Coordinated Seasonal Regulation of Metabolic and Reproductive Hypothalamic Peptides in the Desert Jerboa

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ABSTRACT

Jerboa (*Jaculus orientalis*) is a semi-desert rodent displaying strong seasonal variations in biological functions in order to survive harsh conditions. When environmental conditions become unfavorable in early autumn, it shuts down its reproductive axis, increases its body weight, and finally hibernates. In spring, the jerboa displays opposite regulations, with a reactivation of reproduction and reduction in body weight. This study investigated how genes coding for different hypothalamic peptides involved in the central control of reproduction (*Rfrp* and *Kiss1*) and energy homeostasis (*Pomc*, *Npy*, and *Somatostatin*) are regulated according to seasons in male jerboas captured in the wild in spring or autumn. Remarkably, a coordinated increase

in the mRNA level of *Rfrp* in the dorso/ventromedial hypothalamus and *Kiss1*, *Pomc*, and *Somatostatin* in the arcuate nucleus was observed in jerboas captured in spring as compared to autumn animals. Only *Npy* gene expression in the arcuate nucleus displayed no significant variations between the two seasons. These variations appear in line with the jerboa's seasonal physiology, since the spring increase in *Rfrp* and *Kiss1* expression might be related to sexual reactivation, while the spring increase in genes encoding anorexigenic peptides, POMC, and somatostatin may account for the reduced body weight reported at this time of the year. *J. Comp. Neurol.* 524:3717–3728, 2016.

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Species in their natural environment have to adapt their biological functions to seasonal changes in food resources. In mammals, the nocturnal production of the pineal hormone melatonin is critical for seasonal cue integration and adaptation (Reiter, 1993). Melatonin has been demonstrated to synchronize physiological functions with seasonal changes in order to guarantee species survival and maintain adequate body homeostasis. Reproduction and metabolic activity are crucial melatonin-regulated physiological functions (Barrett and Bolborea, 2012; Dardente, 2012; Hazlerigg and Simonneaux, 2014). Furthermore, considering that reproduction is an energetically demanding process, it is critical that both seasonal reproductive and metabolic activities are synchronized. It is therefore expected that the neuronal mechanisms controlling these functions display seasonal correlations and interact with each other. In fact, in the last decade evidence has suggested that

“reproductive” neuropeptides are also involved in the control of feeding behavior, while “metabolic” neuropeptides can communicate with the reproductive axis (Schneider, 2004; Fernandez-Fernandez et al., 2006; Hill et al., 2008; Garcia-Garcia, 2012; Evans and Anderson, 2012).

Key hypothalamic peptides are known to regulate reproductive activity and metabolism in mammalian species. Kisspeptin (Kp) and RFRP-3 (also named GnIH, for gonadotropin inhibitory hormone) are two RF-amide peptides recently shown to be regulators of

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reproduction in a wide range of mammals (Kriegsfeld et al., 2010; Pinilla et al., 2012). Kp is expressed in neurons located in two hypothalamic regions, the anteroventral periventricular nucleus (AVPV) and the arcuate nucleus (ARC) (Gottsch et al., 2004; Smith et al., 2005; Mikkelsen and Simonneaux, 2009). Kp is a potent activator of GnRH neurons and gonadotropin release in all mammalian species, including humans (Gottsch et al., 2004; Thompson et al., 2004; Dhillon et al., 2005; Messenger et al., 2005). RFRP-3 is expressed in neurons exclusively located in the dorso (DMH) and ventro (VMH)-medial hypothalamus (Kriegsfeld et al., 2006; Revel et al., 2008). Its role in the regulation of reproductive activity appears to depend on species and sex, exhibiting either stimulatory (Ancel et al., 2012; Ubuka et al., 2012) or inhibitory effects (Kriegsfeld et al., 2006; Clarke et al., 2008; Johnson et al., 2007; Pineda et al., 2010). On the other hand, pro-opiomelanocortin (POMC), neuropeptide Y (NPY), and somatostatin are hypothalamic peptides well known to regulate feeding behavior. NPY, synthesized in ARC neurons, is one of the most powerful orexigenic neuropeptides regulating food intake and body weight (Bai et al., 1985; Morley, 1987; Leibowitz, 1991). POMC is a precursor polypeptide synthesized in the ARC, the nucleus tractus solitarius of the brainstem, and the pituitary. POMC produces several biologically active peptides known to play a pivotal role in the regulation of energy balance, among which β -endorphin, ACTH, and α -MSH display strong effects on food intake (Fan et al., 1997; Murphy et al., 1998; Schulz et al., 2010). Somatostatin is a multifunctional neuropeptide produced by the periventricular nucleus of the hypothalamus, the ARC, and other neuronal populations (Guillemin and Gerich, 1976). It binds to receptors located in various brain nuclei involved in feeding regulation, notably the ARC, and it regulates food intake in various mammalian species (Lotter et al., 1981; Danguir, 1988; Fehlmann et al., 2000; Stengel et al., 2010, 2015). Interestingly, independent studies have reported that Kp (Revel et al., 2006a; Smith et al., 2008), RFRP-3 (Revel et al., 2008; Smith et al., 2008), NPY (Lakhdar-Ghazal et al., 1995; Reuss and Olcese, 1995; Skinner and Herbison, 1997), β -endorphin (Roberts et al., 1985; Ebling and Lincoln, 1987), and somatostatin (Webb et al., 1985; Herwig et al., 2012; Klosien et al., 2013; Dumbell et al., 2015) display significant variations in the brain of different mammalian species placed in artificial long or short photoperiods.

