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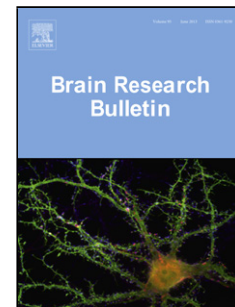


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Differential effects of relaxin-3 and a selective relaxin-3 receptor agonist on food and water intake and hypothalamic neuronal activity in rats

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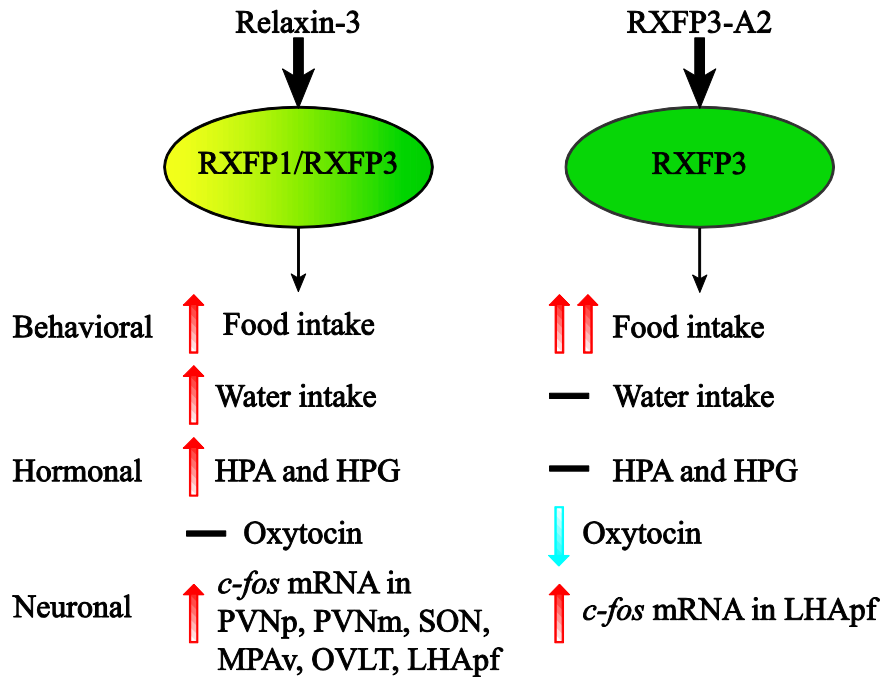
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Graphical abstract



Highlights:

- RXFP3-A2 stimulated food intake more strongly than RLN3
- RLN3 but not RXFP3-A2 increased water intake
- RLN3 but not RXFP3-A2 increased corticosterone and testosterone plasma levels
- RLN3 increased *c-fos* mRNA in the PVNp, PVNm, SON, MPAv, OVLT and LHApf
- RXFP3-A2 increased *c-fos* mRNA in the LHApf and decreased plasma oxytocin

ABSTRACT

The neuropeptide relaxin-3 (RLN3) binds with high affinity to its cognate receptor, relaxin-family peptide receptor 3 (RXFP3), and with lower affinity to RXFP1, the cognate receptor for relaxin. Intracerebroventricular (*icv*) administration of RLN3 in rats strongly

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increases food and water intake and alters the activity of the hypothalamic-pituitary-adrenal (HPA) and -gonadal (HPG) axes, but the relative involvement of RXFP3 and RXFP1 in these effects is not known. Therefore, the effects of *icv* administration of equimolar (1.1 nmol) amounts of RLN3 and the RXFP3-selective agonist RXFP3-A2 on food and water intake, plasma levels of corticosterone, testosterone, and oxytocin, and *c-fos* mRNA expression in key hypothalamic regions in male rats were compared. Food intake was increased by both RLN3 and RXFP3-A2, but the orexigenic effects of RXFP3-A2 were significantly stronger than RLN3 30 and 60 min after injection. Water intake and plasma corticosterone and testosterone levels were significantly increased by RLN3, but not by RXFP3-A2. Conversely, RXFP3-A2 but not RLN3 decreased oxytocin plasma levels. RLN3, but not RXFP3-A2, increased *c-fos* mRNA levels in the parvocellular (PVNp) and magnocellular (PVNm) paraventricular and supraoptic (SON) hypothalamic nuclei, in the ventral medial preoptic area (MPAv), and in the organum vasculosum of the lamina terminalis (OVLT). A significant increase in *c-fos* mRNA expression was induced in the perifornical lateral hypothalamic area (LHApf) by RLN3 and RXFP3-A2. These results suggest that RXFP1 is involved in the RLN3 stimulation of water intake and activation of the HPA and HPG axes. The reduced food intake stimulation by RLN3 compared to RXFP3-A2 may relate to activation of both orexigenic and anorexigenic circuits by RLN3.

Abbreviations:

aCSF – artificial cerebrospinal fluid; BW – body weight; cAMP – cyclic adenosine monophosphate; CRF – corticotropin-releasing factor ; FSH – follicular-stimulating hormone; GnRH – gonadotropin-releasing hormone; HPA – hypothalamic-pituitary-adrenal; HPG – hypothalamic-pituitary-gonadal; LH – luteinizing hormone; LHA_l – lateral part of the lateral hypothalamic area; LHApf – perifornical part of the lateral hypothalamic area; MCH – melanin-

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concentrating hormone; MPA_v – ventral part of the medial preoptic area; NI – nucleus incertus; OVLT – organum vasculosum of the lamina terminalis; PVN_m – magnocellular part of the paraventricular hypothalamic nucleus; PVN_p – parvocellular part of the paraventricular hypothalamic nucleus; RLN3 – relaxin-3; RXFP1 – relaxin-family peptide receptor 1; RXFP3 – relaxin-family peptide receptor 3; RXFP3-A2 – RXFP3-selective agonist relaxin-3 analogue 2; SON – supraoptic hypothalamic nucleus.

Keywords: relaxin-3; food intake; water intake; HPA axis; HPG axis; *c-fos*.

1. INTRODUCTION

Relaxin-3 (RLN3) is the ancestral member of the insulin-relaxin peptide superfamily [1, 2], which is comprised of seven peptides known initially for their major roles in reproduction, growth and development [3-5]. The peptides of this superfamily contain two amino acid chains (A and B) connected by three disulfide bonds [6]. In humans there are three relaxin genes: *RLN1*, encoding human relaxin-1 (H1), *RLN2* encoding human relaxin-2 (H2), and *RLN3* encoding human relaxin-3 [3, 7]. Humans and higher primates have all three relaxin genes, whereas in non-primates, only two relaxin genes are present: *RLN1* and *RLN3*. The product of the *RLN1* gene in non-primates, relaxin, is equivalent to the *RLN2* gene product in humans, H2. In

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mammals, relaxin is produced in several tissues as a paracrine and autocrine factor, and it circulates in the blood during pregnancy [3]. Unlike other relaxin-family peptides that display considerable species heterogeneity, RLN3 sequences are well-conserved across species [8, 9]. Also, unlike other relaxin-family peptides that are strongly expressed in the peripheral organs, RLN3 is primarily a neuropeptide produced almost exclusively in the brain [1], with a particularly strong expression in the pontine nucleus incertus (NI) [10]. NI neuron projections were detected throughout the brain, including areas of the brainstem, hypothalamus, septum, hippocampus, and cerebral cortex [10]. RLN3 binds with high affinity to its cognate receptor, known as relaxin-family peptide receptor 3 (RXFP3), and with a lower affinity to RXFP1, the cognate receptor for relaxin [4, 11]. RLN3 also binds to RXFP4, the cognate receptor for insulin-like peptide 5 (INSL5), but this receptor is a non-functional pseudogene in rats, which therefore limits the brain actions of RLN3 to RXFP3 and RXFP1 in this species [12].

