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RFamide-Related Peptide Gene Is a Melatonin-Driven Photoperiodic Gene

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In seasonal species, various physiological processes including reproduction are organized by photoperiod via melatonin, but the mechanisms of melatonin action are still unknown. In birds, the peptide gonadotropin-inhibiting hormone (GnIH) has been shown to have inhibitory effects on reproductive activity and displays seasonal changes of expression. Here we present evidence in mammals that the gene orthologous to GnIH, the RFamide-related peptide (*RFRP*) gene, expressed in the mediobasal hypothalamus, is strongly regulated by the length of the photoperiod, via melatonin. The level of *RFRP* mRNA and the number of RFRP-immunoreactive cell bodies were reduced in sexually quiescent Syrian and Siberian hamsters acclimated to short-day photoperiod (SD) compared with sexually active animals maintained under long-day photoperiod (LD). This was contrasted in the laboratory Wistar rat, a non-photoperiodic breeder, in which no evidence for *RFRP* photoperiodic modulation was seen. In Syrian hamsters, the reduction of *RFRP* expression in SD was independent from secondary changes in gonadal steroids. By contrast, the photoperiodic variation of *RFRP* expression was abolished in pinealectomized hamsters, and injections of LD hamsters with melatonin for 60 d provoked inhibition of *RFRP* expression down to SD levels, indicating that the regulation is dependent on melatonin. Altogether, these results demonstrate that in these hamster species, the *RFRP* neurons are photoperiodically modulated via a melatonin-dependent process. These observations raise questions on the role of RFRP as a general inhibitor of reproduction and evoke new perspectives for understanding how melatonin controls seasonal processes via hypothalamic targets. (*Endocrinology* 149: 902–912, 2008)

MANY SPECIES OF temperate regions show seasonal variations in several aspects of physiology and behavior, including reproduction, hibernation, molting, and body weight (1–4). These modifications are triggered by the annual changes of photoperiod (day length), which in mammals is decoded by a photo-neuroendocrine system composed of the retina, the suprachiasmatic nucleus (location of the master circadian clock), and the pineal gland (4–7). The latter releases the hormone melatonin exclusively at night, in such a way that duration of secretion varies according to day length and provides an endocrine representation of the photoperiod (4–7). However, although it is well demonstrated that the duration of the nocturnal secretory profile of pineal melatonin is critical for the regulation of seasonal functions (8, 9), the precise mechanisms by which melatonin adjusts seasonal physiology remain to be characterized. Recent data show that melatonin influences seasonal functions by controlling gene expression in various brain loci (10–15). Various lines of evidence indicate that melatonin controls reproductive activity by indirectly modulating gonadotropin secretion through an action in the mediobasal hypothalamus (5). Lesion, binding, and infusion studies have indicated that the region of the dorsomedial hypothalamic (DMH)/ventromedial hypothalamic (VMH) areas in hamsters (16–20) and the premammillary hypothalamic area in sheep (21–23) are important for relaying the melatoninergic message to the reproductive system. However, the neurotransmitters and the signaling mechanisms involved in translating the melatonin signal to the gonadotropic system at this anatomical site are still unknown.

The Syrian hamster (*Mesocricetus auratus*) and the Siberian hamster (*Phodopus sungorus*) are well-known photoperiodic models (2, 4). In both species, sexual activity is promoted by long-day photoperiod (LD), whereas exposure to short-day photoperiod (SD) triggers arrest of the reproductive activity, as manifested by testicular regression and decrease in serum gonadotropin and testosterone levels (4–6). However, if SD exposure is prolonged over 20–30 wk, hamsters become refractory to inhibitory day lengths (SD-R) and a complete recrudescence of the reproductive system occurs spontaneously (4). Recently, we have found that in the arcuate nucleus of the Syrian hamster, expression of the *Kiss1* gene is controlled by photoperiod, via melatonin (24, 25). This gene encodes kisspeptins (Kp), the endogenous ligand(s) of the G protein-coupled receptor 54 (GPR54), which is critical for the regulation of reproduction, both in adults and at puberty onset (26–29). Via GPR54, Kp activate GnRH neurons, which induces GnRH release and downstream gonadotropin se-

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Abbreviations: DMH, Dorsomedial hypothalamic nucleus; GnIH, gonadotropin-inhibiting hormone; GPR54, G protein-coupled receptor 54; hKp10, human kisspeptin 10; Kp, kisspeptin; LD, long-day photoperiod; mKp10, mouse Kp10; Pin-X, pinealectomy; PTWs, paired testicular weights; RFRP, RFamide-related peptide; SD, short-day photoperiod; SD-R, short-day refractory; SSC, saline sodium citrate; VMH, ventromedial hypothalamic nucleus.

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cretion (26–29). We have found that in the Syrian hamster, *Kiss1* expression is down-regulated in SD photoperiod, via a melatonin-dependent mechanism (24, 25). Importantly, we have shown that chronic intracerebroventricular administration of Kp to sexually quiescent SD hamsters is sufficient to restore testicular activity (24, 25), strongly suggesting that *Kiss1* and *GPR54* are critical for mediating the effect of photoperiod on the reproductive axis.

Kp is a member of the Arg-Phe-amide (RFamide) family of peptides (30–33), which also includes the products of the RFamide-related peptide gene (*RFRP*). The mammalian *RFRP* gene is orthologous to the gene encoding the peptide gonadotropin-inhibiting hormone (GnIH) in birds, which possesses inhibitory effects on gonadotropin release from the anterior pituitary (34). The *RFRP* gene gives rise to the two biologically active peptides RFRP-1 and RFRP-3, and intracerebroventricular injections of RFRP-3 reduces plasma levels of LH (35, 36). In contrast to *Kiss1*, which is expressed in the arcuate nucleus (24, 27, 28, 37), *RFRP* is expressed in the area in between the VMH and DMH (33, 35, 36, 38–45). Interestingly, neurons in the same area have earlier been described as Kp immunoreactive (45–47). However, the specificity of this labeling has been highly questioned (27, 28, 45–50), because no *Kiss1* mRNA could be found in this area (27, 28, 37), and antisera cross-reactivity is highly possible due to the close sequence similarity of the peptides.

We found it interesting to examine further this population of neurons in photoperiodic species, because their location matches the site identified in the Syrian hamster for the action of melatonin on reproduction (16–20). We speculated that the gene product(s) recognized in this region by the Kp antisera may be that of another member of the family (39, 51, 52), presumably *RFRP* gene products. We first examined *RFRP* mRNA expression in the brain of two photoperiodic rodents, the Syrian hamster and the Siberian hamster, and we found in accordance with earlier studies in the rat that *RFRP* mRNA is also located in the medial hypothalamus. In these seasonal species, gonadal activity is modulated by day length, and we hypothesized that *RFRP* expression may also change in SD *vs.* LD hamsters. Given the biological roles suggested for *RFRP* and its avian ortholog *GnIH* (*i.e.* inhibitory action of the reproductive axis) (34–36, 53–55), we speculated that *RFRP* expression would increase in the medial hypothalamus of sexually inactive SD hamsters. We observed that *RFRP* displayed a photoperiodic-dependent expression in both Syrian and Siberian hamsters but, surprisingly, exhibited a reduced expression under short photoperiod. Thus, we performed a series of experiments in the Syrian hamster to better characterize this photoperiodic regulation. We examined the influence of gonadal hormones and melatonin and determined that melatonin is responsible for the photoperiodic regulation of *RFRP* expression. We discuss these results in the light of the presumed biological functions of RFRP peptides, and the implications for understanding the mechanisms of seasonal breeding are also exposed.

Materials and Methods

Animals and tissue collection

All experiments were performed in accordance with the rules of the French Department of Agriculture (license no. 67-38 and 67-250), the European Committee Council Directive of November 24, 1986 (86/609/EEC), and the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

The adult male Syrian (*M. auratus*) and Siberian (*P. sungorus*) hamsters used in these experiments were bred in-house. From birth they were maintained in LD photoperiod consisting of 14 h light (200 lux) and 10 h dark (2 lux dim red light) for the Syrian hamsters, or 16 h light and 8 h dark for the Siberian hamsters, with lights off at 1900 h, at 22 ± 2 C with *ad libitum* access to water and food. Adult male Han Wistar rats (250–350 g) were bred and obtained from the Faculté de Médecine (Strasbourg, France). The animals were allowed to adapt to our animal facility for 2 wk under standard laboratory conditions (12 h light, 12 h dark, lights off at 1900 h) before the lighting protocol was changed.

Tissue collection consistently occurred 3–4 h after lights on, except where indicated. Animals were anesthetized with isoflurane vapors and killed by decapitation. Brains were rapidly removed from the skull, snap-frozen at −30 C, and stored at −80 C until *in situ* hybridization. Testes were dissected out, measured, and weighed. When required, trunk blood was collected and centrifuged at 1500 × *g* for 15 min, and plasma was stored at −20 C until assayed.

Experimental design

Photoperiodic modulation of RFRP gene expression. This experiment aimed to examine the photoperiodic expression of *RFRP* in the hypothalamus of two photoperiodic and one non-photoperiodic rodent model. Groups (n = 6) of Syrian and Siberian hamsters were placed in LD (Syrian hamsters, 14 h light, 10 h dark; Siberian hamsters, 16 h light, 8 h dark; for both, lights off at 1900 h) and SD (10 h light, 14 h dark; lights off at 1900 h) photoperiods for 10 wk. Wistar rats were divided into two groups (n = 4 per group) that were transferred for 10 wk into a LD photoperiod consisting of 16 h light, 8 h dark, or into a SD photoperiod consisting of 8 h light, 16 h dark.

The 24-h time course for RFRP gene expression. This experiment aimed to examine whether *RFRP* displays a daily rhythm of expression, under both LD and SD photoperiods. Syrian hamsters were placed in LD and SD for 10 wk, after which they were killed every 4 h over 24 h, starting at 1800 h (n = 5 per group).

