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Original Article

Central injection of relaxin-3 receptor (RXFP3) antagonist peptides reduces motivated food seeking and consumption in C57BL/6J mice

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Abstract

Behavioural arousal in mammals is regulated by various interacting central monoamine- and peptide- neurotransmitter/receptor systems, which function to maintain awake, alert and active states required for performance of goal-directed activities essential for survival, including food seeking. Existing anatomical and functional evidence suggests the highly-conserved neuropeptide, relaxin-3, which signals via its cognate $G_{i/o}$-protein coupled receptor, RXFP3, contributes to behavioural arousal and feeding behaviour in rodents. In studies to investigate this possibility further, adult male C57BL/6J mice were treated with the selective RXFP3 antagonist peptides, R3(B1-22)R/I5(A) and R3(B1-22)R, and motivated food seeking and consumption was assessed as a reflective output of behavioural arousal. Compared to vehicle treatment, intracerebroventricular (icv) injection of RXFP3 antagonists reduced: *(i)* food anticipatory activity before meal time during food restriction; *(ii)* consumption of highly palatable food; *(iii)* consumption of regular chow during the initial dark phase, and; *(iv)* consumption of regular chow after mild (~4-h) food deprivation. Effects were not due to sedation and appeared to be specifically mediated via antagonism of relaxin-3/RXFP3 signalling, as RXFP3 antagonist treatment did not alter locomotor activity in wild-type mice or reduce palatable food intake in relaxin-3 deficient (knock-out) mice. Notably, in contrast to similar studies in the rat, icv injection of RXFP3 agonists and infusion into the paraventricular hypothalamic nucleus did not increase food consumption in mice, suggesting species differences in relaxin-3/RXFP3-related signalling networks. Together, our data provide evidence that endogenous relaxin-3/RXFP3 signalling promotes motivated food seeking and consumption, and in light of the established biological and translational importance of other arousal systems, relaxin-3/RXFP3 networks warrant further experimental investigation.

Keywords Relaxin-3; RXFP3; Arousal; Motivation; Feeding; Neuropeptide

1. Introduction

The mammalian brainstem contains several distinct neural loci which send broad ascending projections to the forebrain, and which have been well studied for their roles in arousal and related behaviours (e.g. [1-3]). These include the locus coeruleus, dorsal and median raphé nuclei, and the pedunculopontine/laterodorsal tegmental nuclei (PPT/LDTg), which contain neurons that release noradrenaline [4], serotonin (5-HT) [5] and acetylcholine [6], respectively [1-3]. A fundamental function of brainstem ascending arousal pathways is the control of alertness and sleep/wake states; but they also modulate levels of motivation, responses to stress, exploratory drive and spatial navigation, and other functions required for animals to survive, avoid predators, and acquire food (e.g. [1-3,5]). The function of these ascending arousal pathways is complemented by other broadly projecting systems, such as orexin (hypocretin)-expressing neurons in the lateral, dorsal and perifornical hypothalamic regions [7], which have been implicated in the control of arousal/sleep, food intake and reward [1,3,8].

Relaxin-3 is a highly conserved neuropeptide [9-12], which signals via its cognate G-protein-coupled receptor, relaxin family peptide 3 receptor (RXFP3; formerly GPCR135 [13,14]), which also has a spatiotemporally conserved expression pattern spanning zebrafish (*Danio rerio*) to mammals [15-19] (see refs [20-22] for review). Existing evidence suggests neurons that express relaxin-3 constitute a newly identified ascending brainstem arousal network [22,23]. For example, relaxin-3 neurons within the pontine *nucleus incertus* (NI) project broadly throughout the brain to innervate septohippocampal, hypothalamic and other limbic circuits in a regional pattern that strongly parallels noradrenaline/locus coeruleus and 5-HT/raphé systems [11,16,18].

Interconnectivity is a hallmark of monoamine/peptide arousal systems, and relaxin-3/NI neurons have strong reciprocal connections with the dorsal raphé nucleus and lateral hypothalamus in the rat [24,25]. Relaxin-3/NI neurons express receptors for 5-HT [26] and orexin [27], and 5-HT depletion increased NI relaxin-3 expression in rats [26], while in a preliminary electrophysiological study, bath application of orexins activated NI relaxin-3 and non-relaxin-3 neurons [28]. Relaxin-3-positive neurons are also present within the pontine raphé nucleus, a region dorsal to the substantial nigra, and in the medial and ventrolateral periaqueductal grey in rat [11,16] and mouse [18] brain. Although studied in less detail, initial reports suggest an ability of these populations to similarly contribute to behavioural arousal. For example, relaxin-3 neurons within the periaqueductal grey project to the thalamic intergeniculate leaflet [29], a key integration node for photic and non-photic information in diurnal and nocturnal species [30]. Application of an RXFP3 agonist was observed to stimulate neuropeptide Y neurons within the intergeniculate leaflet, which project to the suprachiasmatic nucleus to modulate circadian rhythm and arousal [30-32].

The 'classical' transmitter within NI neurons is GABA, reflected by the presence of glutamate decarboxylase [16], although some neurons appear to have an excitatory phenotype and express vesicular glutamate transporter [16,33] (Allen Brain Atlas, <www.brain-maps.org>). The relative contribution that amino acid and peptide transmitters (especially GABA and relaxin-3) make to the overall or specific functions of these neurons is unknown. However, a variety of *in vivo* pharmacological studies in rodents suggest relaxin-3/RXFP3 signalling is capable of significantly modulating neuronal processes and behaviours, in a manner similar to, and perhaps synergistic with, other monoamine and peptide arousal transmitters. These studies initially examined the effect of central injection/infusion of native human relaxin-3 [34,35]; although due to the ability of human relaxin-3 to bind/activate RXFP3 *and* both RXFP1 and RXFP4 *in vitro* and/or *in vivo* [36,37], specific RXFP3 agonist and antagonist peptides have been developed to avoid potential 'cross-reactivity' of relaxin-3 which may not represent endogenous function, but which occurs following central injection of exogenous native peptide [38-40]. Truncation of residues from both the N- and C-terminals of the relaxin-3 A-chain and replacement of a cysteine with an arginine resulted in the formation of a specific RXFP3 agonist (referred to as 'Analogue 2' or RXFP3-A2 [41], and designated here as *R3(B)/R3(A11-24)*). The first selective RXFP3 antagonist developed consisted of a truncated relaxin-3 B-chain with an additional C-terminal arginine, conjugated to the insulin-like peptide 5 A-chain (referred to as R3(BΔ23-27)/I5 [42], designated here as *R3(B1-22)R/I5(A)*). It was subsequently demonstrated that with a serine replacing a cysteine, this truncated relaxin-3 B-chain alone acts as a stable and specific RXFP3 antagonist (designated *R3(B1-22)R*) [43].

Several behavioural studies in rats have demonstrated that pharmacological modulation of RXFP3 by central injection of these peptides can alter arousal-related activities, such as the response to stress [44,45] and hippocampal theta rhythm [46]. In particular, effects on feeding have been well documented (see ref [47] for review). Intracerebroventricular (icv) injection of human relaxin-3 and specific RXFP3 agonists including *R3(B)/R3(A11-24)*, at doses ranging from 0.18-1.1 nmol, increased food consumption during the hour after administration in satiated rats [34,41]. These orexigenic effects were blocked via pre-injection of higher doses (4-4.8 nmol) of RXFP3 antagonists, including *R3(B1-22)R* [41-43]. Although the identity of the population(s) of RXFP3-expressing neurons responsible for mediating these acute effects is unknown, they are likely to reside within the hypothalamus, as local infusion of 0.18 nmol human relaxin-3 into the paraventricular (PVN), arcuate, and other hypothalamic nuclei also stimulates feeding [39].

Near identical neuroanatomical distributions of relaxin-3 and RXFP3 within rats and mice [16,18] predict similar roles for this system in arousal in these species, assuming upstream inputs and downstream signalling components are also similar. In support of this theory, relaxin-3 knockout (KO) mice run less distance on voluntary, free access, home-cage running wheels

compared to wildtype (WT) littermate controls during the dark/active phase [48], suggesting endogenous murine relaxin-3/RXFP3 signalling promotes arousal and motivated behaviour. In comparison to the rat, however, the impact of pharmacological modulation of RXFP3 in mice remains under explored. Such studies are important to determine whether effects are conserved across species, and can utilize transgenic mouse strains, such as relaxin-3 KO mice [48], to assess whether pharmacological effects are specifically due to the blockade of endogenous relaxin-3/RXFP3 signalling.

In studies to explore the role of endogenous murine relaxin-3/RXFP3 signalling in motivated feeding behaviours, we made icv injections of RXFP3 antagonists [34,43] in mice and assessed: (*i*) food seeking behaviour in the period before meal time during food restriction [49,50]; (*ii*) consumption of highly palatable food [51], and; consumption of regular chow (*iii*) during the light phase and initial dark phase and (*iv*) following 'mild' food deprivation. These studies revealed that acute pharmacological RXFP3 antagonism significantly reduced 'motivated' food seeking and consumption in mice. These effects were observed in the absence of effects on locomotor activity in automated locomotor cells, and icv RXFP3 antagonist injection did not alter the behaviour of relaxin-3 KO mice, suggesting pharmacological specificity of the peptide treatments used. Interestingly, icv injection and intra-PVN infusion of RXFP3 agonists did not increase feeding behaviour in satiated or mildly food deprived mice, indicating differences in the role of relaxin-3/RXFP3 signalling in the mouse and the rat. These data provide further evidence that endogenous relaxin-3/RXFP3 signalling drives food consumption and possibly general motivation, in line with a similar role played by other arousal signalling systems.

2. Materials and methods

2.1. Animals

All procedures were undertaken with approval from The Florey Institute of Neuroscience and Mental Health Animal Welfare Committee, in strict compliance with the ethical guidelines of the National Health and Medical Research Council of Australia. Adult male C57BL/6J mice were obtained from the Australian Research Centre (Canning Vale, WA, Australia), while backcrossed male C57BL/6J relaxin-3 knockout (KO) and wildtype (WT) littermate mice (Lexicon Genetics Inc., The Woodlands, TX, USA) were generated from heterozygous pairings and genotypes were identified from tail samples [48]. Mice were provided with at least 3 days between icv injections to allow for recovery and peptide washout. Group housed mice were single-housed after cannulation surgery, and maintained on a 12-h light-dark cycle (lights on 0700-1900 h) with *ad libitum* access to water and standard chow (irradiated mouse food; Ridley Agri-products, Melbourne, VIC, Australia), unless otherwise stated.