RXFP3 and RXFP1 are G-protein-coupled receptors. The activation of RXFP3 by RLN3 induces the inhibition of cAMP accumulation *via* $G_{i/o}$ -protein coupling, while the activation of RXFP1 stimulates adenylate cyclase and increases intracellular cAMP [11, 13]. An increase in cAMP in response to RXFP1 activation stimulates the cAMP-response element that triggers the expression of the immediate early gene *c-fos*, a molecular marker of neuronal activation [14, 15]. The activation of RXFP3 by RLN3 may stimulate the protein kinase C-dependent (PKC) pathway that drives the phosphorylation of extracellular signal-regulated kinase (ERK) 1/2 [16] and subsequently activates *c-fos* gene expression [17]. RXFP1 and RXFP3 mRNAs are expressed in several different brain areas, with an overlapping expression in the paraventricular (PVN) and the supraoptic (SON) hypothalamic nuclei; however, other brain regions, such as the lateral hypothalamic area (LHA), only express RXFP3 [10, 18-20].

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RLN3 plays a role in several physiological functions. The intracerebroventricular (*icv*) administration of this neuropeptide increases food intake [21-23] and water intake [24-26] in rats. Central RLN3 administration also induces the expression of c-Fos protein in the corticotropin-releasing factor (CRF) neurons of the PVN and increases plasma corticosterone levels [22, 27]. RLN3 treatment of hypothalamic explants from male rats stimulates the release of gonadotropin-releasing hormone (GnRH) [28]. In addition, chronic *icv* administration of RLN3 increases plasma levels of luteinizing hormone (LH) and follicular-stimulating hormone (FSH) in male rats [29]; however, the precise involvement of RXFP3 and/or RXFP1 in these physiological functions is not clearly understood. Recently, a high-affinity RXFP3 agonist [R3A(11–24,C15→A)B], referred to as RXFP3-selective analogue 2 (RXFP3-A2), was produced [30]. Similar to RLN3, the *icv* administration of RXFP3-A2 increased food intake in satiated rats [30]. The bath application of RLN3 and RXFP3-A2 produced inhibitory effects on oxytocin and vasopressin PVN neurons *in vitro* [31]. However, the application of RLN3 in the presence of RXFP3 blockade caused an increase in action potential firing, which might represent an excitatory action of RLN3 on these neurons that is mediated by RXFP1 [31]. So far, there is no clear understanding of the differential effects of the specific activation of RXFP3 by its selective agonist *versus* the simultaneous activation of RXFP3 and RXFP1 by RLN3 on food and water intake, plasma hormones, or on the induction of *c-fos* mRNA expression. Given the possible involvement of RLN3 signalling in binge eating, memory, and stress [27, 32-36], further elucidation of the relative contribution of RXFP1 and RXFP3 to the pharmacological effects of RLN3 is required.

Therefore, this study was designed to compare the effects of RLN3, a non-selective agonist at RXFP3 and RXFP1, and RXFP3-A2, a truncated RLN3 analogue with a high

selectivity for RXFP3 over RXFP1 and strong agonist activity [30], on food and water intake and on plasma levels of corticosterone, testosterone, oxytocin, vasopressin, epinephrine, and norepinephrine in male rats. In addition, the activation by RLN3 and RXFP3-A2 of the key hypothalamic regions involved in the regulation of food intake and the hypothalamic-pituitary-adrenal (HPA) and hypothalamic-pituitary-gonadal (HPG) axes activity were investigated by assessing the levels of *c-fos* mRNA expression.

2. MATERIALS AND METHODS

2. 1. Animals

Male Sprague-Dawley rats (postnatal days 57-59, 250-275 g, n = 32) were purchased from the Canadian Breeding Laboratories (St-Constant, QC, Canada). The rats were housed in individual plastic cages lined with wood shavings and maintained on a 12:12 h dark-light cycle (lights on between 6:00 and 18:00 h), with an ambient temperature of $23 \pm 1^{\circ}\text{C}$. Rats were left undisturbed for at least 1 week to adapt to the new environment. Rats had free access to standard laboratory rat chow (2018 Teklab Global 18% Protein Rodent Diet; 3.1 kcal/g, Harlan Teklab, Montreal, QC, Canada) and tap water. All rats were cared for and handled according to the *Canadian Guide for The Care and Use of Laboratory Animals*, and the present protocol was approved by our institutional animal care committee.

2. 2. Peptides

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Rats were infused *icv* with artificial cerebrospinal fluid (aCSF) or 1.1 nmol of human RLN3 (Pharmaceuticals, Belmont, CA, USA) or 1.1 nmol of RXFP3-A2 (Dr MA Hossain, The Florey Institute of Neuroscience and Mental Health, Parkville, Victoria, Australia). RLN3 and RXFP3-A2 were dissolved in aCSF. The dose used was chosen based on an earlier report on the *icv* administration of RLN3 and RXFP3-A2 in rats [30].

2. 3. *Icv* injections

Each rat was deeply anesthetized using 4% isoflurane and implanted with a 26-gauge stainless steel guide cannula (Plastics One, Roanoke, VA, USA) aimed at the lateral ventricle using the coordinates: 0.8 mm caudal to the bregma, 1.5 mm lateral to the midline, and 3.5 mm ventral from the skull surface. After 7 days of postoperative care, all rats were habituated to regular handling over 5 – 7 days. Rats were randomly divided into three groups and all peptides were administered using a between-subject design. *Icv* injections of 5 μ l of RLN3 (n = 10), RXFP3-A2 (n = 12) or aCSF (n = 10) were administered over 1 min via stainless steel injectors (27-gauge) that projected 1 mm below the tip of the guide cannula. After injection, the injector was left in place for 1 min, and then the rats were returned to their home cages. All injections were performed with *ad libitum*-fed rats in the early light phase (8:00 – 11:00). Body weight (BW) was measured immediately before the *icv* injections to assess the food and water intake in milligram per gram BW. Each rat received three injections separated by one week of the same assigned peptide to measure chow intake, water intake, and *c-fos* mRNA expression. One week before the injections, the cannula position was verified by a positive dipsogenic response to human angiotensin II injection (30 ng per rat, Sigma-Aldrich, St. Louis, MO). A dipsogenic

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response was validated if the rat started to drink water without delay and drank continuously for at least 2 min after injection; with a volume of water consumed during the 30 min post-injection >5 ml. Cannula placements were ultimately confirmed histologically at the end of the study. Only rats with correct cannula placement were included in the data analysis.

2. 4. Water and chow intake

Rats were injected *icv* with RLN3 (n = 10), RXFP3-A2 (n = 12) or aCSF (n = 10) and water intake was measured at 0, 30, 60 and 120 min post-injection in home cages without access to chow. Water intake was measured by calculating the difference in the weight of the cage water container before and after the test. One week later, the rats were *icv* injected with RLN3 (n = 10), RXFP3-A2 (n = 12) and aCSF (n = 10) and chow intake was measured at 0, 30, 60 and 120 min post-injection in home cages without access to water. Food intake was measured by calculating the difference in the weight of the chow remaining and that provided immediately before the test.

2. 5. Blood sample and brain preparation

At the end of experiment, rats were sacrificed 30 min after the final *icv* injection of RLN3, RXFP3-A2 or aCSF during the early light phase. Immediately after injection, the rats were transferred to their home cages, where they had no access to chow and water in the 30 min before euthanasia. Rats were deeply anesthetized (60 mg/kg ketamine plus 7.5 mg/kg xylazine), intracardial blood was collected, and they were perfused intracardially with 100 ml of saline followed by 200 ml of a 4% paraformaldehyde solution. Brains were removed and post-fixed in 4% paraformaldehyde for one week. Brains were then transferred to a solution containing 4%

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paraformaldehyde and 20% sucrose, before being sectioned 12 h later using a sliding microtome (HistoSlide 2000, Heidelberg, Germany). Thirty-micron-thick coronal sections were collected and stored at -20°C in a cold sterile cryoprotection solution containing 50 mM sodium phosphate buffer, 30% ethylene glycol, and 20% glycerol.