In this study we chose the jerboa (*Jaculus orientalis*) as a wild animal model for analyzing the neuronal mechanisms involved in the anticipatory adaptation to harsh environmental cues. The jerboa is a semi-desert

rodent whose reproductive and metabolic activities are well adapted to marked environmental changes. This species is sexually active in spring and early summer (Ghobrial and Hodieb, 1973; El Ouezzani et al., 2000; El Qandil et al., 2004) and hibernates at low ambient temperatures (Andjus et al., 1974; El Ouezzani et al., 2011). It displays seasonal variations in the nocturnal production of melatonin (Lakhdar-Ghazal et al., 1992) and melatonin inhibits testicular activity in male jerboas (El Qandil et al., 2004). Additionally, previous studies reported that jerboas display seasonal variations in body weight and food intake (El Ouezzani et al., 2011; Talbi et al., unpublished data). The seasonal changes in reproductive activity are accompanied by variations in the content of hypothalamic peptides involved in the central control of reproductive activity. Thus, gonadotropin releasing hormone (GnRH) displays seasonal variations and melatonin appears to regulate GnRH immunoreactivity in both neurons of the preoptic area and fibers in the median eminence (El Ouezzani et al., 2000; El Qandil et al., 2004). Kp and RFRP-3 immunoreactivity is higher in the hypothalamus of sexually active male jerboas captured in spring as compared to sexually inactive animals of autumn (Janati et al., 2013). Alternatively, β -endorphin displays lower levels in the rostral ARC of jerboas sacrificed during spring-summer as compared to those sacrificed in autumn (El Ouezzani et al., 2005).

In the present study, we investigated seasonal variations in the expression of genes coding for Kp, RFRP-3, POMC, somatostatin, and NPY by comparing their mRNA levels in the hypothalamus of male jerboas captured from the wild in spring or autumn, and correlated these values to the reproductive and metabolic status of the animals.

MATERIALS AND METHODS

Animals

Sexually mature male jerboas weighing 139.4 ± 5.4 g ($n = 8$) were captured in the field of the middle Atlas Mountains of Morocco (sub-desert high plateaus of Ifkerne-Boulmane; altitude 1,565 m, longitude -4.73 , latitude 33.36) in spring (May, $n = 4$) when they are sexually active and autumn (November, $n = 4$) when they are sexually inactive. Males rather than females were chosen in this study because sexual maturation and status are difficult to assess in female jerboa captured in the wild and this could interfere with the seasonal parameters under investigation. The animals were transported to the University of Fes, where they were maintained together in a single cage ($90 \times 60 \times 50$ cm) in a room with open windows and kept under

natural conditions of temperature and photoperiod. Food (sunflower and barley seeds and vegetables) was provided ad-libitum. Animal experimentation was performed in accordance with the international guidelines for the Care and Use of Mammals in Neurosciences and Behavioral Research (2003).

Brain tissue preparation

After 1 week of adaptation, animals were anesthetized with an intraperitoneal injection of ethyl urethane (1 ml/100g, Acros Organics, Morris Plains, NJ) between 10–12 AM. The blood was taken by cardiac puncture, centrifuged at 4000 rpm for 10 minutes and the plasma was extracted and stored at -20°C for testosterone assay. Animals were transcardially perfused with 50 ml of isotonic saline solution (NaCl 9‰) followed by 250 ml of 4% formaldehyde (Sigma-Aldrich, St. Louis, MO) in 0.1 M phosphate buffer at pH 7.6 (PB). Perfused testes were dissected out and weighed. The animals' head was first kept in 4% paraformaldehyde for 1 hour, then the brains were removed, postfixed for 24 hours in the same fixative solution, dehydrated in ethanol baths of increasing concentrations, and then included first in polyethylene glycol 1000 (PEG, Acros Organics) followed by a mixture of PEG 1000/PEG 1500 according to Klosen et al. (1993). Twelve μm coronal brain sections were cut from the suprachiasmatic nucleus to the mammillary bodies using a microtome (Leica 2055 autocut, Rueil-Malmaison, France), mounted on Superfrost ultraplus slides (Menzel-Gläser, Braunschweig, Germany) under RNase free conditions, and stored at -80°C until use for in situ hybridization.