Photoperiodic change of RFRP immunoreactivity. This experiment was to investigate whether the photoperiodic change of *RFRP* mRNA in hamsters is translated at the peptide level. Syrian and Siberian hamsters were raised for 10 wk under LD and SD (n = 5 per group) before they were processed for immunocytochemistry, as described below.

Modulation of RFRP expression by sex steroids. This experiment was to determine whether the photoperiodic modulation of the *RFRP* expression in the Syrian hamster results from the secondary changes in circulating levels of gonadal steroids. Two groups (n = 5 per group) of LD Syrian hamsters were either castrated (LD-cast) or sham operated (LD-sham), and two groups (n = 5 per group) of SD Syrian hamsters were implanted with silastic capsules either filled with testosterone (SD-testo) or left empty (SD-empty). All animals were killed after 4 wk of treatment, after which *RFRP* expression was examined.

Modulation of RFRP expression by melatonin. This experiment was to examine the role of melatonin in the photoperiodic regulation of *RFRP* expression in the Syrian hamster. Two groups of Syrian hamsters (n = 6 per group) were either pinealectomized (Pin-X) to remove the primary source of melatonin or underwent sham surgery (Pin-sham) before being transferred to SD for 10 wk. In addition, six groups of hamsters (n = 6 per group) were left in LD and given daily sc injections for 7, 21, or 60 d of either saline solution (Ringer-5% ethanol) or melatonin (Sigma-Aldrich, Lyon, France) (50 μg in Ringer-5% ethanol) to mimic the effects of SD. Injections occurred 1.5 h before lights-off, as previously described.

RFRP gene expression in photorefractory Syrian hamsters. This experiment aimed to investigate *RFRP* expression in SD-refractory (SD-R) Syrian hamsters, which undergo spontaneous gonadal reactivation after prolonged exposure to SD. Two groups of SD-R hamsters were prepared by leaving the animals in SD for 28 wk, whereas age-matched control hamsters were left in LD (n = 6 per group). The animals were then processed for *in situ* hybridization and immunocytochemistry, as described below.

Surgical procedures

Castration, steroid replacement and Pin-X were performed under anesthesia as previously described (11, 24). For anesthesia, a mixture of Zoletil 20 (Virbac, Carros, France) and Rompun (Bayer Pharma, Puteaux, France) was used. For castration, the gonads of LD animals were removed, whereas control animals left intact underwent sham surgery. For steroid replacement, testosterone (4-androsten-17β-ol-3one; Sigma)-filled silastic capsules (inner diameter, 1.47 mm; outer diameter, 1.95 mm; length, 13 mm) were implanted sc to SD adapted hamsters (testicular regression verified by scrotal palpation), whereas control untreated animals received empty capsules. For Pin-X, a circular hole was drilled in the skull and the pineal gland was extracted or left in place (Pin-sham).

In situ hybridization histochemistry

Coronal brain sections (20 μm) were cut through the mediobasal and posterior hypothalamic regions in seven similar series of sections. The sections were thaw-mounted and stored at −80 C until required. Radioactive *in situ* hybridization was carried out as previously described (11, 56). *RFRP* mRNA was probed with an antisense oligonucleotide labeled with [^{35}S]deoxy-ATP (1250 Ci/mmol; PerkinElmer, Zaventrum, Belgium) using terminal transferase (Roche, Meylan, France). The sequence of the oligonucleotide used for quantitative analysis was 5′-GAC TCA TCT TAA TAT CTT TCT TTG CCC CCC AAT CTT TGA GTT CTT GAA A-3′, based on the rat *RFRP* sequence (bases 181–229 from GenBank accession no. NM_023952). Another nucleotide sequence complementary to the bases 431–480 used by Pertovaara *et al.* (39) produced identical results. The signal obtained with both probes matched that previously described in the literature and was clearly absent from the arcuate nucleus where *Kiss1* is expressed. The sections were rapidly thawed, fixed in 4% paraformaldehyde, acetylated in triethanolamine buffer, and dehydrated in graded ethanols. The radiolabeled oligoprobe was added at a specific activity of 10^7 cpm/ml to the hybridization medium containing 50% formamide (vol/vol), 4× saline sodium citrate (SSC) (1× SSC is 0.15 M NaCl, 0.015 M sodium citrate·2H_2O, pH 7.2), 1× Denhardts solution (0.02% Ficoll, polyvinylpyrrolidone, and BSA), salmon sperm ssDNA (0.5 mg/ml), 0.25 mg/ml yeast tRNA, 10% (wt/vol) dextran sulfate, and 10 mM dithiothreitol. The hybridization mixture was applied to slides, which were coverslipped and incubated overnight at 37 C in a humid chamber. The sections were washed four times for 15 min in 1× SSC at 55 C, followed by two times for 30 min in 1× SSC at room temperature. Finally, the sections were dehydrated, and all were exposed against BioMax MR Film for 7 d together with ^{14}C radioactive standards to allow standardization of densitometric measurements. Selected sections were dipped in EM-1 nuclear emulsion (Amersham Bioscience, Arlington, IL) for 2 wk before being developed for microscopic localization of *RFRP* mRNA transcripts. These sections were counterstained in thionin to determine anatomical structures in details. For semiquantitative analysis, x-ray films were scanned on an Epson 4990 transmittance scanner, and background-subtracted calibrated OD measurements of gene expression were performed using ImageJ (NIH). Integrated density was measured on three sections from the same slide [region situated 2–2.6 mm posterior to the bregma for Syrian hamsters, according to the stereotaxic atlas of the golden hamster brain, Morin and Wood (64)] to calculate the average integrated density per animal. We did not detect any subregional differences for the photoperiodic regulation of *RFRP* expression, and the analysis was performed by measuring the OD of the entire signal for *RFRP*. An observer blind to treatment identity performed all analyses.

Immunohistochemistry

Animals were deeply anesthetized with isoflurane, and transcardially perfused with physiological saline (with heparin) and then with 4% paraformaldehyde in 0.1 M phosphate buffer. The brains were removed from the skull, postfixed overnight, stored in PBS, and cryoprotected in 30% sucrose/PBS for 48 h before sectioning in series of five (Syrian hamsters) and four (Siberian hamsters) at a freezing microtome (40 μm). Immunostaining was performed on free-floating sections as described in a previous report (57), using the avidin-biotin method and diaminobenzidine as chromogen. To avoid animal-animal and section-section variability, one series of sections from all animals were incubated in the same dilutions of the same antisera as indicated below. Briefly before incubating in the primary antiserum, the sections were incubated three times for 5 min in PBS, for 10 min in 1% H_2O_2-PBS, and for 20 min in 5% swine serum mixed in PBS with 1% Triton X-100.

The sections were incubated overnight at 4 C in the primary antiserum. Two commercial polyclonal antisera raised in rabbits were used for this study. One antiserum was raised against YNWNSFGL*RF*-amide or human Kp10 (hKp10) (catalog item H-048-56, lot 00064; Phoenix Pharmaceuticals Inc., Belmont, CA), and the other was raised against FMRF-amide (catalog item RA20002, lot 400206; Neuromics, Edina, NM). The antiserum from Phoenix was diluted 1:1500, and the antiserum from Neuromics was diluted 1:5000. Both antisera produced similar strong staining of neuronal cell bodies in the same area of the hypothalamus, and this staining could be completely abolished by preincubation (10 μM) with RFRP-1, RFRP-3, hKp10, and FMRF-amide. After careful washing in PBS with 0.3% Triton X-100 for three times 10 min, the sections were processed by incubating in a biotinylated donkey antirabbit (dilution 1:1000; Jackson Laboratories, Bar Harbor, ME) and subsequently incubated in an avidin-biotinylated horseradish peroxidase complex (Vector Elite Kit; Vector Labs, Burlingame, CA; diluted 1:125), both for 60 min. Finally, the sections were reacted for peroxidase activity by incubation for 20 min with a solution of 0.125% diaminobenzidine, rinsed twice for 10 min in distilled water, and mounted on gelatinized glass slides. The number of RFRP-immunoreactive cell bodies was counted through the entire length of the mediobasal hypothalamus by an observer not aware of the nature of the animals. The number of cell bodies was counted in a bright-field microscope (Zeiss Imager Z1) at ×20 magnification in one series of sections (every fifth section for Syrians and every fourth for Siberians). The number of positive cells counted from each animal was multiplied by the number of series to give a rough estimate of the total number of cells per brain.

Testosterone assay

Plasma concentrations of testosterone were determined by RIA as described (58). Briefly, 3 ml diethyl ether was added to 50 μl of the blood sample, which was then vortexed for 1 min and centrifuged for 5 min (4 C, 2000 rpm). The ether phase was decanted and evaporated, and the dried extract was redissolved in 300 μl phosphate buffer before tritiated testosterone (1000 cpm) (Amersham Pharmacia Biotech Europe, Orsay, France) was added. The testosterone concentration was determined by using duplicate aliquots of the extracts, which were redissolved in 0.01 M PBS (pH 7.4) containing 0.1% BSA and incubated overnight at 4 C with approximately 6000 cpm of [^{3}H]testosterone and a specific antibody. The specific testosterone antibody was kindly provided by Dr. G. Picaper (Médecine Nucléaire, La Source, France). Bound and free fractions were separated by absorption with dextran-coated charcoal and centrifuged. Aliquots of the bound fractions were counted with a Packard 1600 liquid scintillator counter. The sensitivity of the assay was 50 pg/ml, and the intra- and interassay coefficients of variation were 8 and 9.5%, respectively.

Statistical analyses

Results are shown as mean ± SEM. Data were analyzed by *t* test, Mann-Whitney rank sum test, or two-way ANOVA, followed by Tukey's analysis, as appropriate. Statistical significance was set at $P < 0.05$.

