RFamide-related peptide-3 (RFRP-3) suppresses sexual maturation in a eusocial mammal

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Neuroendocrine mechanisms underlying social inhibition of puberty are not well understood. Here, we use a model exhibiting the most profound case of pubertal suppression among mammals to explore a role for RFamide-related peptide-3 [RFRP-3; mammalian ortholog to gonadotropin-inhibitory hormone (GnIH)] in neuroendocrine control of reproductive development. Naked mole rats (NMRs) live in sizable colonies where breeding is monopolized by two to four dominant animals, and no other members exhibit signs of puberty throughout their lives unless they are removed from the colony. Because of its inhibitory action on the reproductive axis in other vertebrates, we investigated the role of RFRP-3 in social reproductive suppression in NMRs. We report that RFRP-3 immunofluorescence expression patterns and RFRP-3/GnRH cross-talk are largely conserved in the NMR brain, with the exception of the unique presence of RFRP-3 cell bodies in the arcuate nucleus (Arc). Immunofluorescence comparisons revealed that central expression of RFRP-3 is altered by reproductive status, with RFRP-3 immunoreactivity enhanced in the paraventricular nucleus, dorsomedial nucleus, and Arc of reproductively quiescent NMRs. We further observed that exogenous RFRP-3 suppresses gonadal steroidogenesis and mating behavior in NMRs given the opportunity to undergo puberty. Together, our findings establish a role for RFRP-3 in preserving reproductive immaturity, and challenge the view that stimulatory peptides are the ultimate gatekeepers of puberty.

eusocial | GnIH | naked mole rat | puberty | RFRP-3

The social environment profoundly impacts reproductive function, such that social status, stress, and mating opportunities alter puberty onset and fertility in social animals, including humans (1–3). Successful reproduction is contingent upon precise timing and integration of these stimuli, and transduction of environmental cues into neuroendocrine and endocrine signals along the hypothalamic–pituitary–gonadal (HPG) axis. In this system, hypothalamic gonadotropin-releasing hormone (GnRH) plays a major hierarchical role, functioning as the final integrator and chief output pathway for modulation of pituitary gonadotropins that drive the production of gonadal hormones. Hence, the GnRH neuronal population is the final target of diverse and contradictory modulatory signals (4), permitting activation of reproductive function when environmental and physiological conditions are optimal, and inhibition when they are not. Although knowledge of neuroendocrine pathways controlling the timing of reproduction has expanded rapidly in recent years, with stimulatory peptides (i.e., kisspeptins) widely acknowledged as gatekeepers of puberty onset (5–9), the exact mechanisms underlying the timing of sexual maturation and its modification by environmental cues remain obscure.

RFamide-related peptide-3 [RFRP-3; mammalian ortholog to gonadotropin-inhibitory hormone (GnIH)] has emerged as an important component of the pathway of environmentally mediated reproductive inhibition (10), yet no evidence has linked the RFRP-3 system to onset or delay of puberty. In all vertebrates studied, a subset of GnRH neurons express RFRP-3's high-affinity receptor, GPR147 (11, 12), and are apposed by RFRP-3 fibers originating in the dorsomedial nucleus of the hypothalamus (DMH) or paraventricular nucleus (PVN) (13–15). RFRP-3 suppresses gonadotropin secretion through central mechanisms (13, 14, 16–22), but may also act locally in the gonads to drive changes in gonadal functioning (23–25). Accordingly, infusion of RFRP-3 decreases sociosexual behavior in rats and hamsters (16, 26), and endogenous RFRP-3 fluctuates with breeding season across species. In particular, RFRP-3 is commonly elevated during the nonbreeding season and suppressed following exposure to stimulatory photoperiods (brushtail possum: ref. 27; sheep: refs. 28, 29; goats: ref. 30). Likewise, stressors and scarce food availability stimulate RFRP-3 release in mammals (31, 32), with effects of social cues (i.e., nesting site availability) on GnIH activity also demonstrated in birds (33).