Nonradioactive in situ hybridization

Riboprobe preparation and validation

Since none of the available rodent *Rfrp* riboprobes hybridized to the jerboa *Rfrp* mRNA, we cloned the jerboa's *Rfrp* gene. Total RNA was extracted from two fresh jerboa hypothalami (GeneJet RNA purification kit; Thermo Fisher Scientific, Waltham, MA) and reverse transcribed into cDNA (Superscript III, Thermo Fisher Scientific). Touchdown polymerase chain reaction (PCR) was then conducted using forward (CATCTTGTTGACTT-TAGCCACTTC) and reverse (AGAAGATGGCAT-GAAGCTGT) primers designed according to the *Jaculus jaculus* gene sequence (NC_005314.1). The 614 bp cDNA fragment obtained was cloned into the pST Blue1 vector (Perfectly Blunt Cloning Kit, Novagen, Merck, Darmstadt, Germany). Sequencing of the cDNA fragment confirmed that it contained the jerboa's *Rfrp* sequence (LGC Genomics, Germany). Sense and anti-sense jerboa *Rfrp*, Siberian hamster *Kiss1* (380 bp, 99% identical to 187–566 of *Mus musculus*, Genbank

AB_666166.1), rat *Npy* (87–522 of Genbank NM_012614.2), rat *Pomc* (157–731 of Genbank NM_139326) and rat *Somatostatin* (135–465 of Genbank NM_012659) riboprobes were transcribed from linearized plasmids in the presence of digoxigenin-labeled nucleotides according to manufacturer protocol (Dig RNA Labelling Kit, Roche Diagnostics, Mannheim, Germany). The length of all probes was confirmed by formaldehyde-3-[N-morpholino]propanesulfonic acid agarose gel electrophoresis and northern blotting. The specificity of all riboprobes was assessed by the absence of signal with sense probes, resistance of signal to posthybridization RNase treatment, posthybridization under high stringency conditions (SSC 0.1 $\times$, 72°C), and expected neuroanatomical localization (see Results).

Hybridization

Riboprobe hybridization was performed according to a protocol previously validated in our laboratory (Klosen et al., 2013). Brain sections including ARC (for *Kiss1*, *Pomc*, *Npy*, and *Somatostatin*) and DMH/VMH (for *Rfrp*) were postfixed for 10 minutes at room temperature in 4% formaldehyde, rinsed in phosphate buffered saline (PBS, pH 7.6), and then treated with 1 $\mu\text{g}/\text{ml}$ proteinase K (Roche, Meylan, France) for 30 minutes at 37°C . Proteinase K digestion was stopped with 2% phosphate-buffered formaldehyde. Sections were then acetylated twice for 10 minutes with 0.25% acetic anhydride in 100 mM triethanolamine, rinsed twice in PBS containing 0.02% dimethyl-pyrocabonate (DMPC), and preincubated in a solution containing 5 $\times$ saline sodium citrate buffer (SSC), 0.05% Tween-20, and 0.02% DMPC. Hybridization was performed for 40 hours at 60°C with 200 ng/ml of antisense or sense probe in a solution containing 50% formamide, SSC 5 $\times$, Denhardt's solution 5 $\times$, 1 mg/ml salmon sperm DNA, 0.1% Tween-20, and 0.04% DMPC. Posthybridization steps included six rinses of high stringency each for 10 minutes in SSC 0.1 $\times$, 0.05% Tween-20 at 72°C followed by two rinses in A-Dig buffer (Tris (hydroxymethyl) aminomethane 100 mM; NaCl 150 mM; MgCl_2 2.5 mM; 0.05% Tween-20; pH 7.5). Digoxigenin-labeled probes were detected with an alkaline phosphatase-labeled anti-digoxigenin antibody (1/5,000, Roche, Meylan, France). Alkaline phosphatase activity was detected with bromo-chloro-indolyl phosphate and nitroblue tetrazolium. The slides were incubated with the detection solution in the dark at room temperature. The signal level was checked periodically and the development of the alkaline phosphatase signal was stopped before a too strong signal developed in order to avoid saturation of the signal (8 to 10 hours for *Rfrp*, *Pomc*, *Npy*, and *Somatostatin*, and

48 hours for *Kiss1*). All sections and slides to be analyzed for one experiment were processed and developed together under identical conditions. Finally, the slides were mounted with an aqueous mounting medium (CC/MountTM, Sigma, St. Quentin Fallavier, France) and coverslipped with Eukitt (Kindler). For each peptide mRNA, we prepared serial sections throughout the ARC for *Kiss1*, *Pomc*, *Npy*, and *Somatostatin* and the DMH/VMH for *Rfrp*. For these serial sections, we selected 1 section every 12 sections with each series containing 12 sections, thus covering a total rostrocaudal extent of 1,728 μm (12 sections of 12 μm sections of a 1 in 12 series). All spring and autumn animal sections were treated together.