Results

Photoperiodic modulation of RFRP gene expression

In LD Syrian hamsters, expression of *RFRP* was observed in a small region in the medial hypothalamus. The *RFRP*-expressing neurons were distributed in small groups, the largest of which was located in the relatively cell-poor region between the DMH and the VMH (Fig. 1, A–D). A group of *RFRP* mRNA-containing neurons was also observed medial and dorsal to the DMH, inside the boundaries of the DMH as well as in the periventricular area at the same rostrocaudal

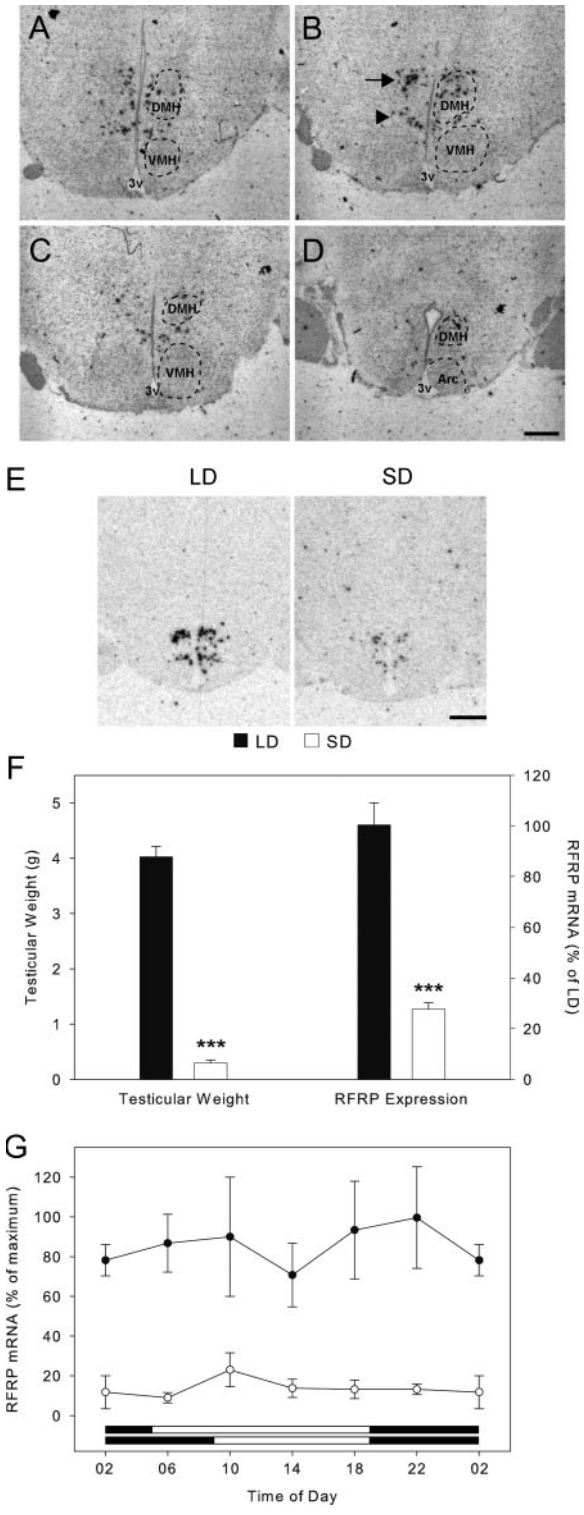

FIG. 1. Effect of photoperiod on *RFRP* expression in the Syrian hamster hypothalamus. *In situ* hybridization was performed on 20-μm brain sections from Syrian hamsters. A–D, Representative photomicrographs from emulsion-coated slides show *RFRP* expression (silver grains) in the mediobasal hypothalamus of a LD Syrian hamster. A–D correspond to different rostrocaudal levels [A, 2.2; B, 2.4; C, 2.6; D, 2.8 mm posterior to bregma, according to the stereotaxic atlas of the golden hamster brain, Morin and Wood (64)]. *RFRP* was expressed in the dorsal part of the DMH (silver grains indicated by *arrow*) and in the interspace between the DMH and the VMH (silver grains indicated by *arrowhead*). 3v, Third ventricle. *Scale bar*, 0.5 mm. E, Representative autoradiograms for *RFRP* expression in the mediobasal hypothalamus of LD and SD hamsters. Higher signal was observed in LD compared with SD hamsters. *Scale bar*, 1 mm. F, *left*, Testicular weight of hamsters maintained under LD or SD photoperiods for 10 wk. Values show mean ± SEM; *right*, quantification of *RFRP* mRNA level showing that *RFRP* expression is reduced in SD compared with LD animals. ***, $P < 0.001$, *t* test; n = 6 per group. Data are expressed as percentage of the LD value and represent the mean ± SEM. G, Level of *RFRP* mRNA over 24 h in LD (●) and SD (○) photoperiods. Regardless of the time of day, *RFRP* expression was constantly higher in LD compared with SD, with no pronounced 24-h rhythm (two-way ANOVA). *Black* and *white bars* represent the dark and light periods, respectively, with the LD photoperiod on *top*. Data are expressed as a percentage of the maximum LD value and represent the mean ± SEM; n = 4–5 per group.

level of the hypothalamus (Fig. 1, A–D). *RFRP* mRNA expression in all groups of neurons was clearly diminished in Syrian hamsters maintained in SD for 10 wk (Fig. 1E). SD animals underwent testicular regression [Fig. 1F; paired testicular weights (PTW): 0.30 ± 0.05 g *vs.* 4.00 ± 0.19 g in LD hamsters] and displayed *RFRP* mRNA level significantly lower than in LD animals (Fig. 1F; $P < 0.001$). The *RFRP* mRNA level in SD was about 28% of that in LD.

To confirm that the photoperiodic expression difference is truly photoperiodic and not diurnal in nature, *RFRP* mRNA level was determined every 4 h over 24 h both in LD and SD. *RFRP* expression was significantly higher in LD relative to SD over 24 h, with no pronounced rhythms (Fig. 1G). By two-way ANOVA, there was an overall effect of photoperiod on the mRNA level ($F = 72.793$; $P < 0.001$), but no effect of time ($F = 0.355$; $P = 0.876$) and no interaction between photoperiod and time ($F = 0.287$; $P = 0.918$). At all time points, multiple pair-wise comparisons showed that LD and SD values were significantly different ($P < 0.01$).

Similar to Syrian hamsters, *RFRP* expression was detected in the same part of the hypothalamus in Siberian hamsters kept under LD, and the mRNA level was highly depressed in SD animals (Fig. 2, A and C; $P < 0.001$). In SD-adapted Siberian hamsters, *RFRP* expression was about 18% of that in LD animals (Fig. 2C). The mean PTW in these animals was 690 ± 50 mg in LD and 40 ± 10 mg in SD.

In contrast to both Syrian and Siberian hamsters, Wistar rats maintained in SD for 10 wk remained sexually active, and *RFRP* expression was detected in both LD and SD animals (Fig. 2B), with no significant variations of *RFRP* mRNA level (Fig. 2B; $P = 0.428$). In the rat, the positive neurons were confined more exclusively to the zone in between the VMH and the DMH (Fig. 2B). In the posterior hypothalamus at the level of the very caudal part of the DMH, a group of *RFRP* mRNA-expressing neurons was present above the third ventricle (not shown).

Photoperiodic change of RFRP immunoreactivity

The male Syrian and Siberian hamsters used for immunohistochemical analysis were maintained in SD for 10 wk, and it was observed that they all underwent testicular regression. For the Syrian hamsters, the PTW was 3.26 ± 0.46 g in LD and 0.3 ± 0.05 g in SD. For the Siberian hamsters, the PTW was 688.33 ± 47.84 mg in LD and 30.93 ± 1.93 mg in SD.

In LD Syrian hamsters, a strong RFRP-immunoreactive

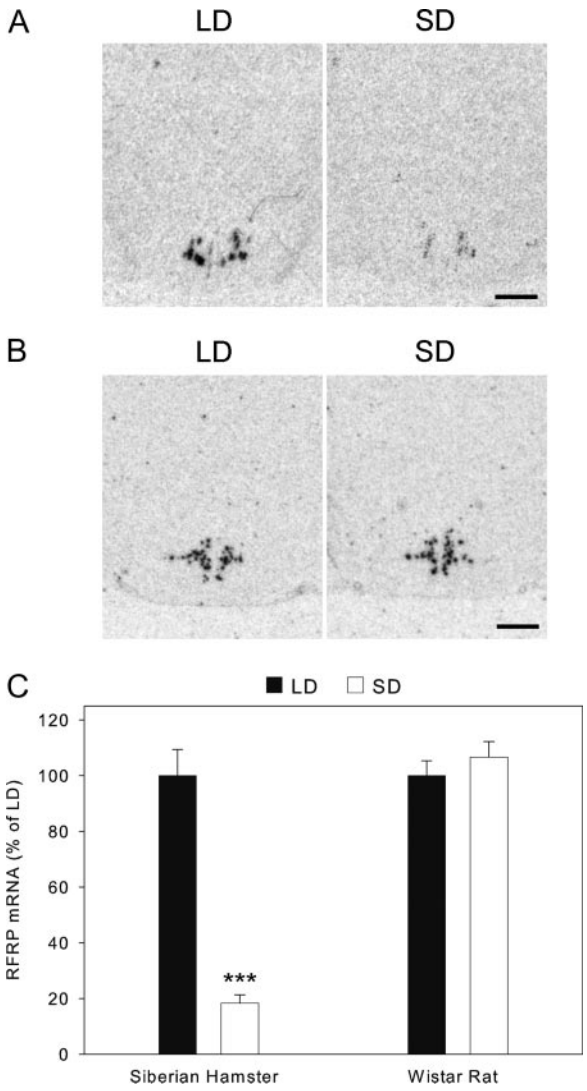

FIG. 2. Effect of photoperiod on *RFRP* expression in the Siberian hamster and in the Wistar rat. A and B, Autoradiograms for *RFRP* probe in the mediobasal hypothalamus. In LD conditions, the expression of *RFRP* was observed in the dorsomedial hypothalamic nuclei of the Siberian hamster (A) and of the Wistar rat (B). In Siberian hamsters (A), but not in rats (B), the signal for *RFRP* was lower in SD compared with LD. *Scale bars*, 1 mm. C, Quantification of *RFRP* mRNA level in Siberian hamsters (*left*; n = 6 per group) and rats (*right*; n = 4 per group) maintained in LD or SD photoperiods for 10 wk. There was an effect of photoperiod on *RFRP* expression in the Siberian hamster (***, $P < 0.001$, *t* test) but not in the rat. Data are expressed as percentage of the LD value and represent the mean ± SEM.

staining was observed in the neuronal cell bodies in the same area as observed to contain *RFRP* mRNA (Fig. 3, A and B). This distribution matches that previously described for RFRP immunoreactivity in rodents (35, 36, 41–44), including the Syrian hamster (36). Furthermore, despite being raised against two different RFamide peptides, the two antisera produced virtually the same staining. Preincubation of both antisera with RFRP-1, RFRP-3, mouse Kp10 (mKp10), and human Kp10 (hKp-10) totally abolished the staining, and application of the same peptides on dot-blots revealed that both antisera bind to all three peptides, although with apparent lower affinity for mKp10 than RFRP-1 (not shown). The quantification shown here was carried out in sections from animals incubated with the antiserum from Phoenix Pharmaceuticals.