2. 6. Determination of plasma concentrations of testosterone, corticosterone, oxytocin, vasopressin, epinephrine, and norepinephrine

Blood samples were centrifuged (3,000 g for 15 min at 4°C) and plasma was stored at -80°C until the determination of corticosterone, testosterone, oxytocin, vasopressin, epinephrine, and norepinephrine plasma levels in duplicate using commercial ELISA kits of corticosterone (Cayman Chemical, Ann Arbor, MI, USA; sensitivity, 5.0 pg/ml), testosterone (Enzo Life Science, Farmingdale, NY, USA; sensitivity, 5.7 pg/ml), oxytocin (Phoenix Pharmaceuticals, Burlingame, CA, USA; sensitivity: 0.13 ng/ml), vasopressin (Enzo Life Science, Farmingdale, NY, USA; sensitivity: 2.84 pg/ml), epinephrine and norepinephrine (Abnova, Taipei City, Taiwan; sensitivity: 10 pg/ml and 36 pg/ml, respectively).

2. 7. *In situ* hybridization for c-fos mRNA

Levels of c-fos mRNA expression in the hypothalamus were detected using *in situ* hybridization, as described [32, 37]. Briefly, the sections were mounted on poly-L-lysine coated slides and fixed for 20 min in 4% paraformaldehyde, digested for 30 min at 37°C with proteinase K (10 $\mu\text{g/ml}$ in 100 mM Tris-HCl containing 50 mM EDTA, pH 8.0), acetylated with acetic anhydride (0.25% in 0.1 M triethanolamine, pH 8.0), and dehydrated through graded

concentrations of ethanol. Sections were incubated overnight with antisense [^{35}S]-labeled cRNA probes (10^7 cpm/ml) for *c-fos* (generated from the 2116-bp fragment of rat *c-fos* cDNA; GenBank accession number X06769.1; Dr I Verma, Salk Institute, La Jolla, CA) at 60°C. Slides were rinsed with sodium chloride-sodium citrate solution, digested with ribonuclease-A (20 $\mu\text{g/ml}$), washed in descending concentrations of sodium chloride-sodium citrate solutions, and dehydrated through an ethanol gradient. Thereafter, slides were defatted in toluene, dipped in nuclear track beta-2 nuclear emulsion (Eastman Kodak, Rochester, NY, USA), and exposed for 2 weeks before developing. Tissues were counterstained with thionine, dehydrated through graded concentrations of ethanol, cleared in toluene and coverslipped with mounting medium.

2. 8. Image analyses

Slides were examined with dark-field microscopy using an Olympus BX61 microscope (Olympus Canada, Richmond Hill, ON, Canada). Images were acquired with a DVC-2000C digital camera (DVC Company Inc., Austin, TX, USA) and analyzed with Stereo Investigator software (MBF Bioscience, Williston, VT, USA). The system was calibrated for each set of analyses to prevent saturation of the integrated signal; and the optical density (OD) reading was normalized to a scale where minimum (0) and maximum (1) values represent pure dark (opaque) and white pixels, respectively. Mean OD was obtained by measuring the OD of pixels of the positive hybridization signal in sections of the parvocellular PVN (PVNp) and the magnocellular PVN (PVNm) (from 1.72 mm to 1.80 mm caudal to bregma), the SON (from 1.32 to 1.56 caudal to bregma) and the OVLT (from 0.15 mm to 0.00 mm rostral to bregma) [38], and subtracting the background readings taken from the areas immediately surrounding the analyzed region. Because of the scattered hybridization signals of *c-fos* mRNA in the MPA (from 0.3 rostral to

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bregma to 0.4 mm caudal to bregma) and the LHA (from 2.9 to 3.4 mm caudal to bregma) [38], *c-fos* mRNA expression was assessed using the cumulative area of the positive hybridization signal. A threshold depicting positive hybridization signal was set for pixels with OD >5 times the background for all micrographs captured with the same luminosity. The OD measurements were performed in the regions of interest outlined bilaterally on two or three consecutive brain sections (four to six values per rat). The individual score of each rat was normalized to the mean value of the aCSF-injected group (mean value of a rat/mean value of aCSF group) to obtain the relative expression of *c-fos* mRNA.

2. 9. Statistical analyses

Results are presented as mean \pm SEM. For cumulative food intake and water intake analysis, two-way repeated-measures ANOVA was used to detect the main and interactive effects of treatment (RLN3, RXFP3-A2 and aCSF) and time (0, 30, 60 or 120 min). For plasma hormones and for *c-fos* mRNA analyses, one-way ANOVA was used to detect the effect of treatment. *Post-hoc* comparisons between the groups were performed using Fisher's protected least significant difference test when the main and interactive ANOVA effects were significant. $P < 0.05$ was considered statistically significant. Statistical analyses were performed using PRISM statistical software, V6.04 (GraphPad Software Inc., La Jolla, CA, USA).

3. RESULTS

3. 1. Food and water intake

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The cumulative food intake was significantly increased by the *icv* administration of RLN3 (5.7 ± 1.3 mg/g BW, 8.9 ± 1.2 mg/g BW, and 12.6 ± 2.6 mg/g BW after 30, 60, and 120 min, respectively) and RXFP3-A2 (11.3 ± 1.6 mg/g BW, 12.6 ± 1.4 mg/g BW, and 13.3 ± 1.4 mg/g BW after 30, 60, and 120 min, respectively) compared to the control injections of aCSF (0.5 ± 0.2 mg/g BW, 0.9 ± 0.5 mg/g BW, and 3.2 ± 1.2 mg/g BW after 30, 60, and 120 min, respectively) (Fig. 1A). The two-way repeated-measures ANOVA revealed significant effects of time ($F_{3, 116} = 31.63$, $p < 0.0001$) and treatment ($F_{2, 116} = 43.16$, $p < 0.0001$) and a significant interaction between time and treatment ($F_{6, 116} = 5.555$, $p < 0.0001$) on food intake. *Post-hoc* analyses revealed that compared to the aCSF control group, the injection of RLN3 significantly increased the cumulated food intake after 30 min ($p < 0.0054$), 60 min ($p < 0.0001$), and 120 min ($p < 0.0001$). Similarly, the administration of RXFP3-A2 significantly increased the cumulative food intake after 30, 60, and 120 min ($p < 0.0001$ at each time-point) compared to the aCSF control. Compared to RLN3, the administration of RXFP3-A2 produced significantly stronger orexigenic effects after 30 min ($p < 0.0023$) and 60 min ($p < 0.0384$), but not after 120 min ($p > 0.05$) following *icv* injections.

The cumulative water intake was significantly increased by RLN3 (12.2 ± 2.7 mg/g BW, 17.4 ± 2.8 mg/g BW, and 27.9 ± 4.9 mg/g BW after 30, 60, and 120 min, respectively) but not by RXFP3-A2 (1.1 ± 0.5 mg/g BW, 5.6 ± 1.4 mg/g BW, and 10.6 ± 2.3 mg/g BW after 30, 60, and 120 min, respectively) compared to the aCSF-injected group (2.8 ± 0.8 mg/g BW, 5.6 ± 1.2 mg/g BW, and 9.5 ± 1.5 mg/g BW after 30, 60, and 120 min, respectively) (Fig. 1B). The two-way repeated-measures ANOVA revealed significant effects of time ($F_{3, 108} = 32.22$, $p < 0.0001$) and treatment ($F_{2, 108} = 30.88$, $p < 0.0001$) and a significant interaction between time and treatment ($F_{6, 108} = 4.342$, $p < 0.0006$) on water intake. *Post-hoc* analyses revealed that compared to the

aCSF control group, the injection of RLN3 significantly increased water intake after 30 min ($p < 0.0018$), 60 min ($p < 0.0001$), and 120 min ($p < 0.0001$). Conversely, no significant difference ($p > 0.05$) was detected between RXFP3-A2 and aCSF-injected groups at any time point. The cumulative water intake was significantly higher in RLN3-injected rats after 30 min ($p < 0.0003$), 60 min ($p < 0.0001$), and 120 min ($p < 0.0001$) compared to RXFP3-A2 administration.