Semi-quantification of hybridized peptide mRNA

Both the number of labeled neurons and the intensity of labeling per neuron were measured in the jerboa's hypothalamus. Neuron counts were performed by two observers unaware of the animal groups. There was no significant difference between either observers; therefore, the data given is the one obtained by the first author. The number of *Kiss1*, *Pomc*, *Somatostatin*, and *Npy* neurons was estimated by counting the labeled cells throughout the entire ARC. The rostral, middle, and caudal parts of the ARC correspond to sections localized between -2,12 and -2,30 mm/bregma, -2,8 and -3,14 mm/bregma, and -3,6 and -3,8 mm/bregma, respectively. These levels are the corresponding levels of the rat brain as defined in the rat brain atlas of Paxinos and Watson (2005). The number of *Rfrp* neurons was also estimated by counting the number of labeled cells throughout the entire the DMH/VMH area. All neurons were counted on both sides of the ventricle at the level of the ARC for *Kiss1*, *Pomc*, *Somatostatin*, and *Npy*, and of the DMH/VMH for *Rfrp*. For each peptide, the total number of neurons was calculated by first multiplying the total number of counted neurons by 12 (1 in 12 sections sampled per series). This gross number of counted neurons was then corrected using Abercrombie's correction factor for split-cell counting errors where $N_{\text{corr}} = N \times [t/t+d]$ (N_{corr} = Total corrected number of neurons; N = counted number of neurons $\times$ 12; t = section thickness; and d = mean neuron diameter) (Abercrombie, 1946). We used the mean diameter of the labeled neurons as an estimate of their rostrocaudal extent. The labeled neurons mostly appeared ovoid or round with no preferential orientation. The mean diameter of the neurons was estimated on 40 labeled cells for each animal and each peptide. The cell contour first was drawn on size-calibrated images in ImageJ (NIH, Bethesda, MD). An ellipse was then fitted on the cell contour using the "Fit

Ellipse" command of ImageJ. Finally, the mean diameter of each cell was then calculated using the maximal and minimal diameters of the fitted ellipse. This mean diameter was then used to calculate Abercrombie's correction factor for each animal and each peptide. The mean diameter for *Kiss1* neurons was: $d_{\text{autmun}} = 11.94 \mu\text{m}$ and $d_{\text{spring}} = 13.59 \mu\text{m}$; *Npy* neurons: $d_{\text{autmun}} = 14.24 \mu\text{m}$ and $d_{\text{spring}} = 15.24 \mu\text{m}$; *Pomc* neurons: $d_{\text{autmun}} = 14.56 \mu\text{m}$ and $d_{\text{spring}} = 15.31 \mu\text{m}$; *Rfrp* neurons: $d_{\text{autmun}} = 15.93 \mu\text{m}$ and $d_{\text{spring}} = 16.11 \mu\text{m}$; and *Somatostatin* neurons: $d_{\text{autmun}} = 12.41 \mu\text{m}$ and $d_{\text{spring}} = 13.61 \mu\text{m}$. A statistically significant difference in mean diameter between seasons was observed only for *Kiss1* cells. The final value given in the figures is the mean number of labeled neurons generated by the Abercrombie's correction in four animals per season.

semi-quantitative analysis of the in situ hybridization labeling intensity was performed as described previously (Klosen et al., 2013). Gray scale images (256 gray level tiff images) were taken on a Leica DMRB microscope (Leica Microsystems, Rueil-Malmaison, France) equipped with an Olympus DP50 digital camera (Olympus France, Rungis, France). All lighting parameters on the microscope and the settings of the Viewfinder Lite (Olympus) acquisition software were standardized to ensure consistent stable lighting throughout the image capture procedure. For each analyzed peptide, images of all autumn and spring animal brains were taken at the same time with the same settings. Images were taken with a 10 $\times$ objective at a resolution of 1392 $\times$ 1040 pixels. A background image of the slide without a brain section was taken for each slide. This background image was subtracted from the corresponding sample image. The images were then analyzed using the ImageJ software. For *Kiss1*, *Npy*, *Pomc*, and *Somatostatin* analysis, both sides of the ventricle at the levels of the rostral, middle, and caudal ARC were sampled, while for *Rfrp* analysis the sampling concerned both sides of the DMH/VMH. Preliminary tests showed that analysis of 60 cells per brain was enough to provide a stable mean labeling intensity for each of the hypothalamic peptides analyzed. Labeling intensity was determined as the mean pixel gray value within a fixed-size circle of 50 μm^2 laid over 60 labeled cells randomly selected throughout the hypothalamic structure. For each animal, a mean value of labeling intensity was calculated from the 60 cells analyzed. For each peptide, labeling intensity was calculated as the mean pixel gray value of the four animals per season.