In SD hamsters, immunostaining within the cytoplasm of individual cells was generally weaker compared with LD hamsters (Fig. 3C). Quantification of the number of cells in LD and SD animals revealed that significantly fewer immunoreactive cells were present in the hypothalamus of SD compared with LD animals (Fig. 3G). In Syrian hamsters, the number of immunoreactive cell bodies was 50% less in SD compared with LD condition (Fig. 3G; LD, 346.0 ± 29.5 cells per brain; SD, 189.0 ± 12.8 cells per brain; $P = 0.001$).

Similarly, the number of cells and the intensity of positive staining in hypothalamic neurons of Siberian hamsters kept under LD (Fig. 3, D and E) was much higher than in animals kept under SD (Fig. 3F). Compared with the Syrian hamster, the staining intensity of individual cells in the Siberian hamster appeared stronger, and the SD-induced decrease in the number of immunoreactive cell bodies was even higher (Fig. 3G; LD, 345.3 ± 28.8 cells per brain; SD, 41.3 ± 12.5 cells per brain; $P < 0.001$). Also, the morphology of the RFRP-immunoreactive neurons in the Syrian hamster was slightly different from the Siberian hamster exposed to LD (Fig. 3, B and E). The positive neurons contained relatively little immunoreactive material in the cell soma in the Syrian hamster compared with the Siberian (Fig. 3, B and E). However, the presence of RFRP-positive nerve fibers was found in about the same quantity and regions in the two species (not shown).

Modulation of photoperiodic RFRP expression by gonadal steroids

The role of gonadal steroids in the photoperiodic modulation of the *RFRP* gene in Syrian hamsters was examined. There was no statistical difference in *RFRP* expression between LD Syrian hamsters that were castrated (LD-cast) or sham-operated (LD-sham), although we observed a nonsignificant tendency for increased expression in castrated animals (Fig. 4A; $P = 0.053$). In addition, two groups of SD-adapted hamsters were implanted for 4 wk with empty (SD-empty) or testosterone-filled (SD-testo) capsules to restore blood concentrations similar to those observed in LD. The plasma testosterone level in SD animals receiving an empty capsule was 0.33 ± 0.13 ng/ml, whereas that of SD animals receiving testosterone was 2.17 ± 0.52 ng/ml. This level in the SD-testo group was not statistically different from LD hamsters (3.24 ± 0.42 ng/ml; $P = 0.219$), whereas both were significantly different from SD-empty animals (LD *vs.* SD-empty, $P = 0.003$; SD-testo *vs.* SD-empty, $P = 0.028$). The expression of *RFRP* in SD hamsters treated with testosterone for 4 wk was not statistically different from control hamsters receiving an empty capsule (Fig. 4B; $P = 0.663$), demonstrating that testosterone treatment alone was not sufficient to restore *RFRP* expression to LD levels. Both SD-empty and SD-testo animals had reduced *RFRP* mRNA level compared with LD control animals ($P < 0.001$). It is concluded from these experiments that the photoperiodic change of *RFRP* expression is not directly modulated by sex steroids.

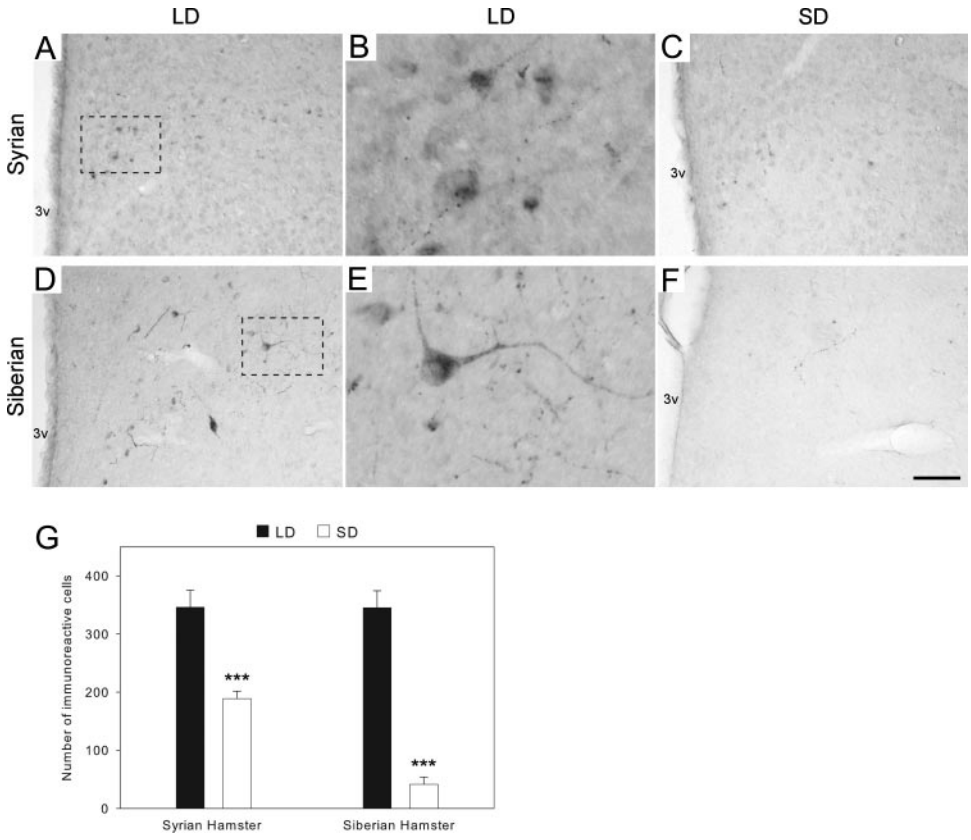

FIG. 3. Effect of photoperiod on RFRP immunoreactivity in the Syrian and Siberian hamsters. A–F, Representative photomicrographs of neurons stained for RFRP immunoreactivity using the Phoenix antiserum. For both Syrian (A) and Siberian (D) hamsters maintained in LD, immunoreactive cells were observed in the region between the VMH and DMH. The *boxed areas* in A and D are magnified in B and E, respectively. In contrast, few immunoreactive cells could be distinguished in SD animals in the same region (C and F, Syrian and Siberian hamsters, respectively). *Scale bar*, 100 μm (A, C, D, and F) and 30 μm (B and E). G, Estimated number of RFRP-immunoreactive cell bodies in LD and SD hamster hypothalami according to photoperiod. For both the Syrian (*left*; n = 5 per group) and the Siberian (*right*; n = 5 per group), the number of immunoreactive cells was lower in SD compared with LD. Values represent the mean ± SEM. ***, $P < 0.001$, *t* test.

Modulation of photoperiodic RFRP expression by melatonin

To examine the role of melatonin in the regulation of RFRP expression in the Syrian hamster, animals were Pin-X to remove the primary source of melatonin before being transferred to SD for 10 wk. This treatment prevented the SD-induced testicular regression (PTW = 3.00 ± 0.55 g) compared with Pin-sham animals (PTW = 0.43 ± 0.06 g). Similarly, the SD-induced decrease in *RFRP* expression was not observed in Pin-X hamsters, compared with SD-sham animals (Fig. 5A; $P < 0.001$). Importantly, the magnitude of difference in *RFRP* expression between Pin-X and Pin-sham animals (Fig. 5A) was comparable to that observed between LD and SD animals.

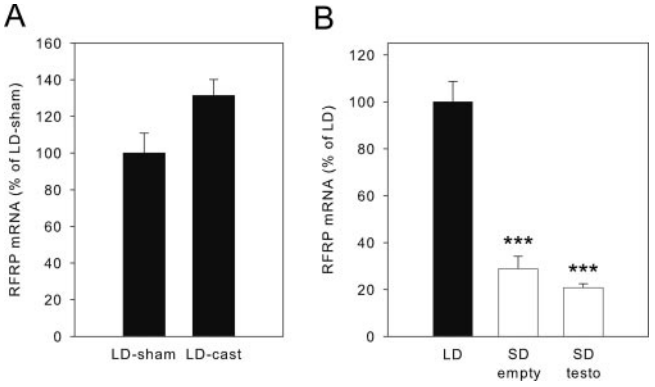

FIG. 4. Effects of gonadal steroids on *RFRP* expression in the Syrian hamster. A, *RFRP* mRNA level was not significantly different between LD-cast and LD-sham. Hamsters (n = 5 per group) were killed 4 wk after the surgical operation, and *RFRP* expression was quantified in the region of the DMH/VMH. Data are expressed as percentage of the LD-sham value and represent the mean ± SEM. B, Expression of *RFRP* in hamsters kept in SD for 8 wk and implanted with a testosterone-filled (SD testo) capsule for 4 additional weeks did not differ significantly from that of control animals receiving an empty capsule (SD empty). In both cases, mRNA level was significantly lower than LD level (***, $P < 0.001$, one-way ANOVA). Data are expressed as percentage of the LD value and represent the mean ± SEM; n = 5 per group.

