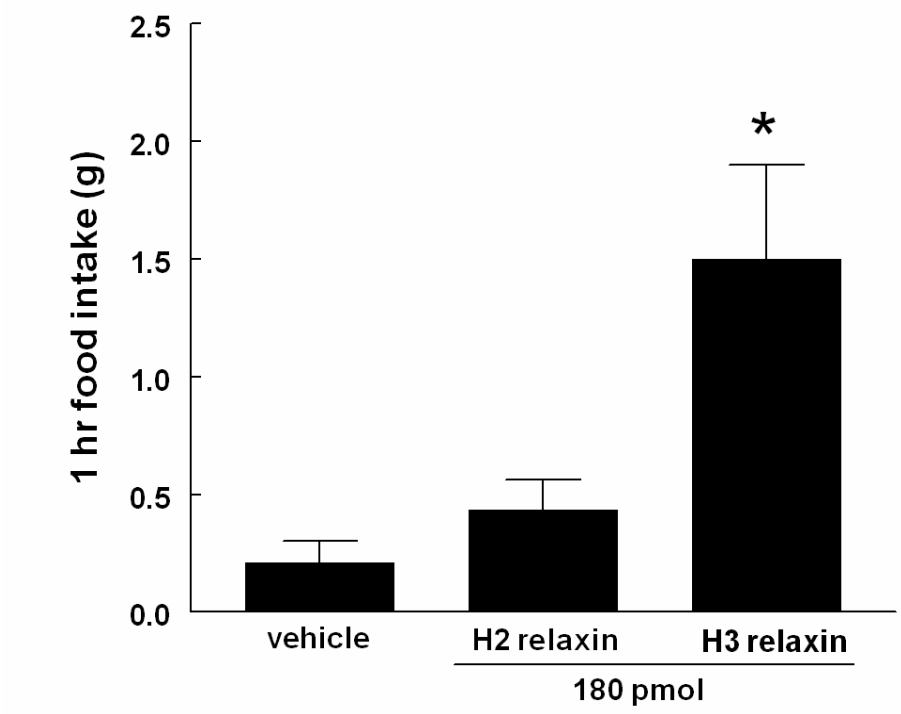


Figure 3

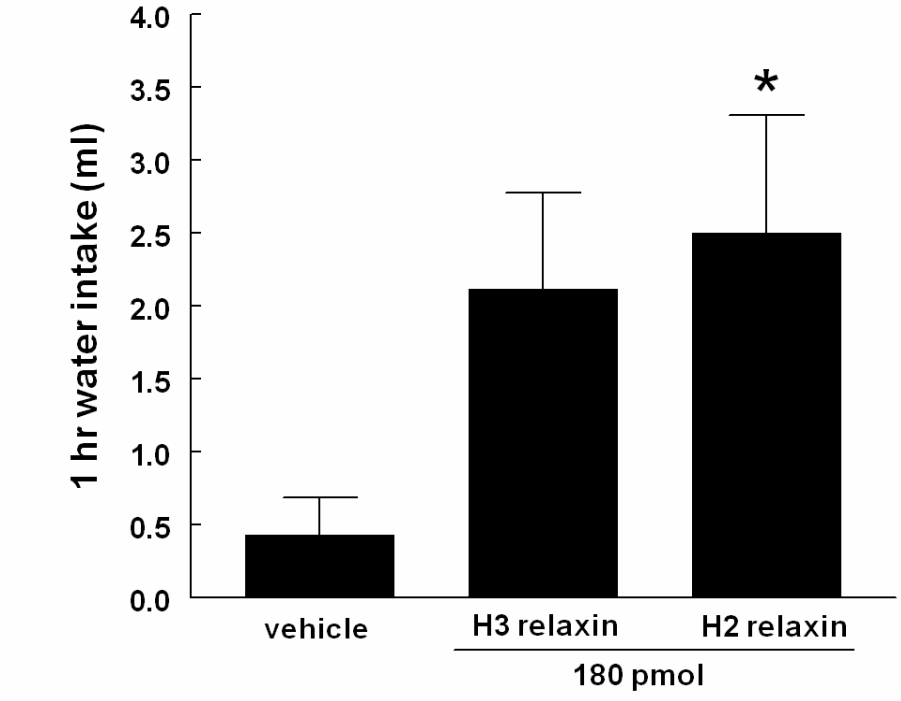
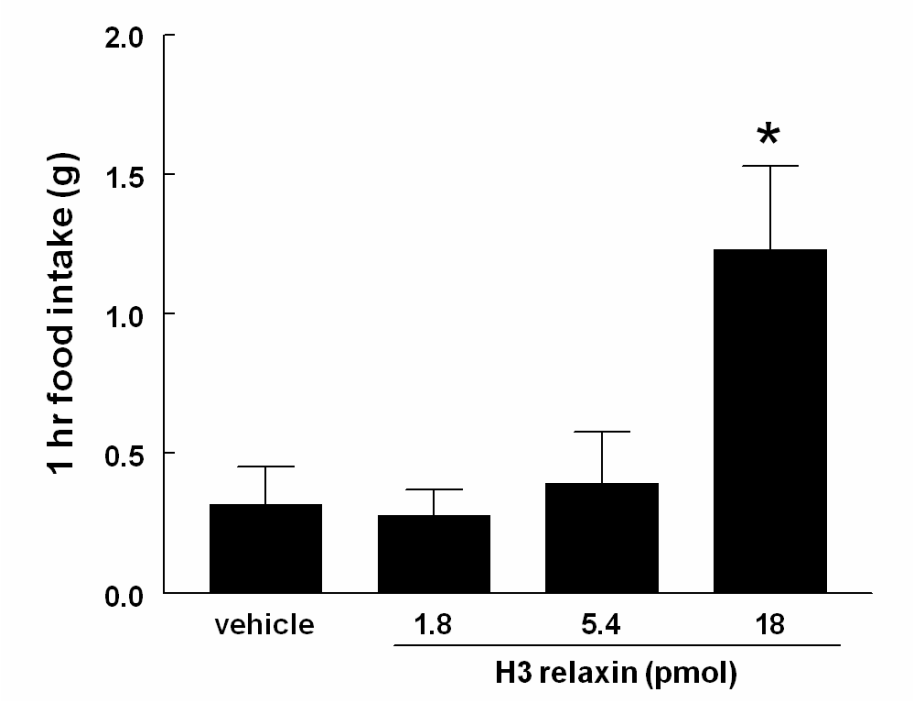


Figure 4

A


2.0
1.5
1.0
0.5
0.0
1 hr food intake (g)
*