2.2. Surgery

Mice were anaesthetized via isoflurane inhalation (Delvet, Seven Hills, NSW, Australia; 4% initially and 2% maintenance at 0.2 l/min), secured in a small animal stereotaxic frame (Kopf Instruments, Tujunga, CA, USA) and administered with analgesic (Meloxicam 20 mg/kg, i.p; Troy Laboratories, Smithfield, NSW, Australia). An incision was made to expose the skull which was cleaned and dried with 6% hydrogen peroxide. Two or three small screws (McCann Optical Parts, Frankston, VIC, Australia) were implanted into pits drilled into the skull proximal to the eventual unilateral cannulation site, to anchor the cannula in place. A small hole was then drilled through the skull (relative to bregma, for icv studies: anterior-posterior -0.46 mm; medial-lateral -1.0 mm; for intra-PVN studies, anterior-posterior -0.9 mm; medial-lateral -0.35 mm) and a stainless-steel guide cannula (11 mm, 24-gauge) was unilaterally inserted so that the base was positioned just dorsal to either the right lateral ventricle (dorsal-ventral -1.6 mm) or right PVN (dorsal-ventral -4.0 mm) [52]. Self-curing acrylic dental cement (Vertex-Dental, Zeist, The Netherlands) was applied to fix the cannula and screws in place.

2.3. Post-operative verification of guide cannula location

For icv studies, after ~5 days recovery mice were injected with 24 pmol angiotensin II (Auspep, Melbourne, VIC, Australia) in 1 µl artificial cerebrospinal fluid (aCSF; 147 mM NaCl, 4 mM KCl, 0.85 mM $MgCl_2$, and 2.3 mM $CaCl_2$) using an 11.5 mm long stainless steel injector (0.3/0.15 mm outer/inner diameter; Small Parts Inc, Miramar, FL, USA) that protruded 0.5 mm below the guide cannula. A dipsogenic response (multiple drinking episodes of >5 s within 3 min) indicated correct injector length [53], and non-responsive mice were re-tested with 12.0 mm and 12.5 mm injectors, as necessary, to allow for variation between mice. Injections of peptides were made using a Hamilton syringe (Harvard Apparatus, Holliston, MA, USA) connected to the injector via polyethylene tubing (Microtube Extrusions, North Rocks, NSW, Australia). For intra-PVN studies, brains were collected at the end of the experiment and a tissue block containing the hypothalamus was isolated and 'post-fixed' (submerged in 4% paraformaldehyde solution) for 48 h, before cryoprotection in 20% sucrose solution for 24 h, and storage at -80°C. Forty (40) µm thick coronal sections were collected through the rostrocaudal extent of the PVN, slide mounted and counterstained with thionin to reveal guide cannula location. Data from mice with incorrect guide cannula placement were excluded from the final analysis.

2.4. Peptide injections

NPY (MW 4273) was purchased from Sigma-Aldrich (St. Louis, MO, USA), while *R3(B1-22)R/I5(A)* (MW 4150) [42], *R3(B1-22)R* (MW 2639) [43], *R3(B)/R3(A11-24)* (MW 4534) [41] and mouse *relaxin-3* (MW 5498) [54] were synthesized as described using solid phase peptide synthesis

and purified using reverse phase HPLC. The identity and purity of each peptide was confirmed by reverse phase HPLC and MALDI-TOF mass spectrometry. The amino acid composition was examined, and absolute quantities measured using amino acid analysis. Receptor binding affinity of each peptide was assessed in competition binding assays and signalling potency was assessed by measuring the effect on cAMP signalling using a cAMP reporter gene assay. The peptide content (purity) of the batches used varied between 60% to ~100%, which was allowed for to ensure that stock solutions constituted equal mass and molar doses of active peptide. One (1) µl injections of aCSF vehicle or peptide were made using an injector which protruded below the ventral extent of the guide cannula by 0.5 mm (intra-PVN studies), or 0.5 - 1.5 mm (icv studies, see above), after mice were temporarily immobilized in a cloth with the guide cannula exposed. The doses of peptides used (see Results) were selected based on doses required to produce behavioural effects in rats [41,43,45].

2.5. Food anticipatory activity studies

Mice were food restricted with access to food limited to a 4 h period ('meal time') during the light phase (12-4 pm). After 8 days, food restricted mice displayed strong food anticipatory activity (FAA) and food seeking behaviour during the hour before meal time compared to free-fed controls, characterized by wakefulness and highly active behaviours. FAA is often quantified using home-cage running wheels, but it has been established in mice that measuring the amount of time spent climbing on the cage lid provides an equivalent quantitative insight [55,56]. Therefore, following entrainment, mice were randomly assigned to receive injection of peptide or vehicle (separate mice used for each treatment) 60 min before meal time. Mice were recorded from above and time spent climbing during the 60 min post-injection was scored by an independent observer blinded to treatments (Fig. 1A).

2.6. Palatable food consumption studies

In initial studies mice had *ad libitum* access to standard chow and on every third day were provided with a pre-weighed pellet of palatable food (consisting of equal parts of fat, sugar and protein; SF03-002, Specialty Feeds, Glen Forrest, WA, Australia) for 60 min, from 2-3 pm. Mice were placed in home-cages fitted with fasting grids (Wiretainers, Preston, VIC, Australia) to aid recovery of food crumbs from bedding when weighing food pellets post-consumption. To limit the variation in time since each mouse last consumed regular chow, food was withheld from all mice for 4 h before testing. (During the light phase, mice occasionally/intermittently wake and consume small amounts of standard chow, and this variance may translate into a source of unwanted variance in the amount of palatable food consumed). Mice were placed into new home-cages without food at 10 am and into another new 'home-cage' with a fasting grid at 1 pm (thus, these mice are 'mildly

food deprived'; see below). Mice were observed to consume near equivalent, stable amounts of palatable food (~0.6 g) during the 60 min test from the 4th exposure onwards. On the 6th and 7th exposures (separated by 3 days), mice were subjected to the routine described, but were injected with either peptide or vehicle at 2 pm (i.e. immediately before receiving palatable food; see Fig. 1B). A 'cross-over' design was employed whereby each mouse received a peptide and vehicle treatment on separate test days, and the order of treatment was randomized.

For an assessment of palatable food consumption in subsequent cohorts (see Results), several changes in protocol were adopted. Mice with *ad libitum* access to standard chow were provided with a pellet of palatable food at 2 pm *every* day for 12 days. This treatment has been shown to invoke FAA, as an adaptive response that allows mice to consume food that is deemed to be of higher nutritional value [57]. To prevent mild food deprivation and ensure undisrupted access to standard chow (which was deemed to be more important than reducing the variation in time since each mouse previously consumed food), mice were not placed in food-free home-cages prior to testing and fasting-grids were not utilised. Although the absence of fasting-grids may have decreased the accuracy of post-consumption pellet weight measurements, we have observed that providing mice with palatable food at the same time they are placed in novel home-cages with fasting grids stimulates exploratory behaviour, which distracts from palatable food consumption. Mice were provided a pellet of palatable food in their home-cage for only 30 min each day, and peptide/vehicle was infused 15 min before exposure, corresponding to a predicted effective time window of peptide action (Fig. 1B). From the fourth exposure/day onwards, mice consumed stable near-equivalent amounts of palatable food (~0.4 g). Peptides and vehicle were tested on day/ exposure 6, 9 and 12, and cross-over design and randomized treatment order were employed. Two separate cohorts were used. In the first, we tested 3.8 nmol *R3(B1-22)R*, 1.1 nmol *R3(B)/R3(A11-24)* and vehicle; while in the second, we tested 1.9 and 2.8 nmol *R3(B1-22)R* and vehicle.

2.7. Standard chow consumption studies

For analysis of standard chow consumption under *satiated* conditions during the light and early dark phase, mice were placed in new home-cages ~24 h before experimentation to remove any extraneous food crumbs from the bedding. For icv studies, cage lids were emptied of food at the time of injection (1-3 pm (light) or 8-11 pm (dark)), and replaced with a pre-weighed pellet of standard chow that was available either 15-45 min post-injection (antagonist studies), or 0-60 min post injection (agonist studies), which was then recovered and weighed. A broader time period was used for agonist studies in an effort to maximize the chance of observing pharmacological effects; while for antagonist studies it was deemed important to examine food intake during a period of predicted high peptide bioactivity and receptor occupancy, as food consumed before or after this

window could mask any subtle pharmacological effects. For intra-PVN studies, mice were transferred to a new home-cage with a pre-weighed pellet of standard chow and observed 0-60 min post-infusion (Fig. 1C).

For analysis of standard chow consumption under *mild food deprivation* conditions, mice were placed in a new home-cage with no food at 10 am and into a second new home-cage with a fasting grid at 1 pm. At 2 pm, mice were infused with peptide or vehicle, and a pellet of pre-weighed standard chow was placed into the home-cage during a period of either 15-45 min post-injection (antagonist studies), or 0-60 min post-injection (agonist studies), which was then recovered and weighed (Fig. 1C). This method is similar to that used in the palatable food studies following mild food deprivation (described above), and was favoured as it is mild enough to observe increased feeding in response to orexigenic peptides such as NPY, but is strong enough to detect decreased feeding in response to anorexigenic agents (such as RXFP3 antagonists; see Results). In contrast, more severe, yet commonly used, overnight food deprivation can result in a 'ceiling' effect that prevents orexigenic agents from further increasing food consumption, while due to the myriad of redundant pathways that are activated to drive this important behaviour, blockade of a single transmitter system is often not sufficient to reduce feeding drive [58]. It should be noted however, that the increase in feeding that is achieved using this mild food deprivation technique is not due merely to the withdrawal of food, as mice rarely eat during the equivalent 4 h of the light phase. Mice vigorously explore the two novel home-cages, which consumes energy, and this is more likely responsible for the observed increase in feeding drive.

Data included from satiated mice was generated using a single cohort, and on each test day individual mice were randomly assigned to either icv treatment. A separate cohort of mice was used to examine the impact of mild food deprivation, and a cross-over design was employed in which mice were tested with the treatments in a randomized order. For intra-PVN studies, in a separate cohort, a cross-over design was utilized whereby each mouse received all three treatments, starting with NPY, while vehicle and *R3(B)/R3(A11-24)* infusions were randomized on the subsequent two test days.

2.8. Automated locomotor cell

Mice were placed in an automated locomotor cell (internal length × width, 27.5 × 27.5 cm; Med Associates, Vermont, USA) for 30 min, illuminated by 70 lux white light during the light phase or dim red light during the early dark phase (8-11 pm), 15-45 min after injection of either vehicle or peptide (Fig. 1D). The distance travelled was tracked by an array of photobeams and analysed via Activity Monitor V6.02 software (Med Associates).

2.9. Graphs and statistics

All graphs were generated using Prism V5.0 software (GraphPad, San Diego, CA, USA). Group sizes (n) are indicated within/above columns on the histograms, and data are expressed as mean ± SEM. Clear outliers (>1.6 standard deviations from the mean) were excluded. Statistical significance was determined using Sigmastat 3.5 software (Aspire Software International, Ashburn, VA, USA) for two-way ANOVA, and SPSS V20 software (IBM, Armonk, New York, NY, USA) for all other tests, utilizing statistical tests appropriate for each data set as indicated in Results (post-tests refer to Bonferroni).