In addition, to confirm the role of melatonin in controlling *RFRP* expression, we investigated in the Syrian hamster the effects of daily melatonin injections for 7, 21, or 60 d. Animals were injected daily with melatonin 1.5 h before lights-off, a strategy well known for inducing a photoperiodic response (SD phenotype) in hamsters maintained in LD (8, 9, 11) by extending artificially the duration of the nocturnal secretory profile of pineal melatonin. In vehicle-treated LD animals, testicular weight was not modified (Fig. 5B). In melatonin-treated LD animals, testicular weight was not changed significantly after 7 or 21 d of treatment (Fig. 5B; $P > 0.05$), whereas it was significantly reduced in hamsters treated for 60 d ($P < 0.001$). We observed that *RFRP* expression was significantly inhibited in LD hamsters treated with melatonin for 21 d ($P = 0.02$ *vs.* vehicle 21 d; $P = 0.03$ *vs.* melatonin 7 d) and 60 d ($P < 0.001$ *vs.* vehicle; $P < 0.001$ *vs.* melatonin 7 and 21 d) but not 7 d ($P > 0.05$ *vs.* vehicle at 7, 21, and 60 d) (Fig. 5C). The decrease of *RFRP* mRNA level in melatonin-treated animals was progressive, and 60 d of treatment were necessary to have the *RFRP* expression level comparable to that observed in SD animals. In contrast, vehicle injections did not alter *RFRP* mRNA levels.

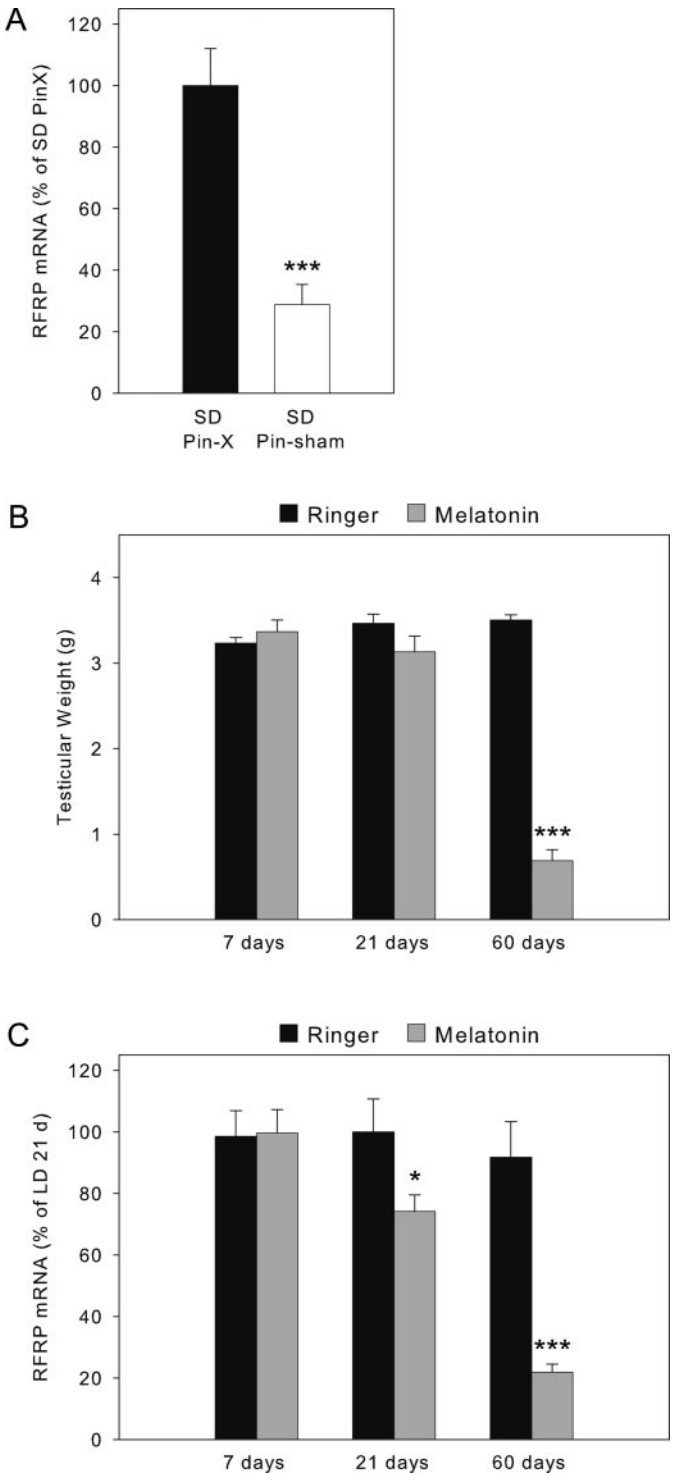

FIG. 5. Regulation of *RFRP* expression by melatonin in the Syrian hamster. A, Effect of Pin-X. SD-induced reduction of *RFRP* expression was prevented by ablating the pineal gland of Syrian hamsters (Pin-X) relative to sham operated animals (Pin-sham). Animals (n = 6 per group) underwent surgery before being transferred from LD to SD and were killed 10 wk later. Data represent the mean ± SEM and are expressed as percentage of the Pin-sham value. ***, $P < 0.001$, *t* test. B and C, Effect of melatonin injections. Groups (n = 6 per group) of Syrian hamsters held in LD were injected daily with ringer (Ringer-5% ethanol) or melatonin (50 μg in Ringer-5% ethanol) 1.5 h before lights off. Animals were treated for 7, 21, or 60 d. B, Testicular weight of Ringer- and melatonin-treated hamsters. Values show mean ± SEM. ***, $P < 0.001$, two-way ANOVA followed by Tukey's analysis. C, Quantification of *RFRP* mRNA level showing that hamsters injected with melatonin for 21 and 60 d had *RFRP* expression significantly lower than vehicle-treated animals. Furthermore, reduction of *RFRP* expression in melatonin-injected hamsters was time dependent, with lower *RFRP* mRNA level in animals treated for 60 d. Data are expressed as percentage of the maximum value observed for the Ringer group at 21 d and represent the mean ± SEM. *, $P < 0.05$; ***, $P < 0.001$, two-way ANOVA followed by Tukey's analysis.

Expression of RFRP in photorefractory Syrian hamster

We examined RFRP expression in SD-R Syrian hamsters. Animals kept in SD for 28 wk became refractory to SD and underwent gonadal recrudescence. Accordingly, testicular weight in SD-R hamsters was not significantly different from that of LD hamsters (PTW = 3.58 ± 0.24 g for SD-R hamsters *vs.* 3.78 ± 0.11 g for LD animals). *RFRP* expression remained low in the DMH of SD-R hamsters (Fig. 6A), with mRNA level being 43% of that in LD animals (Fig. 6C; $P = 0.002$). It tended to be higher than in SD animals, although this was not significant (Fig. 6C; $P = 0.45$). Examination of immunoreactivity to RFRP revealed that the number of stained cell bodies in SD-R animals (256.7 ± 31.0 cells per brain) was not significantly different from that in LD hamsters (Fig. 6, B and C). This number tended to be lower than in LD animals, being intermediate between that of LD and SD hamsters (one-way ANOVA: LD *vs.* SD-R, $P = 0.078$; LD *vs.* SD, $P = 0.004$; SD *vs.* SD-R, $P = 0.205$).

Discussion

Using *in situ* hybridization, we observed that *RFRP* is expressed in the medial hypothalamus similar to that reported in previous studies in other rodents (33, 36, 38, 39, 41, 42), including the Syrian hamster (36), and clearly matched that of RFRP immunoreactivity (this study) (50–57). Importantly, we observed that *RFRP* mRNA level displayed photoperiodic-dependent changes in both Syrian and Siberian hamsters, with reduced expression in SD animals. This was strongly supported by the observation that the number of neurons immunoreactive for RFRP was higher and more intensively stained in hamsters maintained in LD photoperiod, compared with SD. Immunocytochemistry is not a quantitative method, but a reduction of staining to undetectable levels suggests a strong and significant reduction of cytoplasmic peptide stores.

In mammals, only five members of the RFamide family have been identified so far (30–33), and for each member, a specific gene encodes one or more RFamide peptides (30, 31). The *RFRP* (also found as *NPVF*) (32, 33) gene encodes two or three putative peptides (RFRP-1, -2, and -3), depending on species (32, 33, 38). In mammals, only two peptides are processed, RFRP-1 and -3, whereas the predicted amino acid sequence for the C terminus of RFRP-2 that is seen in birds is, instead, included at the N terminus of RFRP-3 (32). Furthermore, in rodents, the *RFRP* gene lacks the sequence for RFRP-2 (32, 36, 38). Several reports have shown that polyclonal antisera raised against members of the RFamide family of peptides are likely to cross-react with other members (36, 45, 47, 50–52). This is also the case in this study, where