3. 2. Plasma concentrations of testosterone, corticosterone, oxytocin, vasopressin, epinephrine and norepinephrine

The levels of plasma hormones were measured at 30 min after the *icv* administration of RLN3 or RXFP3-A2. Testosterone was increased by RLN3 (11.9 ± 1.3 ng/ml) but not by RXFP3-A2 (5.3 ± 0.5 ng/ml) compared to the aCSF-injected group (6.1 ± 0.7 ng/ml) (Fig. 2A). The one-way ANOVA revealed a significant effect of the treatment on plasma testosterone ($F_{2,29} = 17.32$, $p < 0.001$). *Post-hoc* analyses detected significant differences between the RLN3 and aCSF groups ($p < 0.0001$) and between the RLN3 and RXFP3-A2 groups ($p < 0.0001$), but not between the aCSF and RXFP3-A2 groups ($p > 0.05$).

The plasma corticosterone levels were increased by RLN3 (51.9 ± 6.2 ng/ml) but not by RXFP3-A2 (17.1 ± 3.4 ng/ml) compared to the aCSF-injected group (18.4 ± 5.6 ng/ml; Fig. 2B). The one-way ANOVA revealed significant effects of treatment on plasma corticosterone ($F_{2,29} = 14.83$, $p < 0.0001$). According to the *post-hoc* analyses, the plasma corticosterone levels were significantly higher after RLN3 administration compared to RXFP3-A2 ($p < 0.0001$) and aCSF ($p < 0.0001$), but they were not different between the RXFP3-A2- and aCSF-injected groups ($p > 0.05$).

Oxytocin plasma levels were significantly affected by treatments ($F_{2, 29} = 3.89$, $p = 0.0326$, one-way ANOVA; Fig. 2C). *Post-hoc* analyses revealed that oxytocin levels were significantly ($p < 0.01$) decreased by RXFP3-A2 (8.4 ± 0.4 ng/ml) but not by RLN3 ($p > 0.05$; 9.3 ± 0.4 ng/ml) relative to levels in aCSF-injected group (10.8 ± 0.9 ng/ml). However, there was no significant difference ($p > 0.05$) between oxytocin levels in the RLN3- and RXFP3-A2-injected groups.

Although there was a tendency for a decrease in plasma vasopressin after injection of RLN3 (16.4 ± 14.6 pg/ml) or RXFR3-A2 (49.0 ± 18.7 pg/ml) relative to aCSF control injection (103.3 ± 49.3 pg/ml), these changes did not reach significance (Fig. 2D). One-way ANOVA ($F_{2, 29} = 1.95$, $p = 0.1609$) and *post-hoc* analyses did not reveal significant effects of treatments on plasma vasopressin. Similarly, plasma epinephrine ($F_{2, 29} = 0.35$, $p = 0.7096$; Fig. 2E) and norepinephrine ($F_{2, 29} = 1.832$, $p = 0.1817$; Fig. 2F) levels were not affected by *icv* injections of RLN3 or RXFP3-A2.

3. 3. *c-fos* mRNA expression in the PVN and SON

At 30 min after the *icv* administration of RLN3, the expression of *c-fos* mRNA was increased in the magnocellular (Fig. 3A, D) and parvocellular (Fig. 3B, D) parts of the PVN and in the SON (Fig. 3C, E) compared to aCSF-injected rats. However, no difference in the levels of *c-fos* mRNA was observed in these regions between rats that received *icv* injections of RXFP3-A2 and aCSF. One-way ANOVA revealed a significant effect of the treatment on *c-fos* mRNA for the magnocellular ($F_{2, 29} = 26.93$, $p < 0.0001$) and parvocellular ($F_{2, 29} = 3.867$, $p = 0.0324$) parts of PVN and for the SON ($F_{2, 29} = 14.32$, $p < 0.0001$). The *post-hoc* analyses revealed a

significant increase in *c-fos* mRNA expression in the RLN3 group compared to the aCSF group ($p < 0.0001$ for the PVNm, $p < 0.05$ for the PVNp, and $p < 0.001$ for the SON) and compared to the RXFP3-A2 group ($p < 0.0001$ for the PVNm and the SON; $p < 0.05$ for the PVNp) for these three regions. Conversely, no significant variation was observed in the *c-fos* mRNA expression in the RXFP3-A2 group compared to the aCSF-injected rats ($p > 0.05$ for the PVNm, PVNp, and SON).

3. 4. *c-fos* mRNA expression in the LHA

Icv injections of RLN3 and RXFP3-A2 induced *c-fos* mRNA expression in the perifornical LHA (Fig. 4B, C), but not in the lateral LHA (Fig. 4A, C). One-way ANOVA revealed a significant effect of the treatment on the *c-fos* levels in the perifornical part ($F_{2, 23} = 7.547$, $p = 0.003$), but not the lateral part ($F_{2, 26} = 1.123$, $p = 0.3406$) of the LHA. The *post-hoc* analyses revealed a significantly larger increase in *c-fos* mRNA expression in the RLN3 ($p < 0.001$) and the RXFP3-A2 ($p < 0.05$) groups compared to aCSF-injected rats in the perifornical LHA but not lateral LHA ($p > 0.05$). No significant differences in *c-fos* mRNA were observed between the RLN3 and RXFP3-A2 groups in the perifornical ($p > 0.05$) or lateral ($p > 0.05$) LHA.

3. 5. *c-fos* mRNA expression in the ventral MPA and OVLT

The expression of *c-fos* mRNA in the ventral MPA (Fig. 5A, C) and the OVLT (Fig. 5B, C) was altered by the *icv* administration of RLN3. One-way ANOVA revealed a significant

effect of the treatment on the *c-fos* expression for the ventral MPA ($F_{2, 28} = 5.155$, $p = 0.0124$) and the OVLT ($F_{2, 28} = 35.95$, $p < 0.0001$). The *post-hoc* analyses detected a significant increase in *c-fos* mRNA expression in these regions in the RLN3-injected group compared to the aCSF group ($p < 0.01$ for the ventral MPA and $p < 0.001$ for the OVLT) and RXFP3-A2 group ($p < 0.05$ for the ventral MPA and $p < 0.001$ for the OVLT). No significant differences were observed between the RXFP3-A2 and aCSF-control groups in the ventral MPA or OVLT ($p > 0.05$).