3. Results

3.1. Icv injection of an RXFP3 antagonist reduced food anticipatory activity

Food restricted, vehicle-treated mice spent ~36% of 60 min before meal time climbing on the cage lid (indicative of FAA and food seeking behaviour), which was significantly greater (70-fold) than the ~0.5% of time spent climbing by free-fed vehicle controls (one-way ANOVA: post-test, $P < 0.001$; Fig. 2). Icv injection of the RXFP3 antagonist, *R3(B1-22)R/I5(A)* (0.7 nmol) markedly reduced climbing behaviour in food restricted mice by >50% *c.f.* vehicle-treated controls (post-test, $P = 0.021$).

3.2. Icv injection of an RXFP3 antagonist reduced palatable food consumption

Mice were trained by prior exposure to consume stable amounts of palatable food (~0.6 g) when access was provided for 1 h during the light phase. Acute icv injection of the RXFP3 antagonist, *R3(B1-22)R* (3.8 nmol), significantly reduced consumption of palatable food in WT mice by ~25% *c.f.* vehicle controls (two-way RM ANOVA: treatment × genotype interaction, $F_{(1,17)} = 4.20$, $P = 0.041$; post-test between treatments within WT, $P = 0.007$; Fig. 3A). These effects appeared to be due to reduced endogenous relaxin-3/RXFP3 signalling, as no similar reduction was observed in 'life-long' relaxin-3 KO mice [48] (post-test between treatments within KO, $P = 0.859$), which consumed near identical amounts of palatable food as vehicle treated WT littermates (post-test between genotypes within vehicle, $P = 0.934$).

In subsequent studies to further explore these findings, mice were provided with palatable food for 30 min each day and peptide/vehicle injections were conducted 15 min prior, to prevent the consumption of food before or after the predicted time window during which RXFP3 antagonism was expected to alter behaviour. After training exposures, mice consumed stable amounts of palatable food (~0.4 g), which was significantly reduced by ~44% following icv injection of 3.8 nmol *R3(B1-22)R c.f.* vehicle (two-way ANOVA: treatment × dose interaction, $F_{(2,76)} = 0.532$, $P = 0.590$; main effect of treatment, $F_{(1,76)} = 11.68$, $P < 0.001$; post-test between treatments within 3.8

nmol, P = 0.006; Fig. 3B). Consistent with a dose-related relationship, injection of 2.8 nmol *R3(B1-22)R* resulted in a near significant reduction of ~30% (post-test between treatments within 2.8 nmol, P = 0.063), and injection of 1.9 nmol *R3(B1-22)R* produced a modest, non-significant reduction (~16%; post-test between treatments within 1.9 nmol, P = 0.208).

3.3. Icv injection of an RXFP3 antagonist reduced standard chow consumption

Satiated mice consumed low levels of standard chow (~30 mg) during the light-phase period 15-45 min after icv injection of vehicle, which was unaltered by injection of 3.8 nmol *R3(B1-22)R* (two-way ANOVA: treatment × condition interaction, $F_{(2,73)} = 11.68$, $P < 0.001$; post-test between treatments within satiated, P = 0.922; Fig. 4). Satiated vehicle-treated mice increased their food consumption to ~170 mg during the early dark phase, which was reduced by >50% following icv injection of 3.8 nmol *R3(B1-22)R* (post-test between treatments within dark phase conditions, P = 0.029). Mild food deprivation further increased the amount of food consumed by vehicle treated mice during the light phase (~280 mg), and was reduced by >60% following *R3(B1-22)R* treatment (post-test between treatments within mild food deprivation, $P < 0.001$).

3.4. Icv injection of an RXFP3 antagonist did not alter locomotor activity

Icv injection of *R3(B1-22)R* (3.8 nmol) during the light phase did not significantly alter the distance travelled in automated locomotor cells during the period 15-45 min post-injection (81.2 ± 5.1 m, n = 10) relative to vehicle-treated controls (85.3 ± 3.2 m, n =19; unpaired t-test, P = 0.471), suggesting an absence of any 'sedative' effects of the peptide. These findings were reproduced in a separate cohort of mice tested during the early dark phase (*R3(B1-22)R,* 64.2 ± 5.8 m, n =11; vehicle, 58.0 ± 4.3 m, n = 11; unpaired t-test, P = 0.400). Interestingly, we did not observe the expected increase in distance travelled during this period, suggesting the novel environment of the locomotor cell provides a strong locomotor-invoking stimulus, irrespective of normal activity in either phase. In fact, mice tested during the dark phase travelled significantly less distance (two-way ANOVA: treatment × phase interaction, $F_{(1,47)} = 1.316$, P = 0.257; main effect of phase, $F_{(1,47)} = 24.06$, $P < 0.001$); although this finding is unlikely to be physiologically relevant, and instead may reflect variation between cohorts.

3.5. Icv injection and iPVN infusion of RXFP3 agonists did not alter food consumption

In contrast to parallel studies conducted in rats, icv injection of *R3(B)/R3(A11-24)* (1.1 nmol) did not alter standard chow consumption *c.f.* vehicle controls during the hour post-injection in satiated mice (unpaired t-test, P = 0.121; Fig. 5A). Similarly, 5 µg of *R3(B)/R3(A11-24)* (1.1 nmol) or *mouse relaxin-3* (0.9 nmol) did not alter food consumption relative to vehicle, following mild food deprivation (one-way ANOVA: post-tests, P > 0.05). However, mice infused with NPY (5 µg, 1.2 nmol) displayed an ~3-fold increase in food consumption (post-tests with other groups, $P < 0.001$).

Furthermore, icv injection of *R3(B)/R3(A11-24)* (1.1 nmol) did not alter palatable food consumption compared to vehicle (unpaired t-test, P = 0.765, Fig. 5B). In order to ensure that high concentrations of peptide reached the PVN (a presumed site of action for the observed orexigenic effects in rats [39,59]), *R3(B)/R3(A11-24)* was infused into the PVN, using a 0.5 µg (110 pmol) bolus dose that is 10-fold less than that used for the icv studies. Intra-PVN *R3(B)/R3(A11-24)* did not alter food consumption in satiated mice *c.f.* vehicle controls (one-way RM ANOVA: post-test, P = 0.629), but food consumption was increased ~3-fold following intra-PVN injection of 0.5 µg (120 pmol) NPY (post-tests with other groups, P < 0.001; Fig. 6).

4. Discussion

The major finding of this study was that acute icv injection of RXFP3 antagonist peptides reduced motivated food seeking and consumption in adult male mice, which are interpreted as measurable 'outputs' of behavioural arousal. These effects were not due to general sedation, and appeared specifically associated with pharmacological blockade of RXFP3.

Treatment with the chimeric peptide, *R3(B1-22)R/I5(A)* [42], significantly reduced food anticipatory activity (FAA). FAA enables mice to be appropriately aroused and awake (if food is provided during the normally inactive phase), to be able to run, burrow, forage and navigate in order to seek and acquire food that is expected to soon be available [60]. This capacity is largely conferred by monoamine and peptide arousal systems [61], and the present studies suggest endogenous relaxin-3/RXFP3 signalling contributes to this increase in behavioural arousal. The prediction that relaxin-3 expression increases during FAA in mice, in line with the synthesis/release of other arousal transmitters, seems likely given that relaxin-3 expression within the NI is cyclical and has peak and troughs during the dark/active phase in *ad libitum* fed rats [62], and elevated Fos staining is observed in the NI after FAA in rats (Peirs C, Ma S, Gundlach AL, unpublished data). An increase in relaxin-3 mRNA was also observed in female rats at the beginning of the light phase after a repeated 7 week schedule, whereby each week rats were food restricted for 2 days (allowed 60% of normal chow for 24 h), and provided access to a palatable diet supplement for a 2 h period over 3 days that corresponded approximately to the eventual time of brain collection [63].

FAA may be initiated by peripheral humoral signals, such as the hunger-inducing peptide, ghrelin [50,64], which acts via the G-protein coupled receptor, growth hormone secretagogue receptor (GHSR; [65]). Ghrelin, activates brain feeding circuits (NPY and AgRP neurons) via direct and indirect actions, and interacts with leptin signalling; but also activates orexin neurons in lateral hypothalamus [66] that promote behavioural arousal by projecting to and activating other peptide and monoamine arousal pathways [8], which likely includes the relaxin-3/NI system [28]. GHSR is

expressed by other neurons crucial for mediating arousal in response to challenges to energy homeostasis, including glucose-sensitive neurons within the ventromedial hypothalamus [67]; and GHSR is highly expressed within rat and mouse NI [68,69] (Allen Brain Atlas <www.brain-map.org>). Therefore, determining whether endogenous ghrelin and/or orexin are capable of stimulating relaxin-3/NI neurons, and whether this occurs during FAA [64], are important future goals. It would also be of interest to determine if NI and other relaxin-3 pathways function as a food entrainable oscillator (or contribute to this key neural network) [49,64], but studies to determine if the activity of relaxin-3/NI neurons can be synchronized to food availability, and whether this persists on cessation of food restriction, are yet to be conducted.

Repeated access to palatable food during the light/inactive phase can induce FAA in mice, effects that powerfully mimic those achieved by food restriction [57], and are also strongly controlled by ghrelin [70]. Although palatable food was provided every day in some of the present studies, the single-chain, RXFP3 antagonist, *R3(B1-22)R* [43], also reduced consumption of palatable food when it was provided intermittently (every third day), which is not considered sufficient to promote FAA. This antagonist also reduced the consumption of standard chow in satiated mice during the early dark phase, and following mild food deprivation during the light phase, demonstrating that, even in the absence of FAA, endogenous relaxin-3/RXFP3 signalling is capable of promoting behavioural states conducive to consumption of food in mice. Although this RXFP3 antagonist was unable to reduce regular food consumption in satiated mice during the light phase, this likely reflected a 'floor effect' as the amount of food consumed was very low. An ability of RXFP3 antagonists to reduce motivated food consumption is in line with actions of other arousal peptide and monoamine transmitters, as pharmacological antagonism of orexin [71], noradrenaline [72], dopamine [73], and acetylcholine [74] signalling reduces food consumption under various environmental conditions. Notably, in contrast to acute pharmacological RXFP3 antagonism, life-long relaxin-3 deficiency did not alter palatable food consumption *c.f.* WT littermate controls when injected with vehicle. This may be due to compensation during development, as a high level of redundancy exists in the neuronal systems that control feeding [58]. The issue could be addressed in future studies in which RXFP3 is conditionally deleted during adulthood either globally or within discrete RXFP3-rich brain regions.

In the present studies, 0.7 nmol of *R3(B1-22)R/I5(A)* significantly reduced FAA, while a higher dose (3.8 nmol) of *R3(B1-22)R* was required to significantly reduce palatable food consumption (although smaller non-significant effects were observed at lower doses). These data may be related to differences in peptide potency and/or *in vivo* stability, as in competition binding assays, *R3(B1-22)R* displays ~10-fold lower affinity for RXFP3 *c.f. R3(B1-22)R/I5(A)* (pK_i 7.44 vs 8.49,

respectively) [43]. However, differences in assay/test sensitivity and/or the relative involvement of endogenous relaxin-3/RXFP3 signalling in the two types of behaviour may also exist.