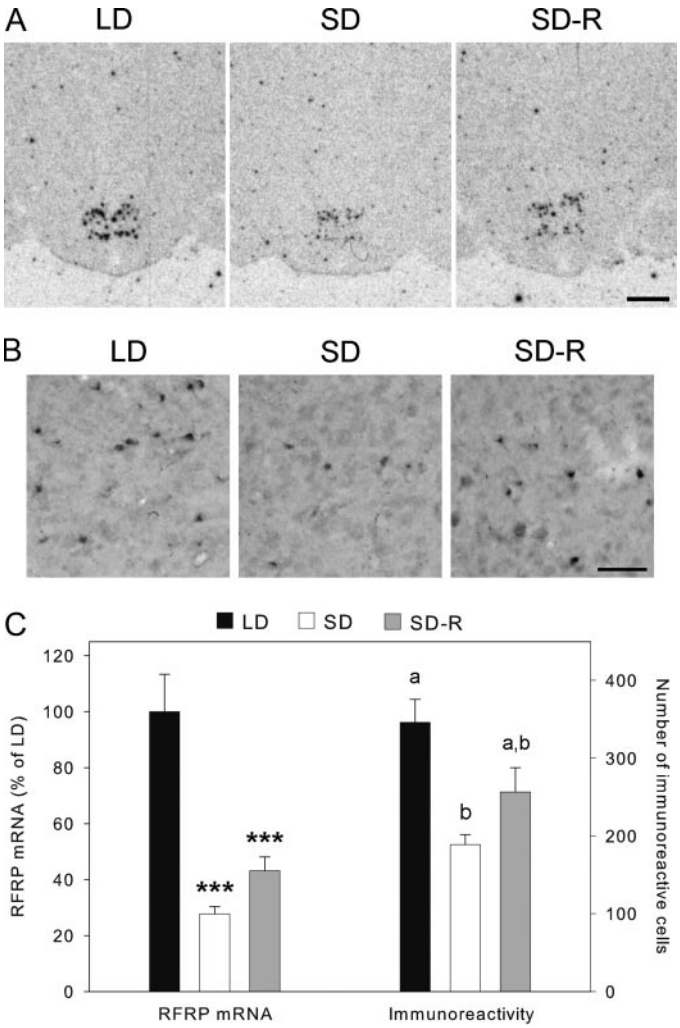

FIG. 6. Expression of *RFRP* in photorefractory Syrian hamsters. A, Autoradiograms showing lower *RFRP* expression in the DMH area of hamsters maintained in SD for 28 wk (SD-R), compared with LD and SD hamsters. *Scale bar*, 1 mm. B, Representative photomicrographs of neurons staining for RFRP using the Phoenix anti-hKp10 antiserum in SD-R animals compared with LD and SD animals. *Scale bar*, 50 μm. C, Quantification of *RFRP* mRNA level (*left*) and counting of immunoreactive cells (*right*) in LD, SD, and SD-R hamsters. *RFRP* gene expression in SD-R hamsters was significantly lower than that in LD animals, in contrast to the number of immunoreactive cells. Data represent the mean ± SEM. For *RFRP* mRNA, values are expressed as a percentage of the LD value. ***, $P \leq 0.001$, one-way ANOVA. For RFRP immunoreactivity, *bars with different letters* differ significantly, one-way ANOVA. n = 4–6 per group.

an antiserum raised against hKp10 cross-reacts with both RFRP-1 and RFRP-3. We have shown that the detected immunoreactivity can be completely abolished after preincubation with RFRP-1 and -3, and the antiserum binds to both RFRP-1, RFRP-3 and mKp10 in dot-blots (unpublished data). This concords with the data published by Brailoiu *et al.* (46), who reported immunoreactive cells in the dorso-hypothalamic hypothalamus using the same anti-hKp10 antiserum. Furthermore, *Kiss1* mRNA has not been detected in the region of the dorsomedial hypothalamus where RFRP neurons are present but in the arcuate nucleus and in the anteroventral periventricular nucleus (24, 27, 28, 37). Based on these data, we can conclude that the immunoreactive neurons very likely contain RFRP-1/RFRP-3 and are accordingly termed RFRP immunoreactive.

So far, there has been limited information on the biological roles of RFRP peptides in mammals. Both peptides may possess feeding modulatory actions (30). In rat, RFRP-1 indirectly stimulates prolactin secretion (33, 59) and modulates nociceptive responses (38, 39). RFRP-3 increases GH secretion and food intake but, importantly, strongly inhibits LH (but not FSH) release and sex behavior (35, 36). Interestingly, the mammalian *RFRP* gene has been found to be orthologous to the *GnIH* gene in birds. This gene encodes for three putative peptides termed GnIH, GnIH-related peptide-1, and GnIH-related peptide-2, designated according to the inhibitory activity of the GnIH peptide on gonadotropin secretion (34, 53–55).

The negative effects on LH release of RFRP-3 in mammals (35, 36) and GnIH in birds (34, 53–55) has suggested that RFRP-3 and GnIH are functional homologs. But in contrast with birds where GnIH affects gonadotropin release directly at the pituitary level, RFRP-3 in mammals may modulate gonadotropin secretion indirectly, via GnRH neurons. Several studies in rodents (including the Syrian hamster) have reported few RFRP-immunoreactive fibers in the outer layer of the median eminence, whereas RFRP-immunoreactive fibers contact a large percentage of GnRH cells (35, 36). Clearly, further studies are needed to understand how RFRP-3 modulates LH release in mammals, in particular to determine whether it affects LH secretion at the pituitary level and/or via GnRH release. In the latter possibility, if RFRP peptides modulate the activity of GnRH neurons, one can speculate that it occurs via direct neuronal connection between RFRP and GnRH neurons, and/or indirectly via contacts to Kp neurons in the arcuate nucleus.

We observed that *RFRP* expression is reduced in SD hamsters, both at the mRNA and peptide level, and this result may appear surprising, for two reasons. First, if the major role of *RFRP* was to inhibit the gonadotropic axis, we would expect *RFRP* expression to increase in SD hamsters, so that the negative drive on reproduction is increased, in parallel to the decrease of the positive drive represented by Kp (24, 25). One can suggest that the role of *RFRP* in hamsters would be to fine-tune the activity of the hypothalamo-pituitary-gonadal axis. This may occur directly on GnRH neurons, and/or via *Kiss1* neurons or other intermediates. This possibility would be particularly relevant, given the extreme potency of Kp as secretagogue of GnRH release. Second, our observation is reversed compared with the results reported for GnIH in quails (see below) (60). Both Syrian and Siberian hamsters and quails are long-day breeders; however, the photoperiod controls reproduction differently in birds and hamsters. Finally, the down-regulation of *RFRP* expression in SD hamsters also raises a number of questions on the role of RFRP as a gonadotropin-inhibitory factor. The biological action of RFRP peptides may extend far beyond the modulation of reproductive activity, and RFRP-1 and RFRP-3 peptides may fulfill different biological roles (33, 35, 38, 39, 59). RFRP-immunoreactive nerve fibers are broadly distributed in the brain (35, 36, 41, 42), suggesting that RFRP peptides may control a large palette of biological functions. The re-

ceptor for RFRP peptides, termed RFRP-R (also found as FF1 or OT7T22), is a G protein-coupled receptor that is widely expressed in the forebrain (38). Importantly, because the RFRP and NPFF peptides (another member of the RFamide peptide family) share significant homology, they have been reported to interact with each other's receptor (38, 39, 54). Consequently, biological actions such as central anti-opioid effect initially attributed to NPFF may occur via RFRP receptor, and may thus reflect endogenous RFRP activity (38). If RFRP peptides possess true pain-modulatory actions, then interesting consequences exist in term of photoperiodic variations.

In addition, the regulation of RFRP mRNA level, RFRP propeptide, and its maturation as RFRP-1 and RFRP-3 should be carefully dissociated. Further experiments using antisera specific for either RFRP-1 or RFRP-3 (35, 41–44) should allow one to distinguish putative differential processing of the two peptides by melatonin. It was recently reported that photoperiod affects processing of the neuropeptide precursor proopiomelanocortin in the hypothalamus of Siberian hamsters by affecting the expression of prohormone convertases 1 and 2 (61). Accordingly, independent from *RFRP* mRNA levels, photoperiodic-dependent regulation of RFRP-1/3 processing may occur as well as differential processing of RFRP-1 and RFRP-3. In SD-R Syrian hamsters, we observed that *RFRP* expression was still reduced compared with LD hamsters, although it was higher than in SD animals. In contrast, the number of RFRP-immunoreactive cell bodies in SD-R hamsters was higher than in SD animals. This differential regulation between mRNA and peptides requires further investigations but suggests that although *RFRP* gene expression might continue to be partially inhibited by melatonin, translation and processing of RFRP peptides may become refractory independently.

The photoperiodic change for *RFRP* expression did not result from a difference in circadian expression profiles between LD and SD, because we did not detect significant 24-h expression rhythms in the Syrian hamster, *RFRP* mRNA level being constantly elevated and reduced in LD and SD animals, respectively. In contrast to hamsters, *RFRP* expression was not influenced by day length in the laboratory Wistar rats that do not display photoperiodic variation in testicular function. These results suggest that only in seasonal species is *RFRP* expression modified by photoperiod. One can therefore assume that further investigations of this differential regulation may help understanding the difference between photoperiodic and non-photoperiodic species.

Hamsters maintained in SD have fully regressed gonads and consequently reduced levels of circulating sex hormones. Given that sex steroids control the expression of a wide range of genes, we verified that the photoperiodic change of *RFRP* expression was not a secondary consequence of the reduction in circulating testosterone in SD animals. Castrated LD hamsters had high *RFRP* mRNA level that was comparable to that of intact animals, whereas *RFRP* expression remained low in SD hamsters implanted with testosterone.

In mammals, the changes in photoperiod are translated by variations in the melatonin daily profile, and in Syrian hamsters, suppression of melatonin through ablation of the pineal gland renders the animals blind to photoperiod, so that exposure to SD is no longer able to inhibit sexual activity. Accordingly, we observed that Pin-X hamsters failed to show a SD-induced decline in *RFRP* mRNA level relative to sham-operated control animals. Altogether, these data strongly suggest that melatonin mediates the photoperiodic regulation of *RFRP* expression in Syrian hamsters without direct influence from gonadal hormones. In addition, we performed melatonin injection experiments in LD Syrian hamsters. Animals injected daily for 60 d with melatonin during the late light phase displayed reduced *RFRP* expression, comparable to SD hamsters, confirming the results of the Pin-X experiment. A 7-d treatment was not sufficient for changing *RFRP* expression in LD hamsters, and a partially reduced mRNA level was noted after 21 d of treatment. Thus, the melatonin-dependent inhibition of *RFRP* expression does not appear to be a fast, early event in the physiological adaptation to short photoperiod. This contrasts with the type 2 deiodinase (*Dio2*) gene, which is expressed in the blood-brain barrier-free zone of the arcuate nucleus. Dramatic inhibition of *Dio2* expression occurs in SD Syrian hamsters, and 7 d (or less) of melatonin treatment is sufficient to suppress *Dio2* mRNA level in LD Syrian hamsters (11). Thus, regulation of *Dio2* and *RFRP* expression by melatonin presumably involves different mechanisms. Whereas *Dio2* may be very sensitive to changes in daily melatonin patterns, inhibition of *RFRP* expression presumably necessitates integrating recurrent long SD melatonin profiles.