vehicle
1.8
5.4
18
H3 relaxin (pmol)

B

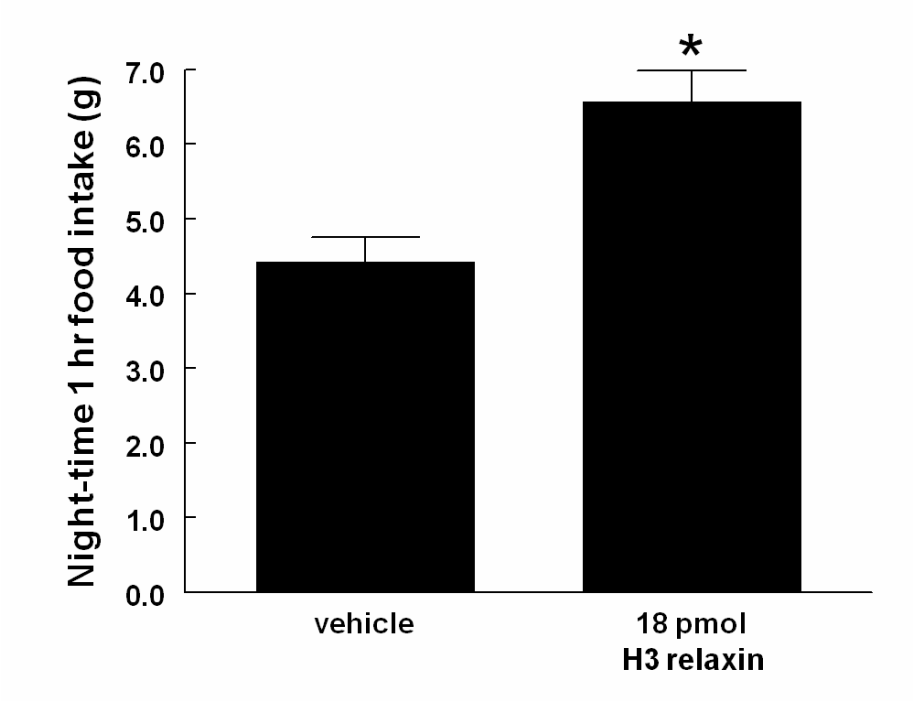

7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
Night-time 1 hr food intake (g)
*
vehicle
18 pmol
H3 relaxin