Although a reduction in food consumption following icv injection of RXFP3 antagonists is yet to be observed in rats at doses of 10 μg for *R3(B1-22)R* (3.8 nmol) and *R3(B1-22)R/I5(A)* (2.4 nmol) [42,43], higher doses may be required in these larger animals. In fact, in a recent study, a trend for such an effect was observed, whereby rats consumed little or no food following injection of 20 μg (4.8 nmol) of the RXFP3 antagonist, 'Analogue 3' (RXFP3-A3), but due to the low amount of food consumed by control rats, which were satiated and tested during the light phase, this reduction was not statistically significant [41]. The molar doses used in previous studies and experiments described here are in line with doses of peptide antagonists used to investigate other neuropeptide systems, such as NPY. For example, icv injection of 10 μg (4.3 nmol) of the NPY Y_1 receptor antagonist, 1229U91, reduced spontaneous food consumption in Zucker fat rats, whereas 30 μg (12.8 nmol) was required in Zucker lean rats [75].

Identifying which RXFP3-rich brain regions are primarily involved in the observed reductions in food seeking and consumption behaviour that occur following icv injection of RXFP3 antagonists in mice remains an important goal. Based on the inability of an RXFP3 agonist infused into the PVN to stimulate feeding, however, it seems unlikely that this region is strongly involved. Anatomical data [76], in conjunction with several functional studies in rats, suggest relaxin-3/ RXFP3 signalling modulates arousal via interactions with thalamic [29], septohippocampal [46], limbic [77], and hypothalamic circuits [44,78]. This suggests the NI and periaqueductal grey relaxin-3 networks can act in both parallel and series with other arousal systems (see Introduction), to drive goal-directed behaviour. The mesolimbic dopaminergic pathway is also an important 'common endpoint circuit' that arousal systems either directly or indirectly target, and which is involved in motivational drive for goals such as consumption of food, and the rewarding sensation when these goals are achieved [79]. Although relaxin-3 positive fibres and RXFP3 mRNA are only weakly present within the ventral tegmental area and nucleus accumbens, their enrichment within limbic structures such as the amygdala that provide strong input to the mesolimbic dopaminergic pathway [80] suggests indirect modulation is possible. The limbic *bed nucleus of the stria terminalis* (BNST) also strongly projects to the ventral tegmental area [80,81] and expresses high levels of RXFP3, and the likely importance of this circuit to overall endogenous relaxin-3/RXFP3 function has been highlighted in a recent study in which injection of the RXFP3 antagonist, *R3(B1-22)R*, icv or directly into the BNST, reduced alcohol-seeking behaviour in rats [82]. Future pharmacological studies should similarly address the brain regions responsible for the behavioural responses to RXFP3 antagonists observed in the present studies, by either local injections into

distinct brain areas, or by chronic RXFP3 depletion/knockdown following Rxfp3 gene deletion in conditional Rxfp3 KO mice.

Notably, in the present studies icv and intra-PVN administration of RXFP3-selective agonist peptides did not increase feeding in adult (satiated) mice, whereas there is a robust effect of such treatments in adult satiated rats [39,41-43,54]. Although our intra-PVN studies involved a large (1 µl) injection volume, which may have activated RXFP3 within adjacent areas such as the periventricular and lateral hypothalamus, it is perhaps unlikely that 'anorexic' effects produced in these nearby regions completely 'negated' any putative orexigenic role of PVN RXFP3. The lack of an observable effect was also not due to any chemical properties of the agonist peptides, as the same batches of mouse relaxin-3 and *R3(B)/R3(A11-24)* were used in concurrent studies in our laboratory and effectively stimulated feeding in rats [41,54]. Furthermore, the lack of an orexigenic effect is unlikely due to insufficient peptide levels. Infusion of as little as 180 pmol and 18 pmol human relaxin-3, icv and intra-PVN respectively, is potently orexigenic in Wistar rats [39], while injection of ~5-fold higher native relaxin-3 or *R3(B)/R3(A11-24)* did not increase food consumption in mice. This finding is quite striking, considering mice are much smaller than rats and a lower bolus dose of centrally-acting compounds is often sufficient to exhibit an equivalent behavioural response. Lower doses of RXFP3 agonists (0.12 and 0.5 nmol) tested also fail to increase food consumption in mice [83]. It therefore seems likely these data represent a true species difference in relaxin-3/RXFP3 signalling and associated downstream signals.

The regional and cellular neural basis of this species difference is currently elusive, but it is possible, for example, that RXFP3 is expressed by different populations of PVN neurons in the rat and mouse. In this regard, it has been postulated that central RXFP3 activation in rats increases feeding by inhibiting the expression and release of the anorexic peptide, oxytocin from neurons within the PVN [47,59,84]. This could be achieved by activation of RXFP3 on PVN oxytocin neurons, as *in vitro* studies predict RXFP3 activation is likely to be inhibitory [20], and therefore functions in a synergistic manner with GABA which is co-released from relaxin-3 neurons [16]. It is therefore possible that within the mouse PVN, RXFP3 is *not* expressed by oxytocin neurons, and hence activation of RXFP3 does not confer orexigenic roles. Detailed anatomical insights into the neural circuitry by which relaxin-3/RXFP3 signalling modulates behaviour have been hampered, in the rat and the mouse, by the lack of suitable RXFP3 antisera or reporter proteins to allow immunological or histological phenotyping of RXFP3-positive neurons in key brain areas/pathways. Such studies, which may require the development of new strains of transgenic mice and other complementary methods, will be important for determining how conserved RXFP3 function is in rodents, with implications for our understanding of the equivalent systems in humans.

5. Conclusions

Correct modulation of behavioural arousal in humans maintains optimal attention, cognition, motivation, sleep patterns and even mood [3,8,85]. Not surprisingly, disruption of transmitter and neuropeptide systems that control arousal and related modalities, such as serotonin, noradrenaline and acetylcholine, and/or orexin and CRF, has been linked to multiple psychiatric diseases, including depression [86-88]. The present studies provide further evidence that relaxin-3 is an arousal transmitter that promotes motivated food seeking and consumption, as these behaviours were reduced following icv injection of RXFP3 antagonists in mice. An important species difference with rats was also identified, as central injection of RXFP3 agonists was not orexigenic in mice. Further exploration of the role relaxin-3/RXFP3 networks play in promoting food consumption and behavioural arousal, and the nature of their interactions with other analogous networks, is an important area of research with considerable translational potential.

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Figure Legends

Fig. 1. Schematic illustrating the timing of peptide/vehicle injections relative to the subsequent time window for RXFP3 antagonist (red shading) studies and for RXFP3 agonist (green shading) studies during which the following different parameters were measured: (A) food anticipatory activity; (B) palatable food consumption; (C) standard chow consumption and; (D) locomotor activity.

Fig. 2. Icv injection of the RXFP3 antagonist, *R3(B1-22)R/I5(A)*, reduced food anticipatory activity. Food restricted mice were icv injected with either 0.7 nmol (3 μg) *R3(B1-22)R/I5(A)* or vehicle 1 h before meal time, returned to their home-cage and their behaviour was recorded by video. Free-fed vehicle treated mice were also examined as controls. The amount of time spent climbing (indicative of food anticipatory activity and food seeking behaviour [55,56]) during the 60 min before meal time was then scored by an independent blinded observer. One-way ANOVA, comparison with food restricted vehicle, * $p < 0.05$; *** $p < 0.001$.

Fig. 3. Icv injection of the RXFP3 antagonist, *R3(B1-22)R*, reduced palatable food consumption. Mice were trained by repeated exposure to consume a stable amount of palatable food when it was provided for either 60 or 30 min during the light phase. (A) C57BL/6J relaxin-3 KO and WT littermate mice were treated with either 3.8 nmol (10 μg) *R3(B1-22)R* or vehicle in new home-cages with fasting grids following mild food deprivation, and the amount of palatable food consumed during the subsequent 1 h was recorded. Two-way RM ANOVA, Bonferroni post-test, ** $p < 0.01$. (B) WT C57BL/6J mice were injected with either 1.9, 2.8 or 3.8 nmol (5, 7.5, 10 μg) *R3(B1-22)R* or vehicle, returned to their home-cage, and the amount of palatable food consumed during the period 15-45 min post-injection was recorded. Data for the 1.9 and 2.8 nmol treatments was achieved in the same cohort, while a different cohort was treated with 3.8 nmol. Two-way ANOVA, Bonferroni post-test: ** $p < 0.01$.

Fig. 4. Icv injection of the RXFP3 antagonist, *R3(B1-22)R*, reduced food consumption in satiated mice during the early dark phase, and in mildly food deprived mice during the light phase; but not in satiated mice during the light phase. Mice were infused with either 3.8 nmol (10 μg) *R3(B1-22)R* or vehicle, and the amount of regular chow consumed during the 15-45 min post injection was recorded. See Methods, section 2.7 for further descriptions. Two-way ANOVA, Bonferroni post-test, * $p < 0.05$; *** $p < 0.001$.