Hormone assays

Serum plasma testosterone was assayed at the Centre of Radioisotopes and Nuclear Medicine of Fes,

Morocco, using a TESTO-CT2 Kit (Cis Bio International, Gif-sur-Yvette, France). This assay is based on the competition between testosterone and a fixed quantity of ^{125}I -labeled testosterone for a limited number of binding sites on testosterone-specific antibody, and provides the quantitative in vitro determination of testosterone. The intra- and interassay values were 4.1% and 5.5%, respectively.

Methodological considerations and statistical analysis

Values of cell count and labeling intensity for each neuropeptide were given as the mean of four jerboas per season. Comparisons were made between two groups of equal size, $n = 4$. According to Zar (1999), when groups are equal then the t -test is fairly robust for moderate to large sample sizes. Thus, all comparisons were made with two-tailed t -tests with $n = 6$ degrees of freedom and $P < 0.05$ being considered statistically significant.

RESULTS

Seasonal variations in male jerboa's testicular activity

Male jerboas captured in spring had significantly larger testes (2.83 ± 0.3 g; $n = 4$) and higher plasma testosterone levels (5.11 ± 0.76 ng/L; $n = 4$) than animals captured in autumn (testes weight was 0.56 ± 0.13 g; plasma testosterone was 0.71 ± 0.17 ng/L; $n = 4$). These results confirm the sexual spring activation and autumn quiescence of the captured male jerboas.

Neuroanatomical distribution of neurons expressing *Rfrp*, *Kiss1*, *Pomc*, *Somatostatin*, or *Npy*

Whatever the season, neurons expressing *Rfrp* mRNA were found only in the DMH/VMH area (Fig. 1A). Neurons expressing *Kiss1* (Fig. 2A), *Pomc* (Fig. 3A), *Somatostatin* (Fig. 4A), and *Npy* (Fig. 5A) mRNA were detected along the rostral, middle, and caudal ARC. Although not shown here, *Kiss1* neurons were also observed in the AVPV and *Somatostatin* neurons in the anterior periventricular hypothalamic area and ventromedial hypothalamic nucleus. The expected neuroanatomical distribution of these neuropeptides, as well as the absence of signal with the sense probes, confirms the specificity of the heterologous probes used to hybridize jerboa's ARC genes.

Seasonal variations in *Rfrp* and *Kiss1* gene expression

Seasonal variations of *Rfrp* expression in the DMH/VMH (Fig. 1A) and *Kiss1* in the ARC (Fig. 2A) were

Figure 1. Seasonal variation in *Rfrp* gene expression in the hypothalamus of male jerboas. **A:** Representative pictures showing nonradioactive in situ hybridization of *Rfrp* mRNA in neurons of the dorso/ventromedial hypothalamus of jerboas captured in autumn (left panel) or spring (right panel), 3v: third ventricle. **B:** Estimation of the total number of *Rfrp* mRNA-expressing neurons per animal throughout the dorso/ventromedial hypothalamus in autumn and spring jerboas. **C:** semi-quantification of labeling intensity of *Rfrp* mRNA measured in individual neurons in the hypothalamus of spring and autumn jerboas; for each animal, a mean pixel gray value is calculated from 60 neurons. Dots show the individual values for each animal and bars the mean value for each group ($n = 4$; ** $P < 0.01$ and *** $P < 0.001$ as compared to autumn value). Scale bar = 50 μm .

examined in jerboas captured in May or November. The number of *Rfrp* mRNA-expressing neurons was significantly higher in spring compared to autumn animals ($P = 0.006$; $t = -4.066$; Fig. 1B). Furthermore, analysis of the labeling intensity per neuron indicated that the level of *Rfrp* mRNA per neuron is significantly higher in spring compared to autumn animals ($P = 0.0005$; $t = -6.595$; Fig. 1C). The number of ARC *Kiss1* mRNA-expressing neurons was not significantly different between spring and autumn animals ($P = 0.8$; $t = -0.217$; Fig. 2B). However, analysis of labeling intensity per neuron indicated a significantly higher level of *Kiss1*

Figure 2. Seasonal variation in *Kiss1* gene expression in the hypothalamus of male jerboas. **A:** Representative pictures showing nonradioactive in situ hybridization of *Kiss1* mRNA in neurons of arcuate nucleus (ARC) of jerboas captured in autumn (left panel) or spring (right panel), 3v: third ventricle. **B:** Estimation of the total number of *Kiss1* mRNA-expressing neurons per animal throughout the ARC in autumn and spring jerboas. **C:** semi-quantification of the labeling intensity of *Kiss1* mRNA measured in individual neurons in the ARC of spring and autumn jerboas; for each animal, a mean pixel gray value is calculated from 60 neurons. Dots show the individual values for each animal and bars the mean value for each group ($n = 4$; $*P < 0.05$ as compared to autumn value). Scale bar = 50 μ M.

mRNA in spring as compared to autumn animals ($P = 0.01$; $t = -3,357$; Fig. 2C).