4. DISCUSSION

This study investigated the effects of the central (*icv*) administration of the selective RXFP3 agonist, RXFP3-A2, and the native RLN3 peptide, which activates RXFP1 and RXFP3 receptors, on food and water intake and on hypothalamic *c-fos* mRNA expression. In line with earlier reports [7, 24-26], this study has demonstrated that the *icv* injection of RLN3 induced a significant increase in food and water intake. Conversely, the administration of RXFP3-A2 strongly stimulated food intake, but did not affect water intake. The observed increase in food intake after the stimulation of RXFP3 is in agreement with the orexigenic effects of RLN3 and RXFP3-A2 that we and others reported earlier [21-23, 30]. However, a direct comparison of the effects of the central administration of equimolar amounts of RLN3 and RXFP3-A2 revealed a significantly higher increase in cumulative food intake in response to RXFP3-A2 at 30 and 60 but not 120 min post-injection than to RLN3. Differential effects of RLN3 and RXFP3-A2 may depend on a difference in their tissue penetration capacity. However, the absence of differences at 120 min after injection may reflect a “ceiling effect” or rats reaching a maximum in food intake. Similarly, different concentrations of RLN3 produced differential effects on cumulative

food intake at 30 and 60 min after injection, but not at 120 min or later [22, 24]. Significant differences in food intake produced by RLN3 and RXFP3-A2 might be rather depend on the binding of RLN3 to its cognate receptor RXFP3 *and* to RXFP1, the native receptor for relaxin, which interferes with or offsets the effects of “pure” RXFP3 activation. In fact, *icv* administration of relaxin has been reported to reduce food intake in male rats [39]. This anorectic effect of relaxin may be mediated *via* actions within the PVN because the administration of relaxin into this region decreased food intake in the early dark phase [39], and there is a high density of RXFP1 in the rat PVN [40]. The present results support the potential involvement of RXFP1 in the activation of the PVN because an increase in *c-fos* mRNA expression was observed in the parvocellular and magnocellular parts of PVN after the *icv* administration of RLN3 but not RXFP3-A2, which might be associated with the activation of both RXFP1 and RXFP3 rather than the activation of RXFP3 alone. In addition, induction of expression of *c-fos* mRNA in the magnocellular neurons may be triggered *via* activation of RXFP1 in the OVLT [41].

RXFP1 and RXFP3 are G-protein-coupled receptors that induce different intracellular responses [3]. The activation of RXFP1 induces an increase in intracellular cAMP, while the activation of RXFP3 induces an inhibition of intracellular cAMP accumulation, suggesting an excitatory and inhibitory impact on target cells, respectively [3, 11]. Recent neurophysiological recordings revealed an inhibition of oxytocin and vasopressin PVN neurons in response to both RLN3 and RXFP3-A2; however, when RXFP3 was blocked by the specific RXFP3 antagonist R3 B1-22R, the bath application of RLN3 caused an increase in action potential firing [31]. RXFP3-induced inhibition was dependent on an outward calcium-dependent potassium current, and the blockade of $G_{i/o}$ -proteins in PVN neurons prevented the inhibitory action of RXFP3-A2

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[31]. In the current study, the *icv* administration of RXFP3-A2 did not induce the expression of *c-fos* mRNA in the PVN, consistent with the inhibition of PVN neurons after the activation of RXFP3. Conversely, the increase in *c-fos* mRNA expression in the PVN in response to RLN3 may be induced by the binding of this peptide to RXFP1 in the OVLT [41]. Parvocellular PVN neurons may affect food intake and energy expenditure due to the involvement of one or more of its several neuron populations, including CRF, oxytocin, and vasopressin neurons [42-44]. Activation of CRF neurons in the PVN is associated with decreases in food intake [44, 45]. Similarly, oxytocin acts as an anorectic signal in both animals and humans [42, 46-48]. Vasopressin is also considered an anorexigenic factor [43], and *icv* or intraperitoneal (*ip*) injections of vasopressin lead to a reduction in food intake [49, 50]. Administration of RLN3 activates the CRF neurons and increases the levels of CRF mRNA in the parvocellular PVN in rats [27]. Conversely, the production of a selective RXFP3 agonist, R3/I5, by an adeno-associated virus within the PVN significantly reduced the expression of oxytocin and vasopressin mRNA [51]. Therefore, the induction of *c-fos* mRNA expression in the PVN in response to RLN3 administration may depend on the RXFP1-related activation of the CRF neurons [27] that would partially blunt orexigenic effects of RLN3. Conversely, the effects of RXFP3-A2 may depend on the activation of the RXFP3 inhibitory pathway via $G_{i/o}$ -protein coupling [11] in oxytocin and vasopressin neurons, leading to a higher increase in food intake. The present results indicates a stronger potential of RXFP3-A2 than RLN3 to decrease plasma oxytocin levels. The levels of vasopressin were not significantly altered by either RLN3 or RXFP3-A2. The lack of significant effects may due to the blood sampling during the early light phase when the plasma levels of vasopressin are at their nadir [52]. Future estimation of the effects of RLN3 and

RXFP3-A2 on the levels of plasma vasopressin should be performed during the late light phase or the beginning of the dark phase, when plasma vasopressin increases and stabilises [52].

In addition to their effects on food intake, the CRF neurons in the parvocellular PVN are involved in the regulation of the HPA axis. This axis includes the hypophysiotropic parvocellular CRF neurons that release CRF from axonal terminals at the level of median eminence from where CRF is transported to the pituitary corticotropic cells, producing adrenocorticotrophic hormone (ACTH), which stimulates adrenal glucocorticoid (cortisol in humans and corticosterone in rats) synthesis and release [44, 53]. In addition to the activation of *c-fos* mRNA expression in the parvocellular PVN, RLN3 injections significantly increased the levels of plasma corticosterone. These results are in agreement with the reported activation of the HPA axis activity in male rats in response to the intracranial administration of RLN3 [22, 27, 36]. The present data suggest that the activation of the HPA axis in response to RLN3 depends on its binding to the RXFP1 because the activation of RXFP3 by its selective agonist RXFP3-A2 did not increase corticosterone plasma levels or *c-fos* mRNA expression in the PVN. This conclusion is supported by an earlier study that reported an increase in plasma corticosterone levels after the *icv* administration of relaxin, the cognate ligand of RXFP1 [36].

Although activation of the NI was associated with heightened arousal and locomotion [54], there is currently no direct evidence that RLN3 alters the activity of the autonomic nervous system. Even though intra-PVN administration of RLN3 significantly decreased the plasma levels of thyroid-stimulating hormone [55], oxygen consumption and respiratory exchange ratio were not affected by intra-PVN RLN3 [55]. Similarly, the current study did not detect significant changes in plasma levels of epinephrine and norepinephrine after *icv* administration of RLN3 or RXFP3-A2.

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In agreement with previous reports [25, 26], a significant increase in water intake after the *icv* administration of RLN3 was observed. However, the present study suggests a particular involvement of RXFP1 in water intake. In fact, injection of RLN3 that activates RXFP1 and RXFP3 led to a strong dipsogenic effect. Conversely, the selective stimulation of RXFP3, but not RXFP1, by RXFP3-A2 did not change the water intake relative to the aCSF-injected control group. Notably, relaxin stimulates water intake but not food intake [39]. The administration of both relaxin and RLN3 increased the expression of *c-fos* mRNA in the magnocellular neurons of SON and PVN [25, 41, 56]. The magnocellular neurons produce vasopressin and oxytocin, which are directly involved in fluid regulation [42, 57, 58]. There is evidence that activation of magnocellular neurons in the SON and PVN in response to relaxin and its dipsogenic effects depend on activation of the circumventricular organs such as the OVLT and subfornical organ (SFO), the small brain areas with a disrupted blood-brain barrier [59]. In fact, ablation of the SFO prevented dipsogenic effects of intravenous infusion of relaxin, while ablation of the OVLT significantly decreased c-Fos expression in magnocellular neurons [41]. The present results do not exclude the possibility that the magnocellular regions were activated by RLN3 *via* the OVLT, as the OVLT displayed a significant induction of *c-fos* mRNA expression in response to *icv* administration of RLN3, but not RXFP3-A2. The OVLT projects directly to the SON and magnocellular PVN and integrity of these connections are important for the activity of magnocellular neurons [60]. Strong activation of *c-fos* mRNA in the magnocellular PVN and SON and in the OVLT in response to RLN3 but not RXFP3-A2, suggests the dipsogenic effects are related to RXFP1, and not RXFP3, activation. This conclusion is supported by observations that *icv* administration of relaxin had comparable [25] or stronger [24] effectiveness on water intake as produced by equimolar amounts of RLN3 in rats.