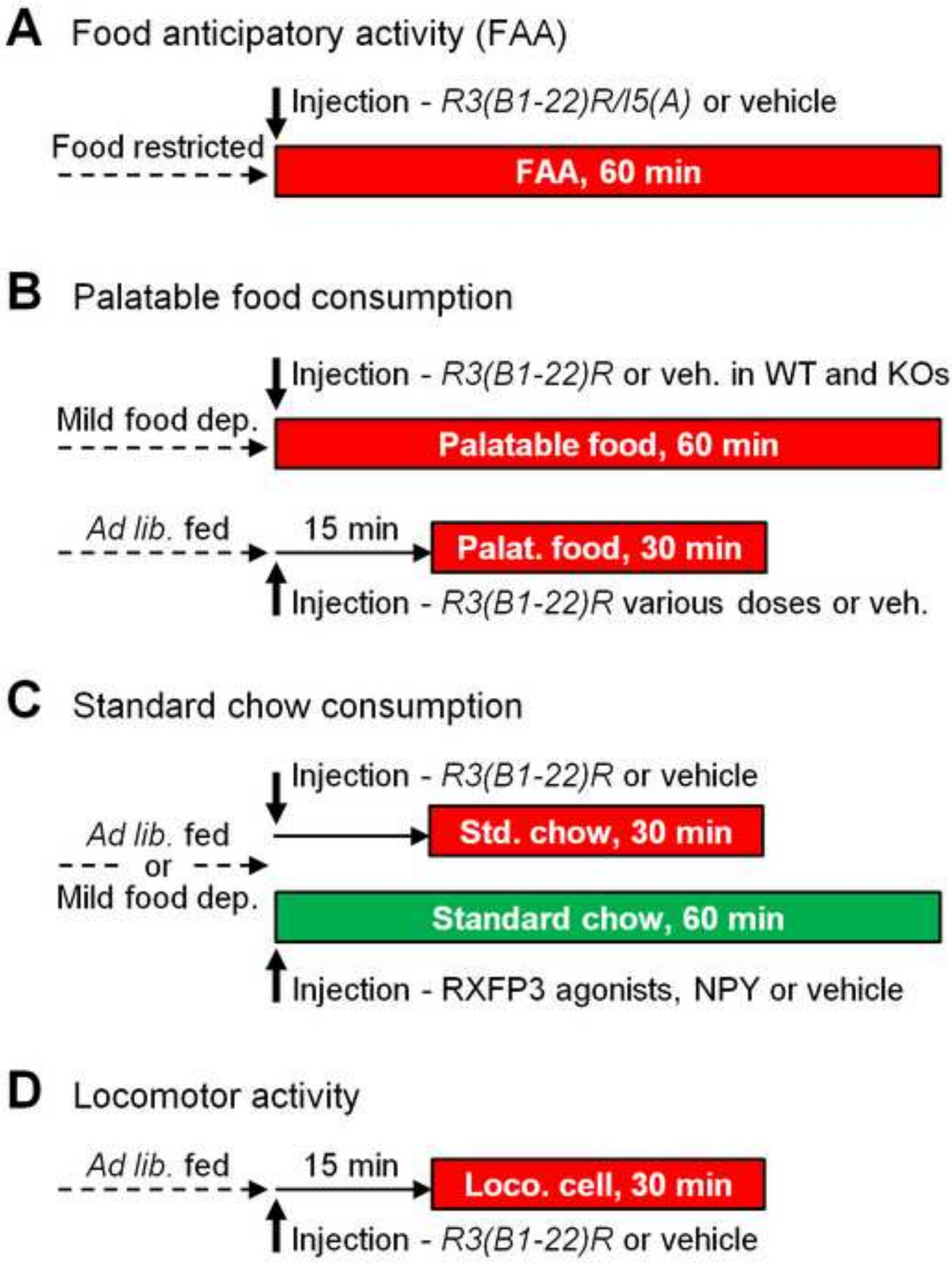
Fig. 5. Icv injection of RXFP3 agonists did not alter food consumption. (A) Mice were injected during the light phase with 5 μg of either *R3(B)/R3(A11-24)* (1.1 nmol), *mouse R3 (relaxin-3)* (0.9

nmol) or vehicle, and the amount of regular chow consumed during 60 min post-injection was recorded. Mice were tested during the light phase under both satiated/home-cage conditions, and following mild food deprivation. Mildly food deprived mice were also infused with NPY (5 µg, 1.2 nmol) as a positive control. One-way RM ANOVA, Bonferroni post-test, NPY versus other mild food deprivation groups, *** $p < 0.001$. (B) Mice were trained by previous exposure to consume a stable amount of palatable food when it was provided for 30 min during the light phase, and were icv infused with either 1.1 nmol *R3(B)/R3(A11-24)* or vehicle, returned to their home-cage, and the palatable food consumed during the period 15-45 min post-injection was recorded.

Fig. 6. Intra-PVN infusion of an RXFP3 agonist did not alter food consumption. Mice were infused (iPVN) during the light phase with 0.5 µg of either *R3(B)/R3(A11-24)* (110 pmol), NPY (120 pmol) or vehicle, placed in new cages, and the amount of regular chow consumed during 1 h post-infusion was recorded. One-way RM ANOVA, Bonferroni post-test, NPY versus other groups, *** $p < 0.001$.

Highlights

- Relaxin-3/RXFP3 system widely distributed throughout mammalian brain
- Study assessed effect of pharmacological modulation of RXFP3 on feeding in mice
- Icv RXFP3 antagonists reduced motivated food seeking and consumption
- Icv and intra-PVN RXFP3 agonists not orexigenic in mice, in contrast to in rats
- Data support hypothesis that endogenous relaxin-3/RXFP3 signalling promotes arousal



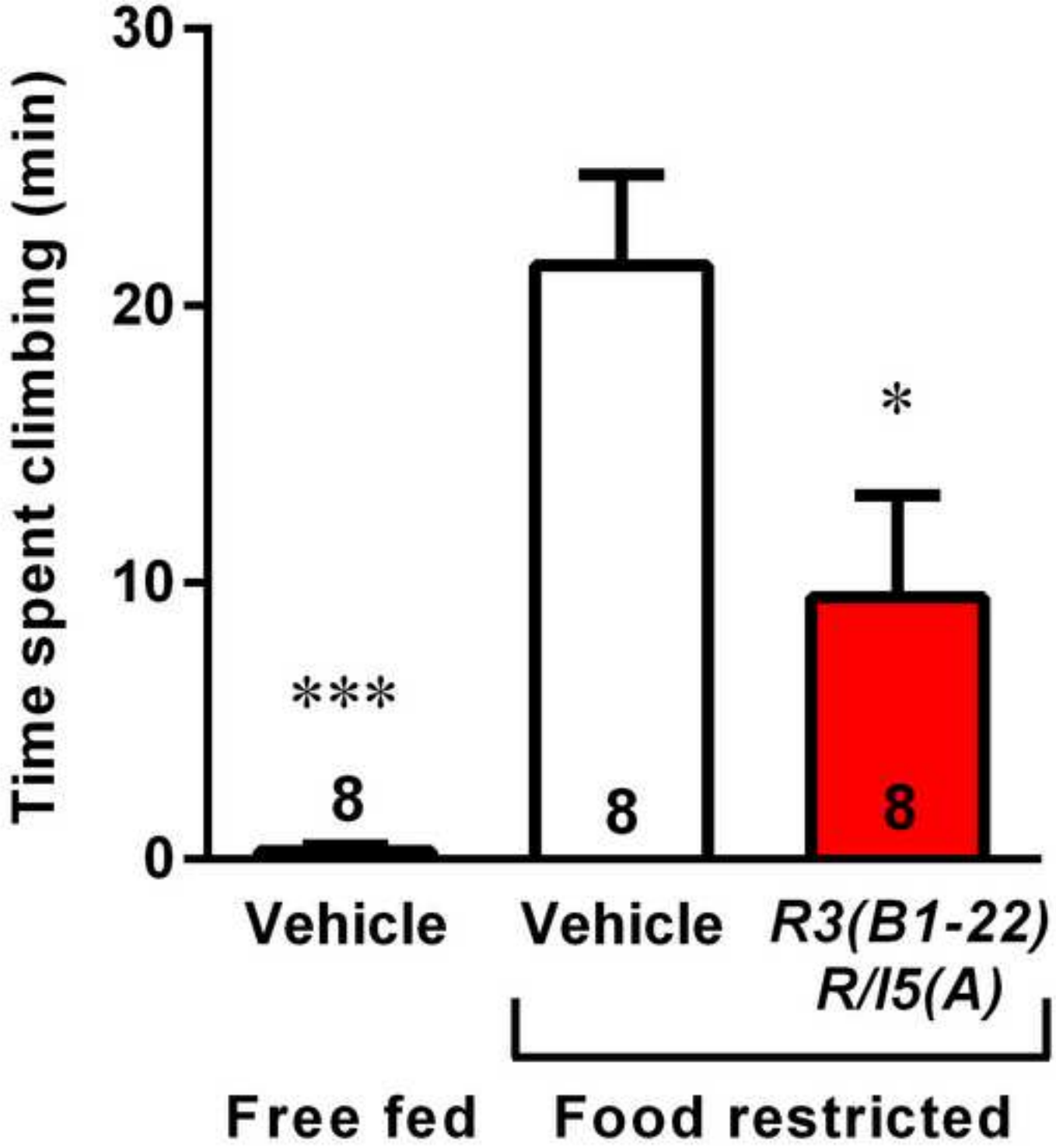


Figure 3

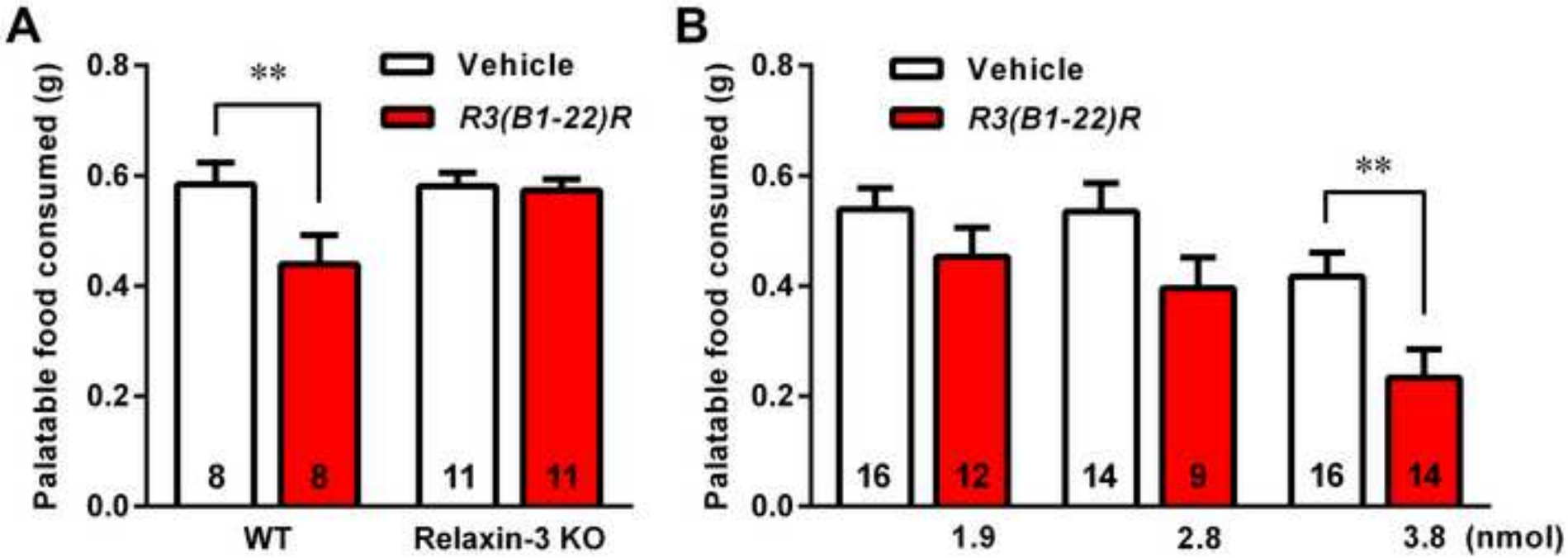

A
Palatable food consumed (g)
0.8
0.6
0.4
0.2
0.0
**
Vehicle
R3(B1-22)R
8
8
11
11
WT
Relaxin-3 KO
B
Palatable food consumed (g)
0.8
0.6
0.4
0.2
0.0
Vehicle
R3(B1-22)R
**
16
12
14
9
16
14
1.9
2.8
3.8 (nmol)