Seasonal variations in *Pomc*, *Somatostatin*, and *Npy* gene expression

Expression of genes coding for the anorexigenic POMC (Fig. 3A) and somatostatin (Fig. 4A) and the orexigenic NPY (Fig. 5A) was analyzed in the ARC of spring and autumn jerboas. The number of neurons expressing *Pomc* mRNA ($P = 0.004$, $t = -4,346$; Fig. 3B) or *Somatostatin* mRNA ($P = 0.01$; $t = -3,531$; Fig.

Figure 3. Seasonal variation in *Pomc* gene expression in the hypothalamus of male jerboas. **A:** Representative pictures showing nonradioactive in situ hybridization of *pomc* mRNA in neurons of arcuate nucleus (ARC) of jerboas captured in autumn (left panel) or spring (right panel), 3v: third ventricle, ME: median eminence. **B:** Estimation of the total number of *Pomc* mRNA-expressing neurons per animal throughout the ARC in autumn and spring jerboas. **C:** semi-quantification of the labeling intensity of *Pomc* mRNA measured in individual neurons in the ARC of spring and autumn jerboas; for each animal, a mean pixel gray value is calculated from 60 neurons. Dots show the individual values for each animal and bars the mean value for each group ($n = 4$; $**P < 0.01$ and $***P < 0.001$ as compared to autumn value). Scale bar = 50 μ M.

4B) was markedly higher in spring as compared to autumn. Similarly, the intensity of hybridization signal per neuron was higher in spring as compared to autumn for both *Pomc* ($P = 0.0005$, $t = -6,723$; Fig. 3C) and *Somatostatin* ($P = 0.02$; $t = -3,022$; Fig. 4C) neurons. Analysis of seasonal variations of the mean number of *Npy* mRNA-expressing neurons ($P = 0.1$; $t = 1,809$; Fig. 5B) as well as the mean staining intensity per neuron ($P = 0.1$, $t = 1,648$; Fig. 5C) indicates no significant variations between either season.

Figure 4. Seasonal variation in *Somatostatin* gene expression in the hypothalamus of male jerboas. **A:** Representative pictures showing nonradioactive in situ hybridization of *Somatostatin* mRNA in neurons of arcuate nucleus (ARC) of jerboas captured in autumn (left panel) or spring (right panel), 3v: third ventricle, ME: median eminence. **B:** Estimation of the total number of *Somatostatin* mRNA-expressing neurons per animal throughout the ARC in autumn and spring jerboas. **C:** semi-quantification of the labeling intensity of *Somatostatin* mRNA labeling measured in individual neurons in the ARC of spring and autumn jerboas; for each animal, a mean pixel gray value is calculated from 60 neurons. Dots show the individual values for each animal and bars the mean value for each group ($n = 4$; * $P < 0.05$ as compared to autumn value). Scale bar = 50 μ M.

DISCUSSION

This study is the first to report seasonal regulation of genes encoding several hypothalamic peptides involved in the central control of reproduction and energy balance in a wild rodent species. Notably, our results indicate a coordinated springtime increase in the expression of four peptides involved in activation of reproduction and food intake inhibition in the desert jerboa.

The jerboa is a desert rodent of the family of North African Dipodidae. It is a wild animal that lives in an environment characterized by large fluctuations in

Figure 5. Seasonal variation in *Npy* gene expression in the hypothalamus of male jerboas. **A:** Representative pictures showing nonradioactive in situ hybridization of *Npy* mRNA in neurons of arcuate nucleus (ARC) of jerboas captured in autumn (left panel) or spring (right panel), 3v: third ventricle, ME: median eminence. **B:** Estimation of the total number of *Npy* mRNA-expressing neurons per animal throughout the ARC in autumn and spring jerboas. **C:** semi-quantification of the labeling intensity of *Npy* mRNA labeling measured in individual neurons in the ARC of spring and autumn jerboas; for each animal, a mean pixel gray value is calculated from 60 neurons. Dots show the individual values for each animal and bars the mean value for each group ($n = 4$). Scale bar = 50 μ M.

external temperature, food, and water supply. Therefore, it appears to be a better model compared to other laboratory animals to assess the possible link between the seasonal regulation of hypothalamic peptides involved in the central control of reproduction and metabolism. The jerboa is a species known to develop interesting strategies to ensure its adaptation to seasonal changes. In its natural environment, it anticipates seasonal stressors and adjusts its reproductive and metabolic axes accordingly. In autumn, the male jerboa shuts down its reproduction, as seen by lower GnRH immunoreactivity (El Ouezani et al., 2000) and reduced testicular activity (El Qandil et al., 2004; this study). In

the female jerboa, body weight (El Ouezzani et al., 2011) and food intake (Talbi et al., unpublished data) increase in autumn–winter in order to anticipate the upcoming winter hibernation, a state accompanied by hypothermia and low metabolism (El Hilali and Veillat, 1975; El Ouezzani et al., 2001; Malan, 2014). In contrast, when favorable conditions return in spring, these physiological states are reversed, with the reactivation of the reproductive axis (El Ouezzani et al., 2000; El Qandil et al., 2004; this study) and a decrease in body weight and food intake (El Ouezzani et al., 2011; Talbi et al., unpublished data). The present work investigated whether expression of genes encoding peptides regulating reproduction (Kp and RFRP-3) and energy balance (POMC, somatostatin, and NPY) are altered by seasonal changes of the environment in the male jerboa.