The central administration of RLN3 and RXFP3-A2 may stimulate food intake due to actions within the LHA, the brain region strongly involved in appetite regulation. The electrical stimulation of LHA, traditionally called the “hunger center,” increases appetite, while the inactivation of LHA produces anorectic effects [61, 62]. The LHA neurons express orexin and melanin-concentrating hormone (MCH), which are neuropeptides with strong orexigenic effects [63, 64]. The LHA contains RXFP3 but not RXFP1 binding sites [20] that may limit the effects of RLN3 to RXFP3 in this region. In the present study, *c-fos* mRNA expression was significantly increased in the perifornical but not in the lateral subdivision of LHA after *icv* administration of both RLN3 and RXFP3-A2. Both perifornical and lateral LHA contain orexin and MCH neurons [23], but there is evidence of a particular role of the LHA sub-regions in eating behaviors. Thus, hyperphagic obesity-prone rats have more orexin-positive neurons in the perifornical than in the lateral LHA [65]. Moreover, obesity induced by a high-fat diet significantly increased the number of orexin neurons in the perifornical but not the lateral LHA [65]. In addition, an increased activity of orexin neurons in the perifornical but not the lateral LHA was detected in rats during the dark active circadian phase when their eating behavior increases [66]. Accordingly, rats that overeat a high-fat diet displayed elevated behavioral arousal and an increased expression of orexin mRNA in the perifornical but not the lateral LHA [67]. The direct administration of RLN3 into the LHA did not affect food intake [21], but these injections were aimed at the lateral LHA, where a significant effect of RLN3 or RXFP3-A2 administration on feeding activity was not observed. It is also possible that induction of *c-fos* mRNA expression in the LHA in response to RLN3 and RXFP2-A2 may depend on multisynaptic circuitry. The present results particularly implicate the perifornical LHA, which is strongly interconnected with the brain structures of the gustatory and metabolic networks including the infralimbic and

agranular insular areas, the central nucleus of the amygdala, the bed nucleus of the stria terminalis, the substantia innominata and the parabrachial nucleus [68]. In a previous study, the *icv* administration of 800 pmol RLN3 in male rats increased *c-fos* mRNA expression in the perifornical LHA by 14% compared to the control [23]; however, this increase was not statistically significant. In the present study, a higher (1.1 nmol) amount of RLN3 significantly increased *c-fos* mRNA expression in the perifornical LHA by 47%, which was statistically significant compared to the control aCSF-injected group. The same dose (1.1 nmol) of RXFP3-A2 also significantly increased *c-fos* mRNA expression in the perifornical LHA. The activation by RXFP3-A2 of perifornical LHA neurons, which contain the orexigenic neuropeptides orexin and MCH, and no activation of the PVN, which contains anorexigenic CRF, oxytocin, and vasopressin neurons, may reinforce its orexigenic effects. Conversely, a coinciding activation of the PVN and the LHA by RLN3 may counteract its orexigenic effects, which were lower than those of RXFP3-A2.

RLN3 has also a potential role in reproductive function. The *icv* administration of RLN3 stimulates the HPG axis by increasing the LH [28] and FSH [29] plasma levels in male rats. These effects likely depend on the stimulation of GnRH neurons because the administration of RLN3 increases GnRH release from hypothalamic explants, and an increase in plasma LH and testosterone induced by the *icv* injection of RLN3 was blocked by pre-treatment with a peripheral GnRH antagonist [28]. GnRH is expressed by neurons in the MPA, and in this region, only a low number of GnRH-immunoreactive neurons (about 3%) co-express RXFP3 mRNA [29]. In addition to RXFP3, MPA neurons express RXFP1 mRNA [19, 20]. The induction of *c-fos* mRNA expression in the MPA in response to the *icv* administration of RLN3 but not RXFP3-A2 suggests that RXFP1 may be involved in MPA activation. The activation of the MPA by

RLN3 was accompanied by a strong increase in plasma testosterone. Conversely, the *icv* administration of an equimolar dose of RXFP3-A2 did not affect plasma levels of testosterone. Although the present data suggest that RXFP1 plays a role in the activation of the HPG axis by RLN3, the *icv* administration of relaxin, which selectively activates RXFP1, produced a consistent but statistically non-significant increase in LH and testosterone plasma levels [28], suggesting that a synergistic effect of RXFP1 and RXFP3 may be required for the effects of RLN3 within the MPA on the HPG axis.

5. CONCLUSIONS

In summary, this study has further implicated RXFP1 in the regulation of water intake and activation of the HPA and HPG axes by RLN3. A strong stimulation of food intake produced by RXFP3-A2 may involve the activation of the perifornical LHA but not the brain regions that drive anorexigenic effects. The reduced stimulation of food intake produced by RLN3 compared to RXFP3-A2 may depend on the broader neuronal activation produced by RLN3, which involves the neuronal groups that produce orexigenic *and* anorexigenic effects.

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FIGURE LEGENDS

Figure 1. Chow (A) and water (B) intake relative to body weight (BW) at 30, 60, and 120 min following *icv* administration of aCSF (n = 10), RLN3 (n = 10), or RXFP3-A2 (n = 12). **, p < 0.01; ***, p < 0.001: significantly different from the aCSF-injected rats. #, p < 0.05; ##, p < 0.01; ###, p < 0.001: significantly different from the RXFP3-A2-injected rats.

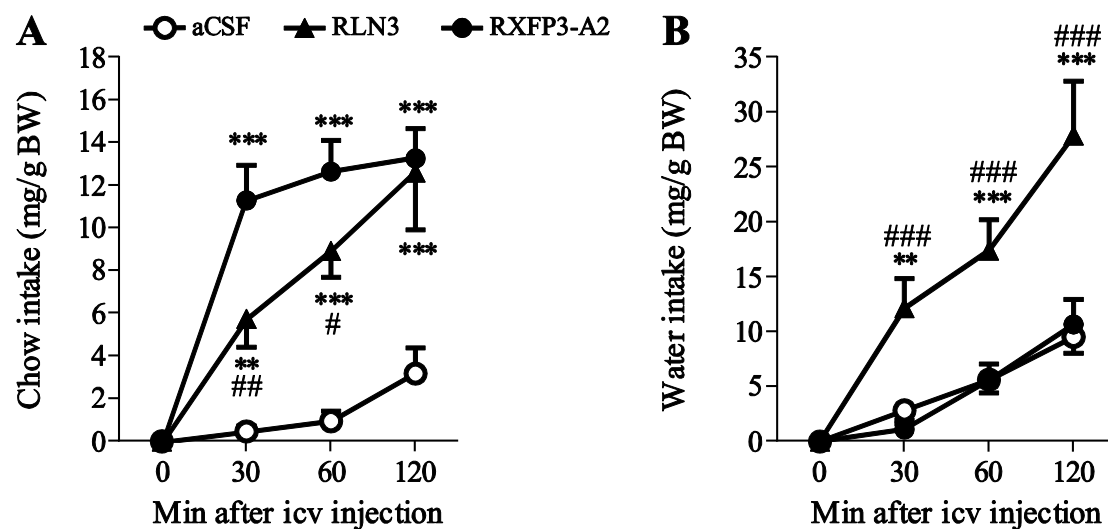


Figure 2. Plasma levels of testosterone (A), corticosterone (B), oxytocin (C), vasopressin (D), epinephrine (E), and norepinephrine (F) measured 30 min after *icv* administration of aCSF, RLN3 or RXFP3-A2. **, p < 0.01; ***, p < 0.001: significantly different from the aCSF-injected rats. ###, p < 0.001: significantly different from the RXFP3-A2-injected rats.