Figure 4

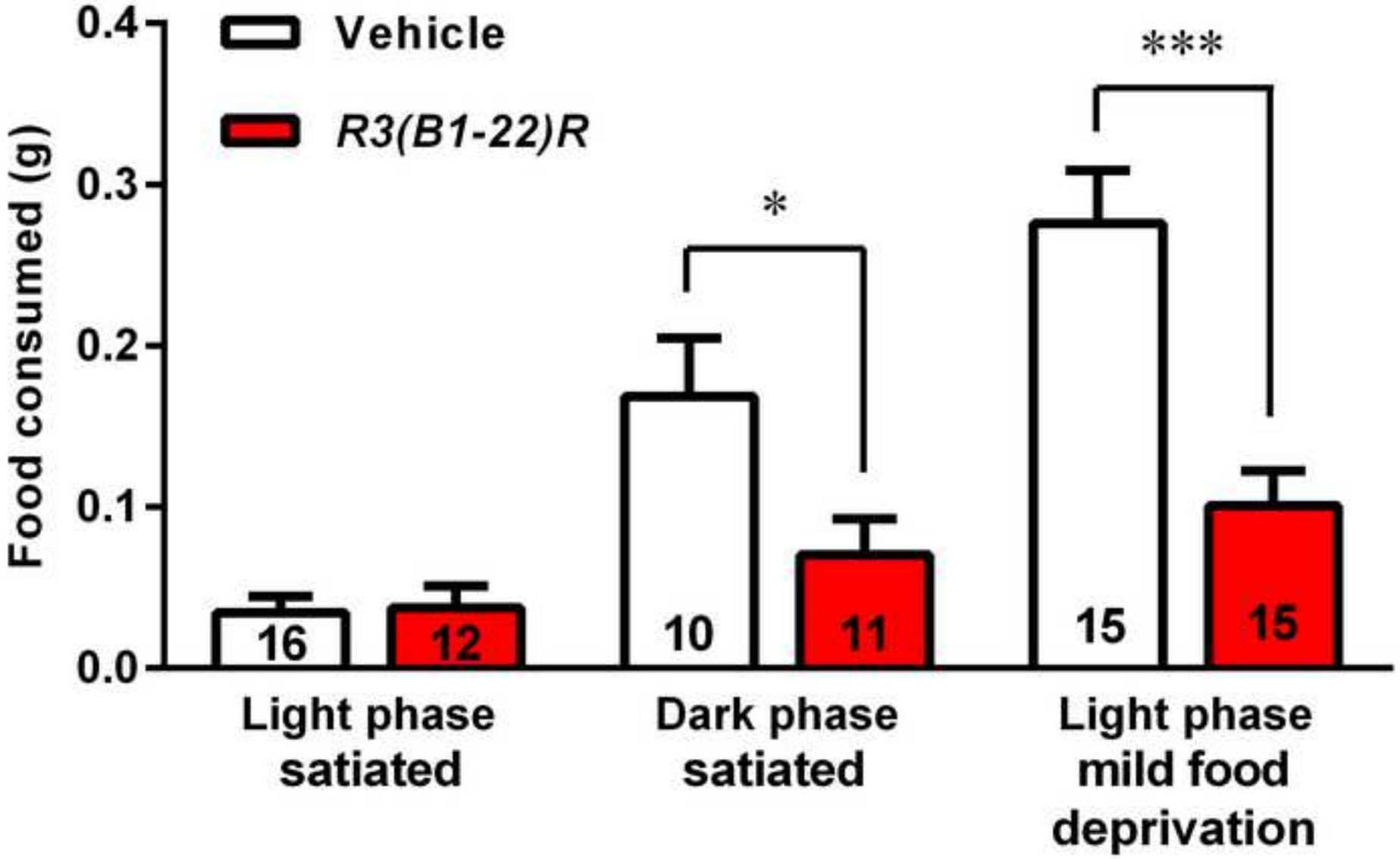

Vehicle
R3(B1-22)R
Food consumed (g)
0.4
0.3
0.2
0.1
0.0
*
***
16
12
10
11
15
15
Light phase satiated
Dark phase satiated
Light phase mild food deprivation

Figure 5

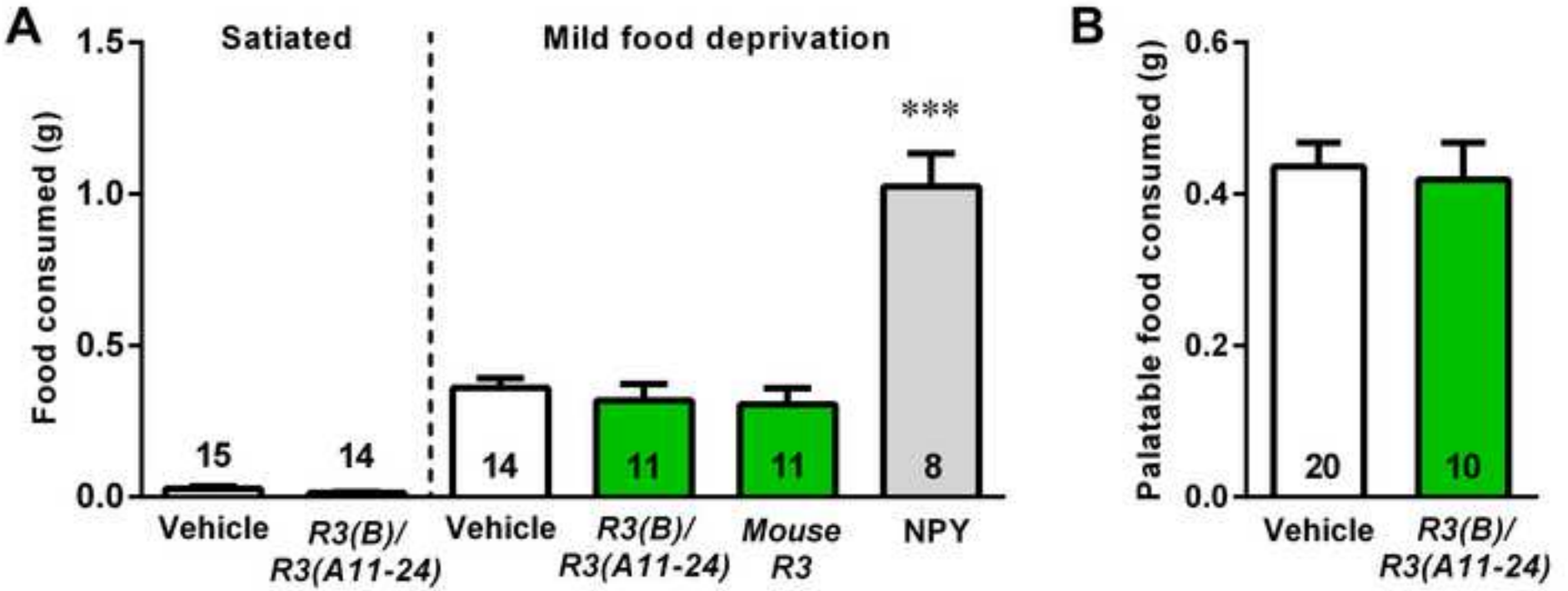
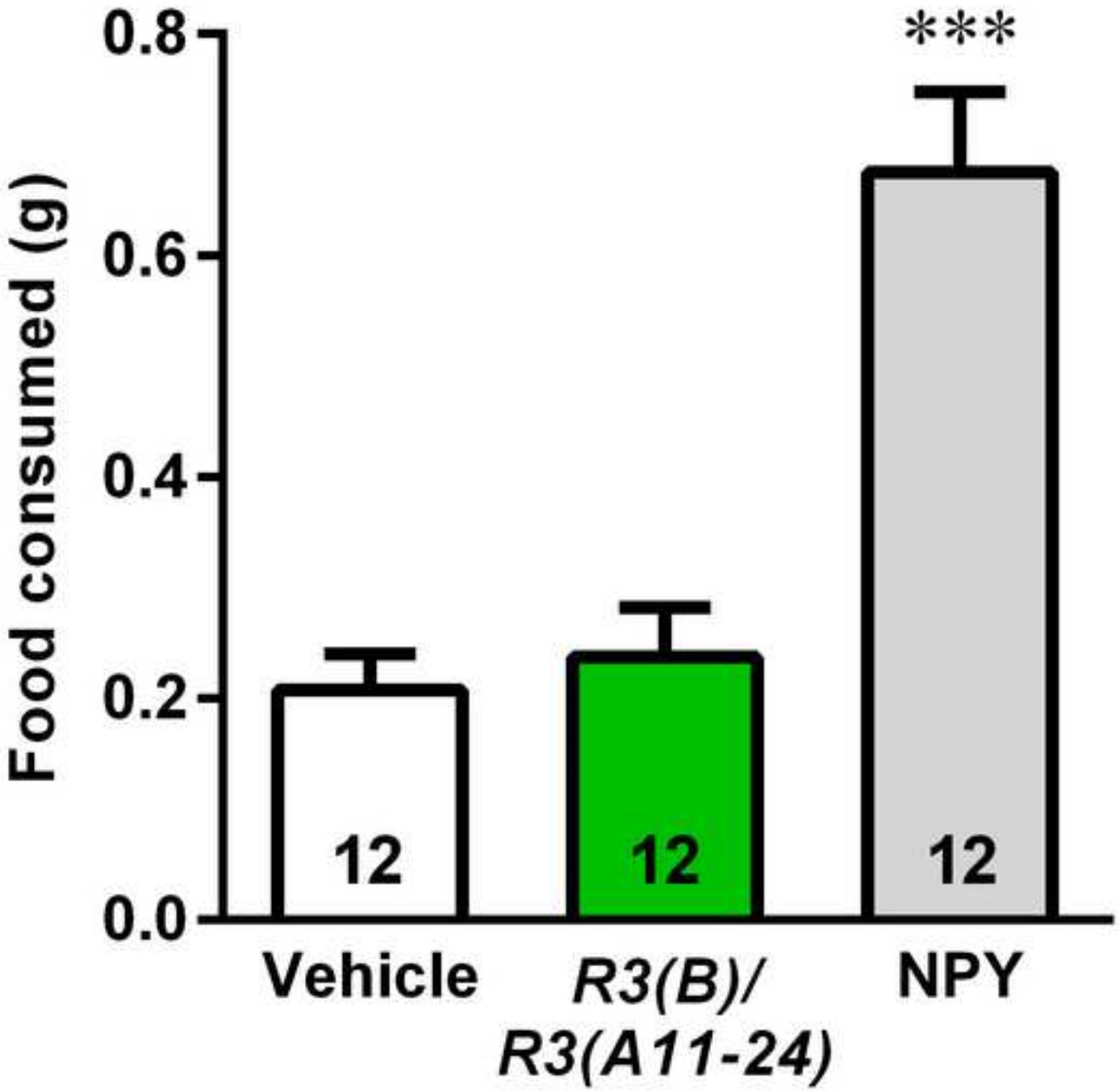
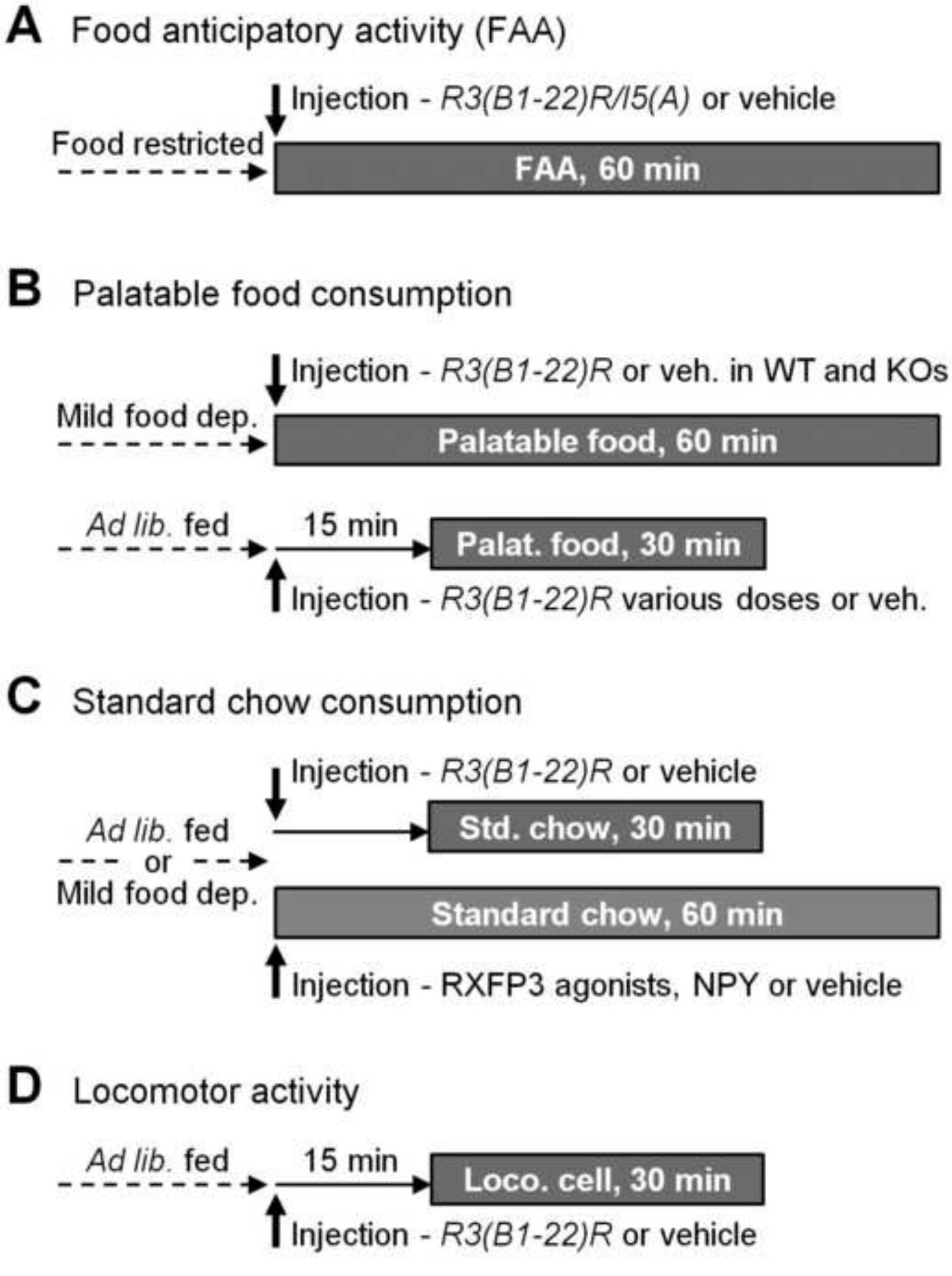