Figure 5

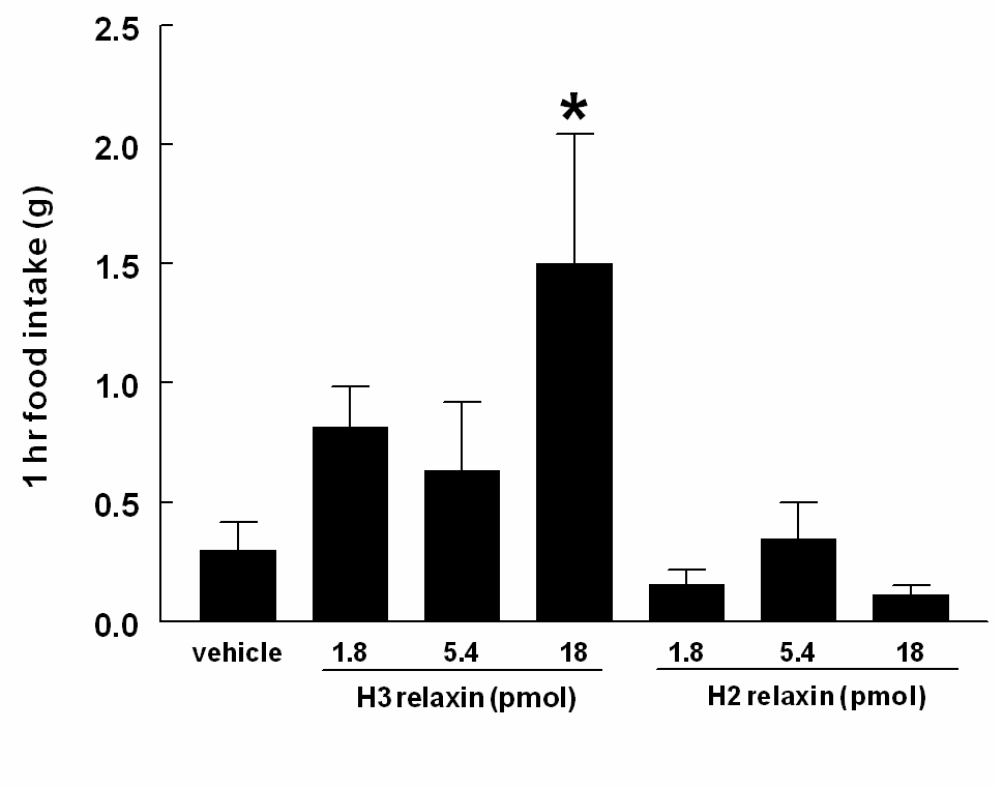

2.5
2.0
1.5
1.0
0.5
0.0
1 hr food intake (g)
*
vehicle
1.8
5.4
18
1.8
5.4
18
H3 relaxin (pmol)
H2 relaxin (pmol)

Table 1:

Behavior	Vehicle	Relaxin-3 (18pmol H3)	Relaxin-3 (180pmol H3)
Feeding	2 [0-4]	2 [2-5]	6 [4-7] *
Drinking	0 [0]	0 [0]	0 [0]
Grooming	7 [1-8]	6.5 [4.25 -8]	6 [5-6]
Burrowing	0 [0]	0 [0]	0 [0]
Rearing	8 [3-10]	5 [4-9.75]	6 [4-8]
Locomotion	4 [2-4]	3 [3-4]	3 [1-5]
Sleep	0 [0-3]	0 [0-2.25]	0 [0-8]
Head down	10 [5-19]	14.5 [6.5-16]	9 [6-9]
Tremor	0 [0]	0 [0-1]	0 [0]

Table 1: Effect of iPVN administration of relaxin-3 (18 pmol or 180 pmol) on behavior in the first hour following injection. Behavior was classified into one of nine categories. Each rat was observed for three 3 sec periods every 6 min and the behavior in each period scored as previously described (19). Behavioral data are expressed as median number of occurrences of behavior (interquartile ranges are expressed in square brackets), * = $p < 0.05$ vs vehicle.