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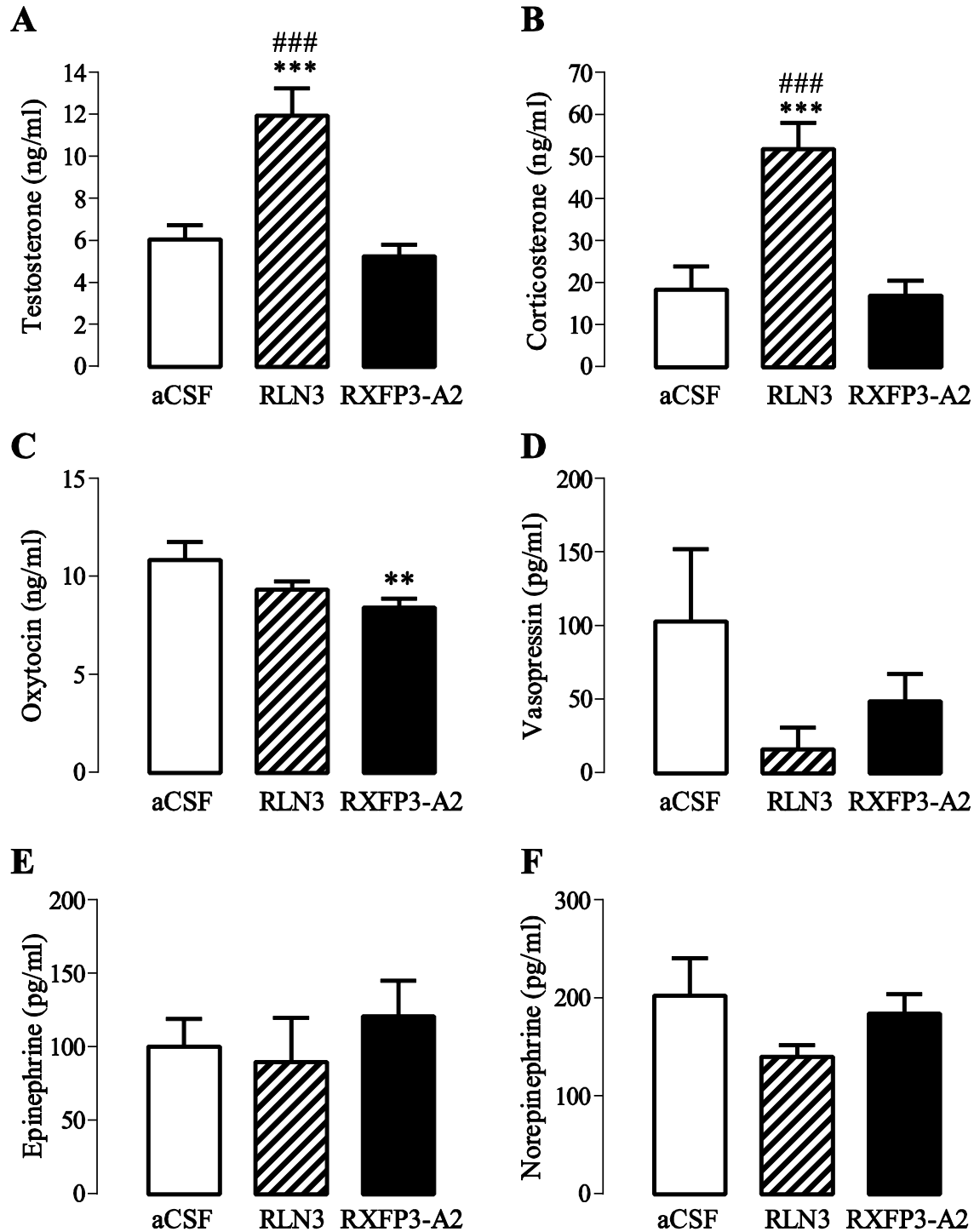
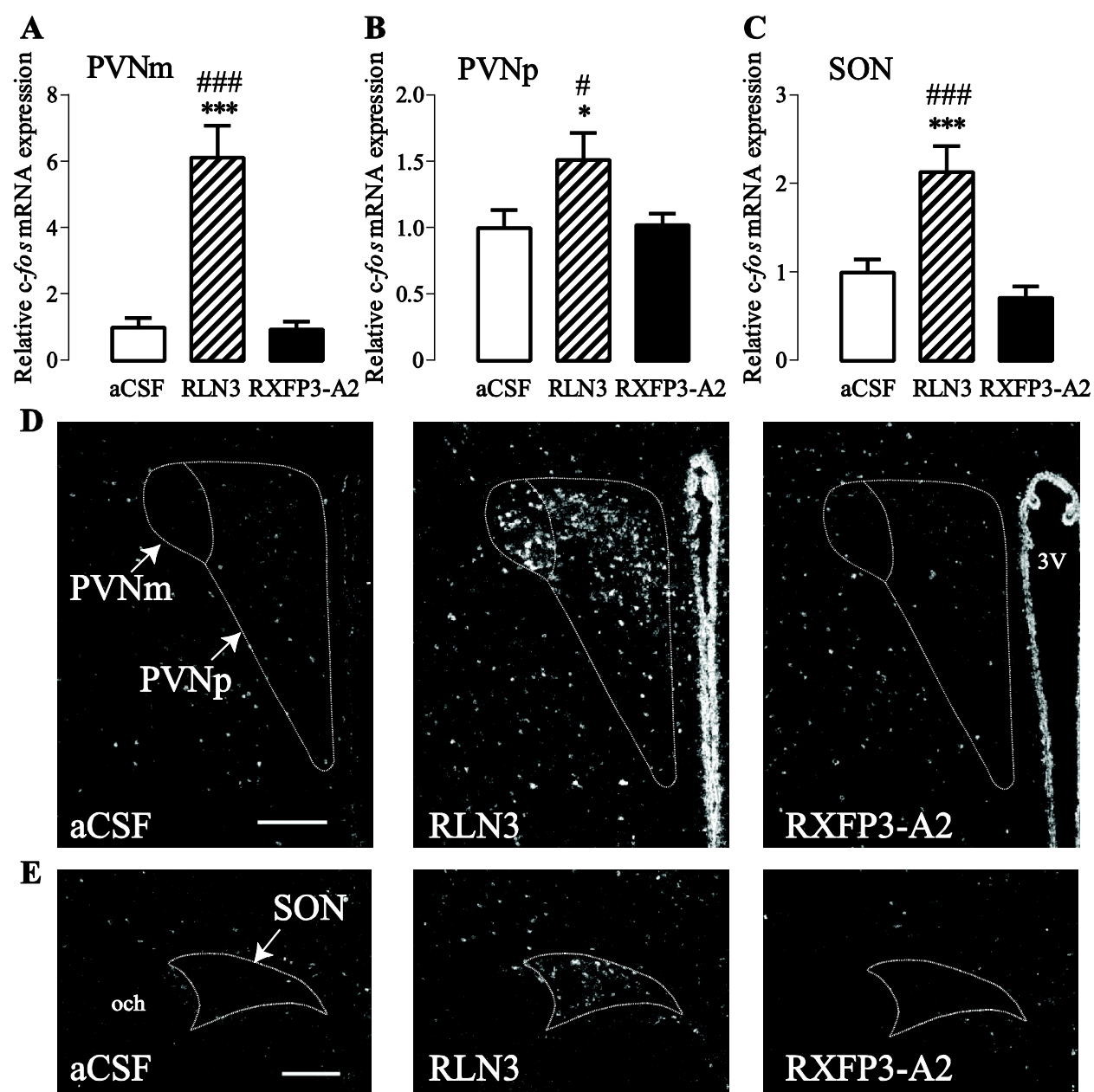


Figure 3. Relative levels of *c-fos* mRNA expression in the magnocellular (PVNm, A) and parvocellular (PVNp, B) hypothalamic paraventricular nucleus and the supraoptic (SON, C) hypothalamic nucleus 30 min after *icv* administration of aCSF (n = 10), RLN3 (n = 10), or

RXFP3-A2 (n = 12). Dark-field micrographs illustrate the positive hybridization signal of *c-fos* mRNA in the PVNm and PVNp (D) and SON (E). *, p < 0.05; ***, p < 0.001: significantly different from the aCSF-injected rats. #, p < 0.05; ###, p < 0.001: significantly different from the RXFP3-A2-injected rats. 3V, third ventricle; och, optic chiasm. Scale bars, 250 μ m.