In birds, the *GnIH* gene is expressed in the paraventricular hypothalamic nuclei (53, 62) and, interestingly, also displays seasonal-dependent variations (60, 62, 63). In sparrows, the GnIH-immunoreactive neuron area was reported to be greater at the onset of photorefractoriness to LD compared with photosensitive (SD) or photostimulated (LD) birds (63). In addition, *GnIH* expression and GnIH cell content both increase in quails under SD compared with LD photostimulated birds (60). Importantly, Ubuka *et al.* (60) demonstrated that this photoperiodic modulation of *GnIH* expression is fully dependent upon the melatonin released from the pineal gland and the eyes, and they showed that GnIH-immunoreactive neurons in the paraventricular hypothalamus coexpress the Mel1c melatonin receptor (63). This suggested that melatonin acts directly on *GnIH*-expressing neurons through Mel1c-mediated mechanisms to induce *GnIH* expression in SD. Similar to GnIH in birds, we demonstrate here that melatonin also controls *RFRP* expression in hamsters (but not in Wistar rats), although the direction of this regulation appears to be opposite, possibly due to several reasons (see above). Whether melatonin acts directly on *RFRP*-expressing cells of hamsters will necessitate further investigations. Interestingly, the location of these neurons strikingly corresponds to the site that in Syrian hamsters mediates the action of melatonin on reproduction (16–20). Not only have melatonin-binding sites been reported in the dorsomedial hypothalamus, but also bilateral lesion of this area results in the loss of gonadotropic response to melatonin (19, 20). Thus, it is likely that the photoperiodic change in *RFRP* expression is mediated by those melatonin receptors described in the same zone (18–20).

One can further speculate that in cells expressing *RFRP*,

additional genes could be regulated, either activated or inhibited, by melatonin, with a dynamic possibly different from that of *RFRP*. So far, the identification of the melatonin-responsive cells controlling reproduction has been remarkably challenging (4–6, 9). Our results now make it possible to spot a defined population of cells in the medial hypothalamus that respond to melatonin, thus opening new tracks for investigating the molecular bases of melatonin action in the brain.

Finally, *RFRP* expression may be modulated by factors other than melatonin. One study reported that RFRP-immunoreactive cells express estrogen receptor-α and exhibit c-Fos expression shortly after estradiol injections (36). In our experiments, however, we did not observe long-term effects of gonadal steroids on *RFRP* expression. Clearly, further investigations should be carried out to examine whether and how additional factors can influence *RFRP* expression.

In summary, we show that in both Syrian and Siberian hamsters, the *RFRP* gene is modulated by photoperiod, with low mRNA levels and fewer immunoreactive cell bodies in SD compared with LD animals. This photoperiodic variation in the Syrian hamster is independent from secondary changes in gonadal steroids. Rather, *RFRP* expression is inhibited by SD melatoninergic signals. In contrast, there was no evidence for photoperiodic modulation of *RFRP* in the Wistar rat, a strain of rat showing no photoperiodic variation in testicular function. These results show for the first time in mammals that *RFRP* is a gene regulated by photoperiod, via melatonin. Identification of such melatonin-responsive cells located in the dorsal and medial hypothalamus of hamsters opens exciting perspectives for understanding how melatonin controls seasonal processes at the molecular level.

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References

1. **Bronson FH, Heideman PD** 1994 Seasonal regulation of reproduction in mammals. In: Knobil E, Neill JD, eds. The physiology of reproduction. 2nd ed. New York: Raven Press; 541–583
2. **Morgan PJ, Ross AW, Mercer JG, Barrett P** 2003 Photoperiodic programming of body weight through the neuroendocrine hypothalamus. J Endocrinol 177: 27–34
3. **Dawson A, King VM, Bentley GE, Ball GF** 2001 Photoperiodic control of seasonality in birds. J Biol Rhythms 16:365–380
4. **Goldman BD** 2001 Mammalian photoperiodic system: formal properties and neuroendocrine mechanisms of photoperiodic time measurement. J Biol Rhythms 16:283–301
5. **Malpaux B, Migaud M, Tricoire H, Chemineau P** 2001 Biology of mammalian photoperiodism and the critical role of the pineal gland and melatonin. J Biol Rhythms 16:336–347
6. **Pevet P** 1988 The role of the pineal gland in the photoperiodic control of reproduction in different hamster species. Reprod Nutr Dev 28:443–458
7. **Simonneaux V, Ribelayga C** 2003 Generation of the melatonin endocrine message in mammals: a review of the complex regulation of melatonin synthesis by norepinephrine, peptides, and other pineal transmitters. Pharmacol Rev 55:325–395
8. **Pitrosky B, Pevet P** 1997 The photoperiodic response in Syrian hamsters depends upon a melatonin-driven rhythm of sensitivity to melatonin. Biol Signals 6:264–271
9. **Bartness TJ, Powers JB, Hastings MH, Bittman EL, Goldman BD** 1993 The timed infusion paradigm for melatonin delivery: what has it taught us about the melatonin signal, its reception, and the photoperiodic control of seasonal responses? J Pineal Res 15:161–190
10. **Johnston JD** 2004 Photoperiodic regulation of prolactin secretion: changes in intra-pituitary signalling and lactotroph heterogeneity. J Endocrinol 180:351–356
11. **Revel FG, Saboureau M, Pevet P, Mikkelsen JD, Simonneaux V** 2006 Melatonin regulates type 2 deiodinase gene expression in the Syrian hamster. Endocrinology 147:4680–4687
12. **Watanabe T, Yamamura T, Watanabe M, Yasuo S, Nakao N, Dawson A, Ebihara S, Yoshimura T** 2007 Hypothalamic expression of thyroid hormone-activating and -inactivating enzyme genes in relation to photorefractoriness in birds and mammals. Am J Physiol Regul Integr Comp Physiol 292:R568–R572
13. **Barrett P, Ross AW, Balik A, Littlewood PA, Mercer JG, Moar KM, Sallmen T, Kaslin J, Panula P, Schuhler S, Ebling FJ, Ubeaud C, Morgan PJ** 2005 Photoperiodic regulation of histamine H3 receptor and VGF messenger ribonucleic acid in the arcuate nucleus of the Siberian hamster. Endocrinology 146:1930–1939
14. **Ross AW, Bell LM, Littlewood PA, Mercer JG, Barrett P, Morgan PJ** 2005 Temporal changes in gene expression in the arcuate nucleus precede seasonal responses in adiposity and reproduction. Endocrinology 146:1940–1947
15. **Ross AW, Webster CA, Mercer JG, Moar KM, Ebling FJ, Schuhler S, Barrett P, Morgan PJ** 2004 Photoperiodic regulation of hypothalamic retinoid signaling: association of retinoid X receptor γ with body weight. Endocrinology 145:13–20
16. **Lewis D, Freeman DA, Dark J, Wynne-Edwards KE, Zucker I** 2002 Photoperiodic control of oestrous cycles in Syrian hamsters: mediation by the mediobasal hypothalamus. J Neuroendocrinol 14:294–299
17. **Bae HH, Mangels RA, Cho BS, Dark J, Yellon SM, Zucker I** 1999 Ventromedial hypothalamic mediation of photoperiodic gonadal responses in male Syrian hamsters. J Biol Rhythms 14:391–401
18. **Hastings MH, Walker AP, Roberts AC, Herbert J** 1988 Intra-hypothalamic melatonin blocks photoperiodic responsiveness in the male Syrian hamster. Neuroscience 24:987–991
19. **Maywood ES, Bittman EL, Hastings MH** 1996 Lesions of the melatonin- and androgen-responsive tissue of the dorsomedial nucleus of the hypothalamus block the gonadal response of male Syrian hamsters to programmed infusions of melatonin. Biol Reprod 54:470–477
20. **Maywood ES, Hastings MH** 1995 Lesions of the iodomelatonin-binding sites of the mediobasal hypothalamus spare the lactotropic, but block the gonadotropic response of male Syrian hamsters to short photoperiod and to melatonin. Endocrinology 136:144–153
21. **Lincoln GA, Maeda K** 1992 Effects of placing micro-implants of melatonin in the mediobasal hypothalamus and preoptic area on the secretion of prolactin and β-endorphin in rams. J Endocrinol 134:437–448
22. **Malpaux B, Daveau A, Maurice F, Gayrard V, Thiery JC** 1993 Short-day effects of melatonin on luteinizing hormone secretion in the ewe: evidence for central sites of action in the mediobasal hypothalamus. Biol Reprod 48:752–760
23. **Malpaux B, Daveau A, Maurice-Mandon F, Duarte G, Chemineau P** 1998 Evidence that melatonin acts in the premammillary hypothalamic area to control reproduction in the ewe: presence of binding sites and stimulation of luteinizing hormone secretion by in situ microimplant delivery. Endocrinology 139:1508–1516
24. **Revel FG, Saboureau M, Masson-Pevet M, Pevet P, Mikkelsen JD, Simonneaux V** 2006 Kisspeptin mediates the photoperiodic control of reproduction in hamsters. Curr Biol 16:1730–1735
25. **Revel FG, Ansel L, Klosen P, Saboureau M, Pevet P, Mikkelsen JD, Simonneaux V** 2007 Kisspeptin: A key link to seasonal breeding. Rev Endocr Metab Disord 8:57–65
26. **Navarro VM, Castellano JM, Garcia-Galiano D, Tena-Sempere M** 2007 Neuroendocrine factors in the initiation of puberty: the emergent role of kisspeptin. Rev Endocr Metab Disord 8:11–20
27. **Smith JT, Clarke IJ** 2007 Kisspeptin expression in the brain: catalyst for the initiation of puberty. Rev Endocr Metab Disord 8:1–9
28. **Smith JT, Clifton DK, Steiner RA** 2006 Regulation of the neuroendocrine reproductive axis by kisspeptin-GPR54 signaling. Reproduction 131:623–630
29. **Maeda K, Adachi S, Inoue K, Ohkura S, Tsukamura H** 2007 Metastin/kisspeptin and control of estrous cycle in rats. Rev Endocr Metab Disord 8:21–29
30. **Bechtold DA, Luckman SM** 2007 The role of RFamide peptides in feeding. J Endocrinol 192:3–15
31. **Dockray GJ** 2004 The expanding family of -RFamide peptides and their effects on feeding behaviour. Exp Physiol 89:229–235
32. **Fukusumi S, Fujii R, Hinuma S** 2006 Recent advances in mammalian RFamide