A
Satiated
Mild food deprivation
Food consumed (g)
1.5
1.0
0.5
0.0
***
15
14
14
11
11
8
Vehicle
R3(B)/ R3(A11-24)
Vehicle
R3(B)/ R3(A11-24)
Mouse R3
NPY
B
Palatable food consumed (g)
0.6
0.4
0.2
0.0
20
10
Vehicle
R3(B)/ R3(A11-24)

Figure 6





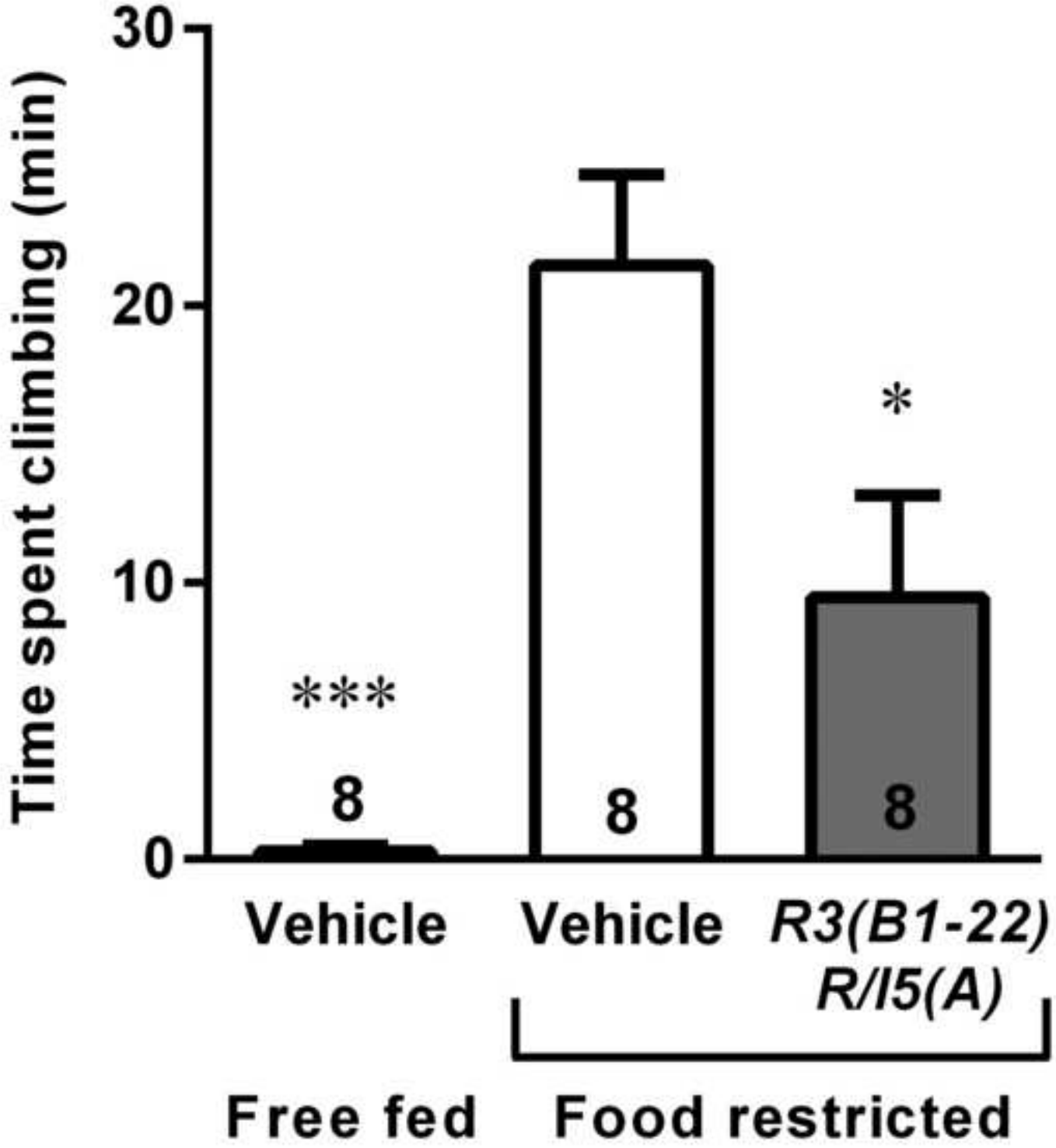


Figure 3 greyscale