Expression of neuropeptides regulating reproduction is influenced by the seasonal environment. The *Rfrp* mRNA level in the DMH/VMH was higher in spring as compared to autumn, seen in both the mean number of *Rfrp* mRNA-expressing neurons and the mean staining intensity of individual neurons. *Kiss1* gene analysis in the ARC revealed that the labeling intensity per neuron was significantly increased in spring compared to autumn; however, no apparent significant difference in the number of *Kiss1* mRNA-expressing neurons was observed between the two seasons. We previously reported that the number of Kp-immunoreactive (ir) neurons in the ARC of male jerboas is increased in spring as compared to autumn (Janati et al., 2013). This discrepancy between the seasonal variation in Kp-ir and *Kiss1* mRNA-expressing neurons may be caused by differences in the sensitivity of the technical approaches. Alternatively, it may result from seasonal variation in post-transcriptional processing since *Kiss1* mRNA is always detected in ARC neurons whatever the season, but the synthesis rate reflected by the level of *Kiss1* mRNA per neuron displays seasonal variation. The coincident upregulation of mRNA levels and immunostaining clearly shows that the increase in Kp and RFRP-3 immunostaining is not due to lack of secretion and neuronal accumulation of these peptides, but the synthesis of these neuropeptides is increased in spring. Based on these findings, it is tempting to speculate that in the jerboa, like in other seasonal rodents, the autumn and winter increases nighttime melatonin production (Lakhdar-Ghazal et al., 1992) and inhibits *Rfrp* and *Kiss1* gene expression via a reduction of thyroid hormone synthesis in the basal hypothalamus (Revel et al., 2006a,b, 2008; Barrett et al., 2007; Ubuka et al., 2012; Klosen et al., 2013). The difference in the amplitude of the seasonal variations between *Rfrp* and *Kiss1* gene expression could result from a differential peptide

response to the sex steroid feedback. Indeed, the negative feedback exerted by sex steroids on ARC Kp neurons is well documented in many species (Smith et al., 2005; Revel et al., 2006; Kauffman et al., 2007; Ansel et al., 2010), whereas the sex steroid feedback on RFRP-3 neurons is absent in rat (Quennell et al., 2010), sheep (Smith et al., 2008), Syrian (Revel et al., 2008) and Siberian (Ubuka et al., 2012) hamsters, or moderate in mice (Molnár et al., 2011; Poling et al., 2012).

Altogether, our results indicate that in spring there is an increased synthesis of both RFRP-3 and Kp in the jerboa's hypothalamus that correlates with a gonadal activation. Kp is well established as a potent activator of GnRH release in mammalian species (Pinilla et al., 2012, for review) and its increase in spring may drive the seasonal activation of jerboa's reproduction, as already demonstrated for other seasonal species like the Syrian (Revel et al., 2006a) and Siberian (Mason et al., 2007) hamsters and sheep (Caraty et al., 2007). Preliminary data obtained in the male jerboa indeed confirm that a single icv injection of 3 nmole of Kp increases testosterone production in both spring and autumn (Talbi et al., in preparation). In contrast, the effect of RFRP-3 on reproductive activity is controversial, being inhibitory in sheep (Clarke et al., 2008), rat (Johnson et al., 2007), and mice (Ducret et al., 2009) but stimulatory in the male Syrian (Ancel et al., 2012) and short-day-adapted Siberian hamsters (Ubuka et al., 2012). The marked springtime increase in *Rfrp* expression in sexually active jerboas is in line with what has been observed in other seasonal species (Simonneaux and Ancel, 2012) and suggests a potential activatory effect of RFRP-3 on the reproductive axis of male jerboa.