peptides: the discovery and functional analyses of PrRP, RFRPs and QRFP. Peptides 27:1073–1086
33. **Hinuma S, Shintani Y, Fukusumi S, Iijima N, Matsumoto Y, Hosoya M, Fujii R, Watanabe T, Kikuchi K, Terao Y, Yano T, Yamamoto T, Kawamata Y, Habata Y, Asada M, Kitada C, Kurokawa T, Onda H, Nishimura O, Tanaka M, Ibata Y, Fujino M** 2000 New neuropeptides containing carboxy-terminal RFamide and their receptor in mammals. Nat Cell Biol 2:703–708
34. **Tsutsui K, Saigoh E, Ukena K, Teranishi H, Fujisawa Y, Kikuchi M, Ishii S, Sharp PJ** 2000 A novel avian hypothalamic peptide inhibiting gonadotropin release. Biochem Biophys Res Commun 275:661–667
35. **Johnson MA, Tsutsui K, Fraley GS** 2007 Rat RFamide-related peptide-3 stimulates GH secretion, inhibits LH secretion, and has variable effects on sex behavior in the adult male rat. Horm Behav 51:171–180
36. **Kriegsfeld LJ, Mei DF, Bentley GE, Ubuka T, Mason AO, Inoue K, Ukena K, Tsutsui K, Silver R** 2006 Identification and characterization of a gonadotropin-inhibitory system in the brains of mammals. Proc Natl Acad Sci USA 103:2410–2415
37. **Gottsch ML, Cunningham MJ, Smith JT, Popa SM, Acohido BV, Crowley WF, Seminara S, Clifton DK, Steiner RA** 2004 A role for kisspeptins in the regulation of gonadotropin secretion in the mouse. Endocrinology 145:4073–4077
38. **Liu Q, Guan XM, Martin WJ, McDonald TP, Clements MK, Jiang Q, Zeng Z, Jacobson M, Williams Jr DL, Yu H, Bomford D, Figueroa D, Mallee J, Wang R, Evans J, Gould R, Austin CP** 2001 Identification and characterization of novel mammalian neuropeptide FF-like peptides that attenuate morphine-induced antinociception. J Biol Chem 276:36961–36969
39. **Pertovaara A, Ostergard M, Anko ML, Lehti-Koivunen S, Brandt A, Hong W, Korpi ER, Panula P** 2005 RFamide-related peptides signal through the neuropeptide FF receptor and regulate pain-related responses in the rat. Neuroscience 134:1023–1032
40. **Ukena K, Tsutsui K** 2001 Distribution of novel RFamide-related peptide-like immunoreactivity in the mouse central nervous system. Neurosci Lett 300: 153–156
41. **Yano T, Iijima N, Hinuma S, Tanaka M, Ibata Y** 2004 Developmental expression of RFamide-related peptides in the rat central nervous system. Brain Res Dev Brain Res 152:109–120
42. **Yano T, Iijima N, Kakihara K, Hinuma S, Tanaka M, Ibata Y** 2003 Localization and neuronal response of RFamide related peptides in the rat central nervous system. Brain Res 982:156–167
43. **Yoshida H, Habata Y, Hosoya M, Kawamata Y, Kitada C, Hinuma S** 2003 Molecular properties of endogenous RFamide-related peptide-3 and its interaction with receptors. Biochim Biophys Acta 1593:151–157
44. **Fukusumi S, Habata Y, Yoshida H, Iijima N, Kawamata Y, Hosoya M, Fujii R, Hinuma S, Kitada C, Shintani Y, Suenaga M, Onda H, Nishimura O, Tanaka M, Ibata Y, Fujino M** 2001 Characteristics and distribution of endogenous RFamide-related peptide-1. Biochim Biophys Acta 1540:221–232
45. **Greives TJ, Mason AO, Scotti MA, Levine J, Ketterson ED, Kriegsfeld LJ, Demas GE** 2006 Environmental control of kisspeptin: implications for seasonal reproduction. Endocrinology 148:1158–1166
46. **Brailoiu GC, Dun SL, Ohsawa M, Yin D, Yang J, Chang JK, Brailoiu E, Dun NJ** 2005 KiSS-1 expression and metastin-like immunoreactivity in the rat brain. J Comp Neurol 481:314–329
47. **Revel FG, Masson-Pevet M, Pevet P, Simonneaux V, Horvarth T, Mikkelsen JD,** Distribution of metastin (KiSS-1)-immunoreactive neurons in the male rat brain. Proc 35th Annual Meeting of the Society for Neuroscience, Washington, DC, 2005 (Abstract 763.3)
48. **Clarkson J, Herbison AE** 2006 Postnatal development of kisspeptin neurons in mouse hypothalamus: sexual dimorphism and projections to gonadotropin-releasing hormone neurons. Endocrinology 147:5817–5825
49. **Franceschini I, Lomet D, Cateau M, Delsol G, Tillet Y, Caraty A** 2006 Kisspeptin immunoreactive cells of the ovine preoptic area and arcuate nucleus co-express estrogen receptor α. Neurosci Lett 401:225–230
50. **Mikkelsen JD, Revel FG, Larsen L, Caraty A, Simonneaux V,** Distribution and function of kisspeptins and their receptor GPR54 in the rodent brain. Proc 36th Annual Meeting of the Society for Neuroscience, Atlanta, GA, 2006 (Abstract 658.13)
51. **Panula P, Kivipelto L, Nieminen O, Majane EA, Yang HY** 1987 Neuroanatomy of morphine-modulating peptides. Med Biol 65:127–135
52. **Vilim FS, Aarnisalo AA, Nieminen ML, Lintunen M, Karlstedt K, Kontinen VK, Kalso E, States B, Panula P, Ziff E** 1999 Gene for pain modulatory neuropeptide NPFF: induction in spinal cord by noxious stimuli. Mol Pharmacol 55:804–811
53. **Tsutsui K, Bentley GE, Ubuka T, Saigoh E, Yin H, Osugi T, Inoue K, Chowdhury VS, Ukena K, Ciccone N, Sharp PJ, Wingfield JC** 2007 The general and comparative biology of gonadotropin-inhibitory hormone (GnIH). Gen Comp Endocrinol 153:365–370
54. **Ukena K, Tsutsui K** 2005 A new member of the hypothalamic RF-amide peptide family, LPXRF-amide peptides: structure, localization, and function. Mass Spectrom Rev 24:469–486
55. **Satake H, Hisada M, Kawada T, Minakata H, Ukena K, Tsutsui K** 2001 Characterization of a cDNA encoding a novel avian hypothalamic neuropeptide exerting an inhibitory effect on gonadotropin release. Biochem J 354:379–385
56. **Mikkelsen JD, Woldbye D, Kragh J, Larsen PJ, Bolwig TG** 1994 Electroconvulsive shocks increase the expression of neuropeptide Y (NPY) mRNA in the piriform cortex and the dentate gyrus. Brain Res Mol Brain Res 23:317–322
57. **Mikkelsen JD, Hauser F, deLecea L, Sutcliffe JG, Kilduff TS, Calgari C, Pevet P, Simonneaux V** 2001 Hypocretin (orexin) in the rat pineal gland: a central transmitter with effects on noradrenaline-induced release of melatonin. Eur J Neurosci 14:419–425
58. **Parisot M, Tanvez A, Lacroix A, Vallet E, Beguin N, Leboucher G** 2005 Social competition and plasma testosterone profile in domesticated canaries: an experimental test of the challenge hypothesis. Horm Behav 48:225–232
59. **Samson WK, Keown C, Samson CK, Samson HW, Lane B, Baker JR, Taylor MM** 2003 Prolactin-releasing peptide and its homolog RFRP-1 act in hypothalamus but not in anterior pituitary gland to stimulate stress hormone secretion. Endocrine 20:59–66
60. **Ubuka T, Bentley GE, Ukena K, Wingfield JC, Tsutsui K** 2005 Melatonin induces the expression of gonadotropin-inhibitory hormone in the avian brain. Proc Natl Acad Sci USA 102:3052–3057
61. **Helwig M, Khorooshi RM, Tups A, Barrett P, Archer ZA, Exner C, Rozman J, Braulke LJ, Mercer JG, Klingenspor M** 2006 PC1/3 and PC2 gene expression and post-translational endoproteolytic pro-opiomelanocortin processing is regulated by photoperiod in the seasonal siberian hamster (*Phodopus sungorus*). J Neuroendocrinol 18:413–425
62. **Bentley GE, Kriegsfeld LJ, Osugi T, Ukena K, O'Brien S, Perfito N, Moore IT, Tsutsui K, Wingfield JC** 2006 Interactions of gonadotropin-releasing hormone (GnRH) and gonadotropin-inhibitory hormone (GnIH) in birds and mammals. J Exp Zoolog A Comp Exp Biol 305:807–814
63. **Bentley GE, Perfito N, Ukena K, Tsutsui K, Wingfield JC** 2003 Gonadotropin-inhibitory peptide in song sparrows (*Melospiza melodia*) in different reproductive conditions, and in house sparrows (*Passer domesticus*) relative to chicken-gonadotropin-releasing hormone. J Neuroendocrinol 15:794–802
64. **Morin LP, Wood RI** 2001 A stereotaxic atlas of the golden hamster brain. San Diego/London: Academic Press

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