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Figure 4. Relative levels of *c-fos* mRNA expression in the lateral (LHA1, A) and perifornical (LHApf, B) regions of the lateral hypothalamic area 30 min after *icv* administration of aCSF (n = 10), RLN3 (n = 10), or RXFP3-A2 (n = 12). Dark-field micrographs illustrate the positive hybridization signal of *c-fos* mRNA in the LHA1 and LHApf (C). *, $p < 0.05$; ***, $p < 0.001$: significantly different from the aCSF-injected rats. 3V, third ventricle; f, fornix. Scale bar, 500 μm .

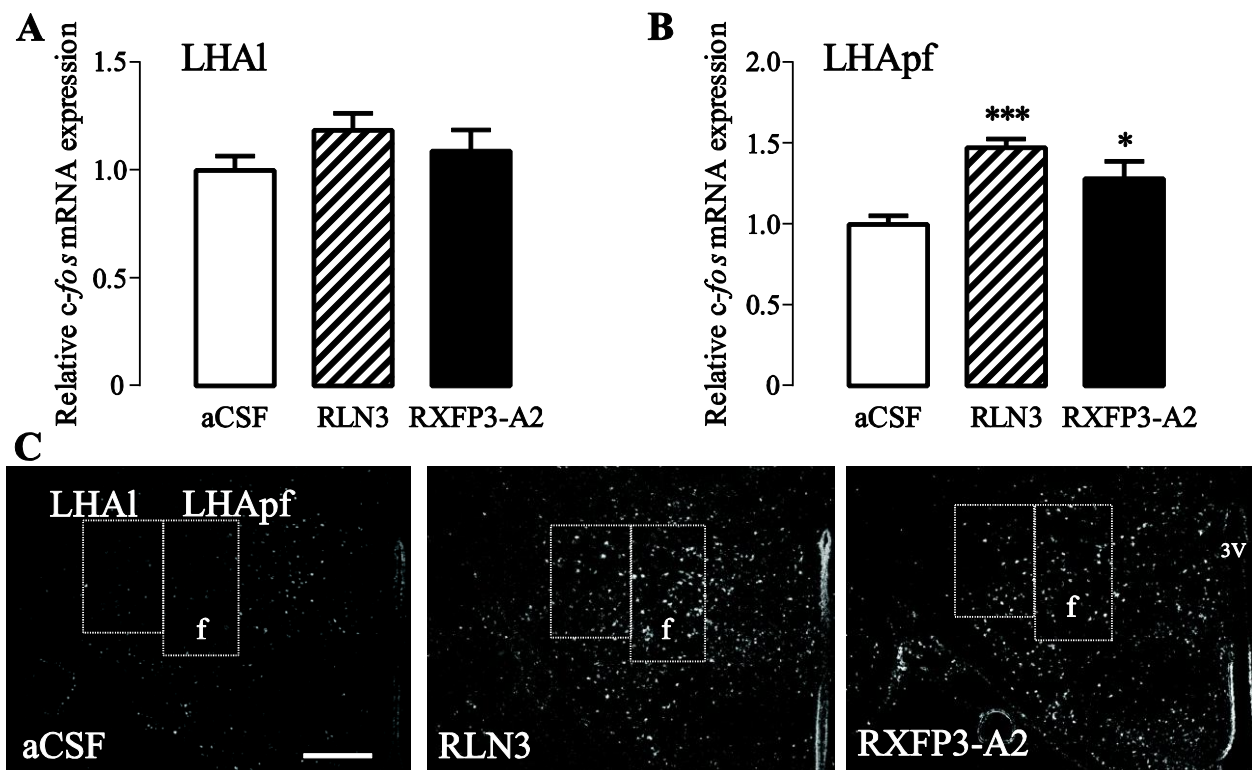
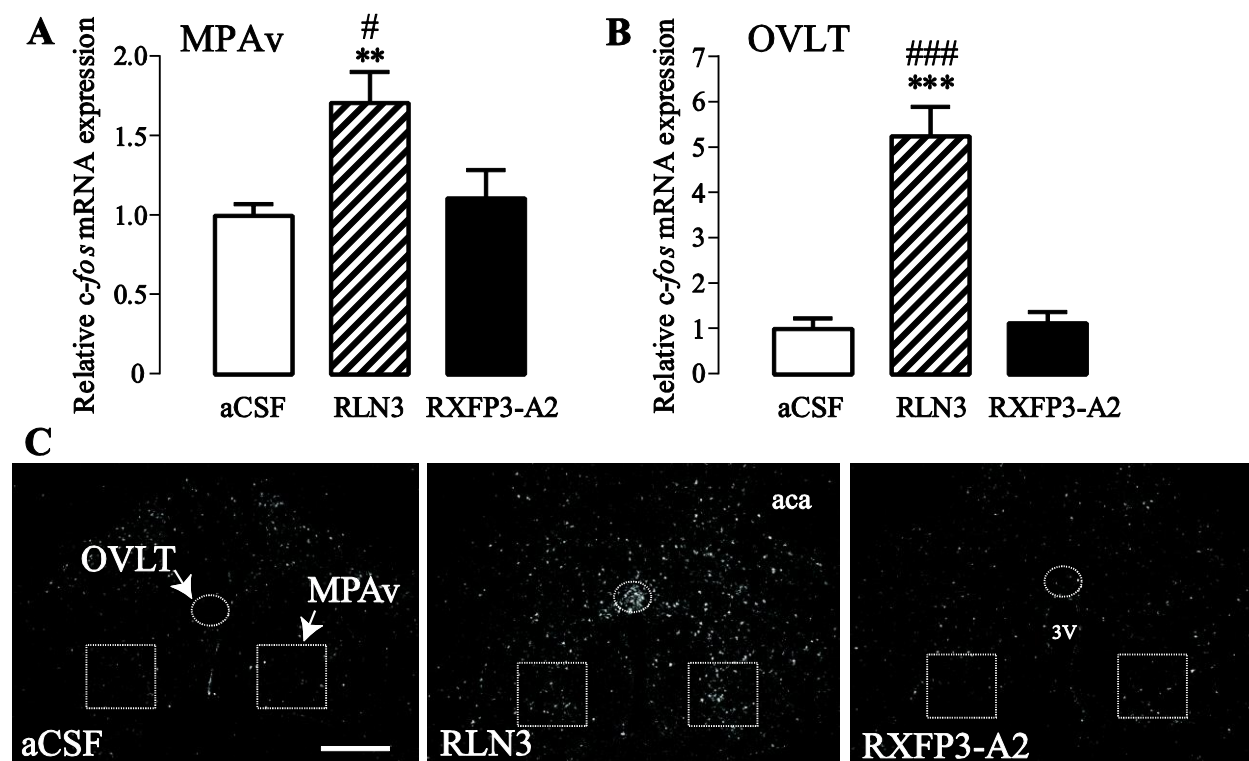


Figure 5. Relative levels *c-fos* mRNA expression in the ventral region of the medial preoptic area (MPAv, A) and the organum vasculosum of the lamina terminalis (OVLT, B) 30 min after *icv* administration of aCSF (n = 10), RLN3 (n = 10), or RXFP3-A2 (n = 12). Dark-field micrographs illustrate the positive hybridization signal of *c-fos* mRNA in the MPAv and the OVLT 30 min after *icv* injection of aCSF, RLN3 or RXFP3-A2 (C). **, $p < 0.01$, ***, $p < 0.001$:

significantly different from the aCSF-injected rats. #, $p < 0.05$, ###, $p < 0.001$: significantly different from the RXFP3-A2-injected rats. aca, anterior part of the anterior commissure; 3V, third ventricle. Scale bar, 500 μm .



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