The seasonal regulation of genes encoding peptides considered as key molecules in the control of feeding behavior was also investigated in the male jerboa. Expression of the gene coding for the anorexigenic POMC, seen in both the number of labeled neurons and the mean staining intensity of individual POMC neurons in the hypothalamus, was markedly increased in spring as compared to autumn. In the Siberian hamster, ARC *Pomc* gene expression was also reported higher in long-day as compared to short-day-adapted animals (Mercer et al., 2000). Seasonal variation in *Pomc* expression was also reported in the ewe, where the number of POMC cells and the density of grains per cell increased during the anestrus season (Clarke et al., 2000). α -MSH, one of the peptides processed from POMC, is a strong inhibitor of food intake (Fan et al., 1997; Brown et al., 1998; McMinn et al., 2000). Therefore, our observation of lower *Pomc* expression in autumn jerboas could lead to a reduced synthesis in α -MSH, which

could account for the increase in body weight observed at this time of the year. Somatostatin is a hypothalamic neuropeptide known for inhibiting the secretion of the pituitary growth hormone (Vale et al., 1972; Brazeau et al., 1974; Rogers et al., 1988) and food intake in rats and baboons (Lotter et al., 1981). Recent studies have reported its involvement in the seasonal regulation of body mass and metabolism. Indeed, Siberian hamsters adapted to a short photoperiod display a decrease in body weight associated with an increase in *Somatostatin* gene expression (Herwig et al., 2012; Dumbell et al., 2015). In the present study, we observed a significant increase in *Somatostatin* gene expression in spring as compared to autumn, which is in line with the reported decrease in the jerboa's body weight in spring and summer (El Ouezzani et al., 2011). Therefore, Siberian hamster and jerboa appear to display opposite but coordinated seasonal variations in both *Somatostatin* gene expression and body weight. Regarding NPY expression in the jerboa's ARC, the level of *Npy* mRNA was found very strong in both seasons. No significant variation between spring and autumn in the number of NPY cells or the staining intensity of individual NPY neurons was observed. A similar lack of photoperiodic variation in NPY expression was previously reported in the Siberian hamster (Mercer et al., 2000). By contrast in the ewe, both the number of NPY-ir cells (Skinner and Herbison, 1997) and the level of *Npy* gene expression in the ARC (Clarke et al., 2000) were reported to be higher in May as compared to September, and were suggested to be related to a seasonal regulation of food intake. Besides its strong orexigenic function, studies have reported its involvement in the regulation of GnRH neurons activity (Li et al., 1999). NPY can either activate (Kalra and Crowley, 1984; Khorram et al., 1987) or inhibit (Khorram et al., 1987; McDonald et al., 1989; Kaynard et al., 1990) the reproductive axis, with possible environment and species dependence.

Two genes encoding neuropeptides involved in food intake and body weight regulation, *Pomc* and *Somatostatin*, thus display a strong reduction of expression in autumn. We recently reported in the Siberian hamster that the seasonal variation in *Somatostatin* gene expression, like *Kiss1* and *Rfrp*, is driven by a melatonin-dependent TSH mechanism (Klosen et al., 2013). Further studies should examine whether the parallel reduction in *Kiss1*, *Rfrp*, *Somatostatin* but also *Pomc* gene expression observed in autumn in jerboa is related to similar melatonin-dependent mechanisms. Additionally, peptide interaction cannot be excluded to explain such correlated seasonal variations. Fu and van den Pol (2010) reported Kp fiber projection to POMC neurons

and showed a direct excitation of POMC neurons by Kp, suggesting a possible indirect modulation of food intake by Kp through POMC neurons, while a direct role of Kp in the regulation of metabolism has also been suggested (De Bond and Smith, 2014, for review). On the other hand, there is a bidirectional communication between somatostatin and POMC neurons since somatostatin receptors are located on POMC ARC neurons and POMC neurons project to a majority of somatostatin neurons in the periventricular nucleus, indicating that somatostatin effects on growth could be under the influence of melanocortins (Fodor et al., 1998).

In conclusion, this study reports a general springtime activation of genes encoding hypothalamic peptides involved in the activation of reproduction and inhibition of food intake in the male jerboa. These variations are in line with the seasonal physiology of the jerboa, since genes encoding RFRP and Kp are both increased in spring when the animals become sexually active and similarly genes encoding the anorexigenic POMC and somatostatin are also increased in spring when the animals reduce their body weight. Based on these results, it is hypothesized that reproduction and energy balance are modulated oppositely in the jerboa. The increase in body weight during autumn could be a long-term anticipatory adaptive process allowing the animal's preparation for the upcoming reproductive activity in early spring. The possibility that the seasonal changes in melatonin production orchestrate this coordinated seasonal variation in neuropeptides should be considered. Furthermore, the possible interactions among these neuropeptides to synchronize the balance between the seasonal reproduction and the metabolic activity should also be investigated.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

ROLE OF AUTHORS

All authors had full access to the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. Study concept and design: RT, VS, SEO. Acquisition of data: RT, PK, MP.

Analysis and interpretation of data: RT, VS, PK, SEO. Drafting of article: RT, VS, SEO. Revision of the article for important intellectual content: RT, VS, PK, SEO. Statistical analysis: RT, PK. Obtained funding: VS, SEO. Technical and material support: RT, PK, MP. Study supervision: VS, SEO.

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