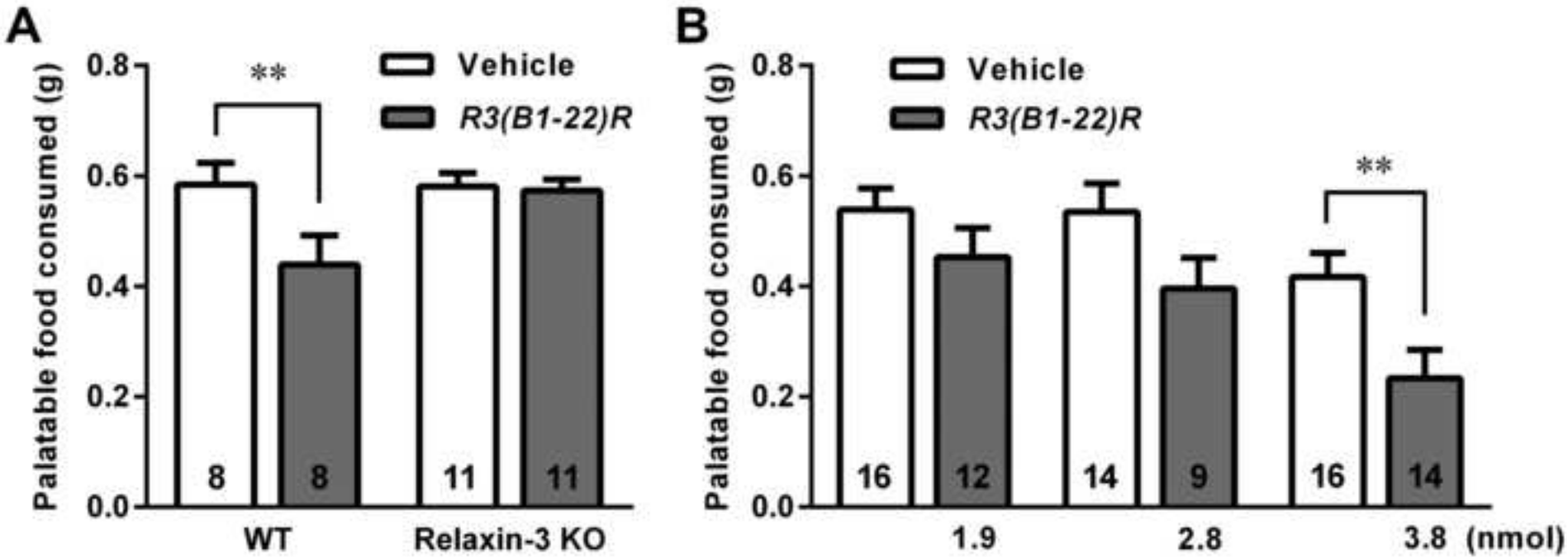


Figure 4 greyscale

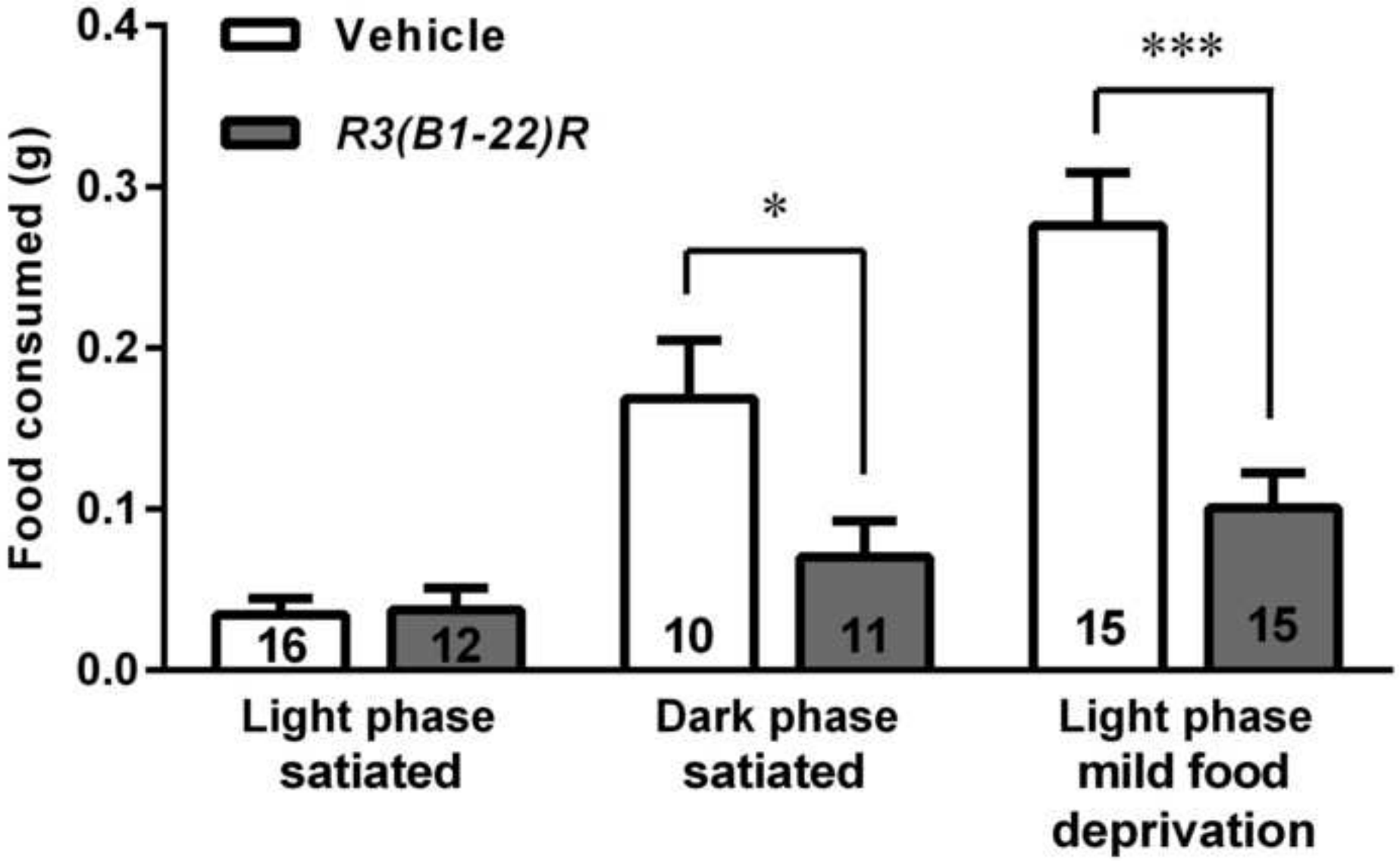


Figure 5 greyscale

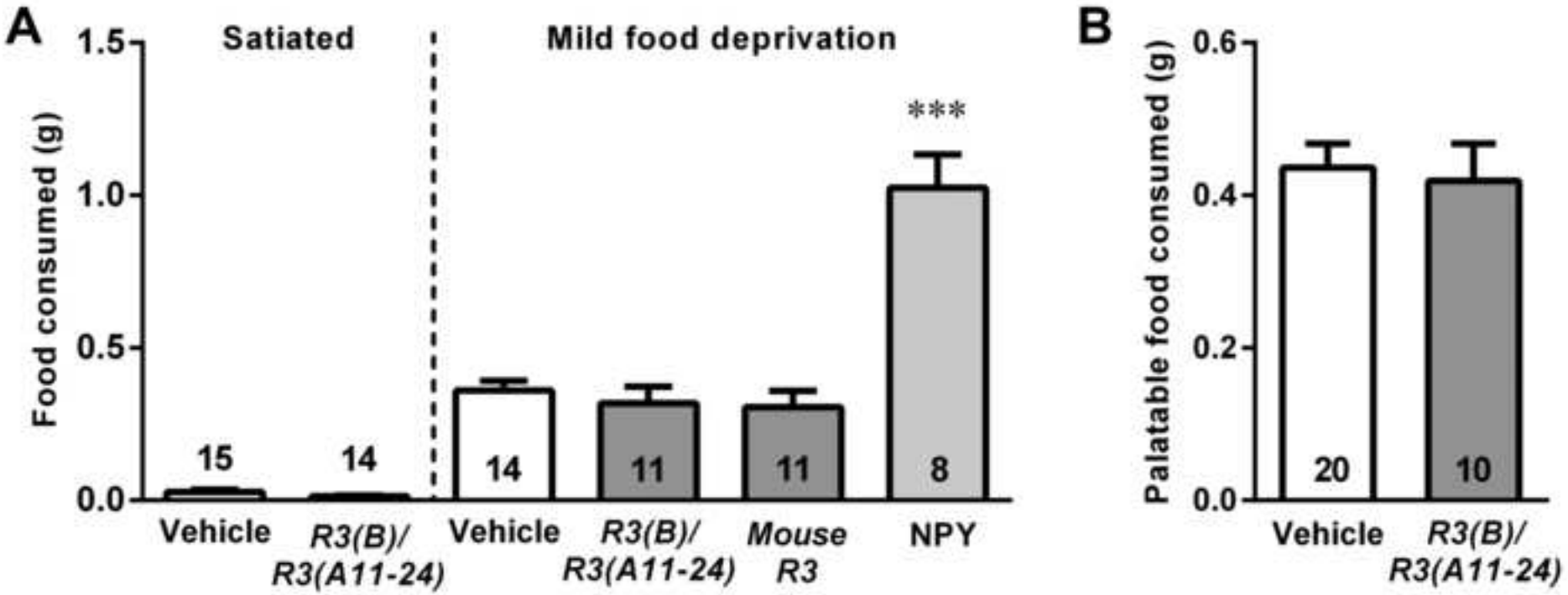
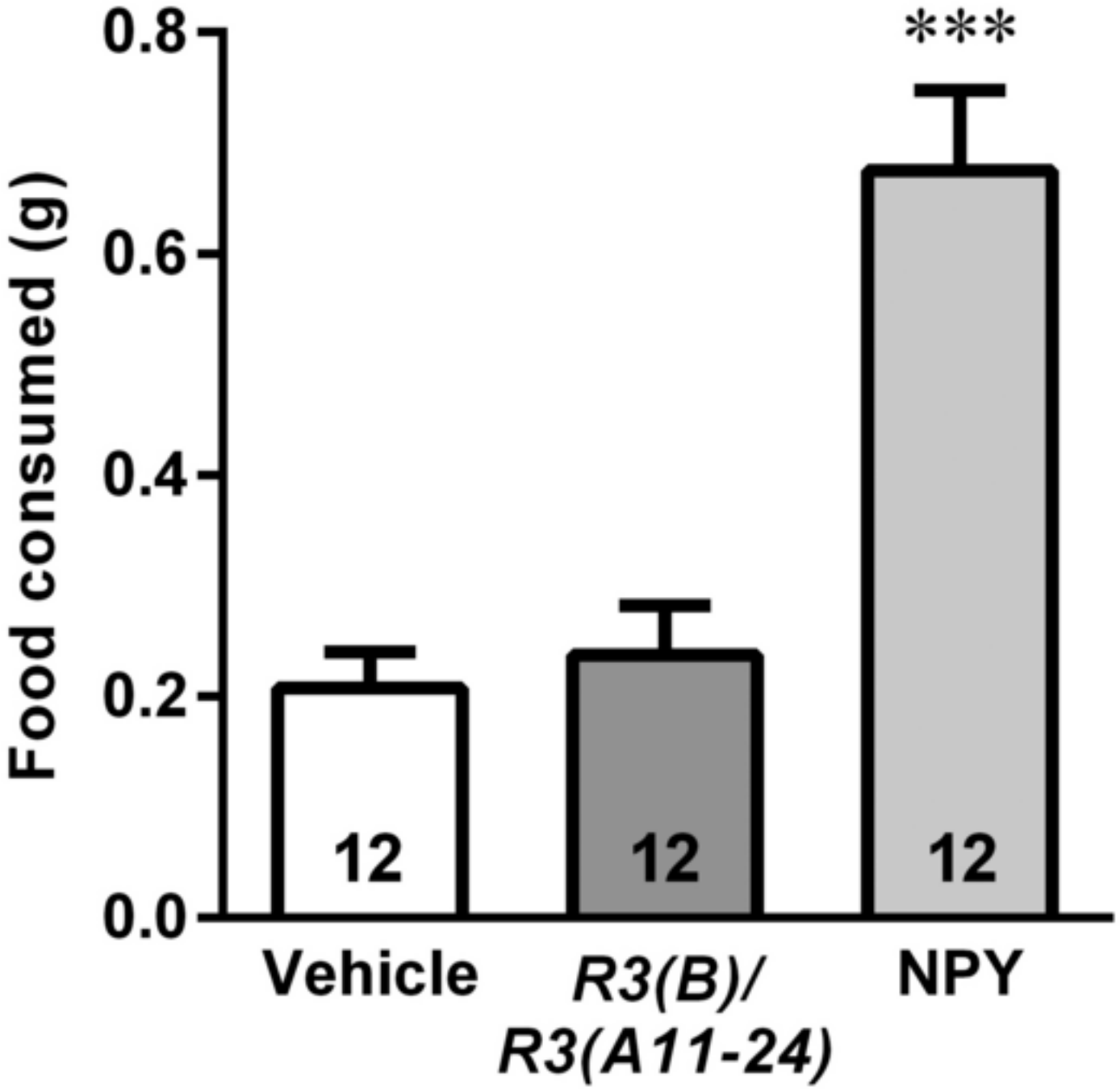


Figure 6 greyscale



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Date:
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