Given its pronounced inhibitory role in regulation of reproduction, RFRP-3 represents an attractive candidate system for suppressing HPG function before puberty, yet the vast majority of research on this peptide has used sexually mature adults. What limited developmental research exists is heterogeneous. Whereas knockdown of production and receptors for RFRP-3 does not appear to alter pubertal development in peripubertal mice (34, 35), RFRP-3–aided inhibition of gonadotropin secretion has been observed in prepubertal animals (36), confirming bioactivity of this peptide at younger nonadult ages. These findings point to a potential role for RFRP-3 in maintaining the prepubertal reproductive axis in a quiescent state until environmental conditions are permissive for breeding. To resolve this gap in mechanistic understanding, we must move beyond examinations of seasonally

Significance

Naked mole rats exhibit the most profound case of socially induced infertility among mammals. They live in sizable colonies where breeding is monopolized by two to four dominant animals; all other colony members retain prepubertal gonadal quiescence until death or removal from the colony. Proximate mechanisms underlying this phenomenon are unknown but presumably involve integration of environmental cues by the brain into neuroendocrine signals that impact the gonadotropin-releasing hormone (GnRH) system. The role of RFamide-related peptide-3 (RFRP-3) in modulating the reproductive axis in vertebrates has been well documented, making it a prime candidate as a gatekeeper for puberty onset. Here, we present evidence for RFRP-3–mediated social delay of puberty and define a previously unidentified neuroendocrine component of social control of sexual maturation.

breeding adults, to nontraditional species, where variance in sexual maturation is especially visible and profound.

Naked mole rats (NMRs; *Heterocephalus glaber*) do not fit the traditional framework of mammalian sexual development and are characterized by the strictest social and reproductive hierarchy among mammals. Most individuals fail to achieve breeding status and remain in a juvenile-like prepubescent state for the duration of their lives, making NMRs a powerful model system for studying neuroendocrine regulation of pubertal timing and progression. In colonies of up to 300 individuals, breeding is restricted to one dominant female (the queen) and one to three males, with other colony members being socially subordinate and reproductively suppressed. Though pronounced alterations in neural and endocrine function accompany the change (37–47), male and female subordinates are capable of transitioning to breeding status following the death/removal of breeders or separation from the colony. Indeed, although colony-housed subordinates have low progesterone (P), testosterone (T), and luteinizing hormone (LH) concentrations compared with same-sex breeders (37, 38), they show elevated urinary P and T levels within 1 wk of separation from the colony (37, 40), with females developing a perforated vagina during that time (37). At longer intervals, sexually maturing individuals begin to exhibit mating behavior, as well as alterations in sociosexual neural circuits (41–44, 46). Contrary to expectations, NMRs do not show substantial variation in GnRH or kisspeptin expression according to sex or reproductive state, with differences in kisspeptin immunoreactivity confined to breeder and subordinate females (44). To clarify the mechanisms preserving gonadal quiescence in both male and female subordinates, we characterized RFRP-3 expression throughout the NMR brain and compared RFRP-3 expression among sexually mature and immature adults. To directly assess RFRP-3 regulation of puberty onset, we centrally infused RFRP-3 in reproductively quiescent adults that had been removed from their colony and given the opportunity to undergo sexual maturation.

Results

In study 1, we characterized RFRP-3 localization and distribution in reproductively suppressed subordinates, with the aim of isolating unique expression patterns, and areas of RFRP-3–GnRH cross-talk. In common with other rodent species (14), RFRP-immunoreactive (ir) cell bodies and fibers were present in the DMH. We also report the presence of RFRP-ir cell bodies in the arcuate nucleus (Arc). RFRP-ir cell bodies were absent from other regions, yet fibers were densely packed in the anteroventral periventricular nucleus, bed nucleus of the stria terminalis (BNST), medial preoptic area (MPOA), median eminence (ME), nucleus accumbens shell (NACs), posterior portion of the septum, PVN, Arc, and periaqueductal gray, with sparse fibers present in the olfactory tubercle and ventral portion of the diagonal band (DB). In line with GnRH characterization in this species (44), GnRH neurons in the DB and MPOA received close appositions of RFRP-3 neuronal fiber terminals (Fig. 1), suggesting direct GnRH modulation by RFRP-3.

In study 2, RFRP-ir in the NACs, PVN, DMH, and Arc was quantified to characterize differences among reproductively active
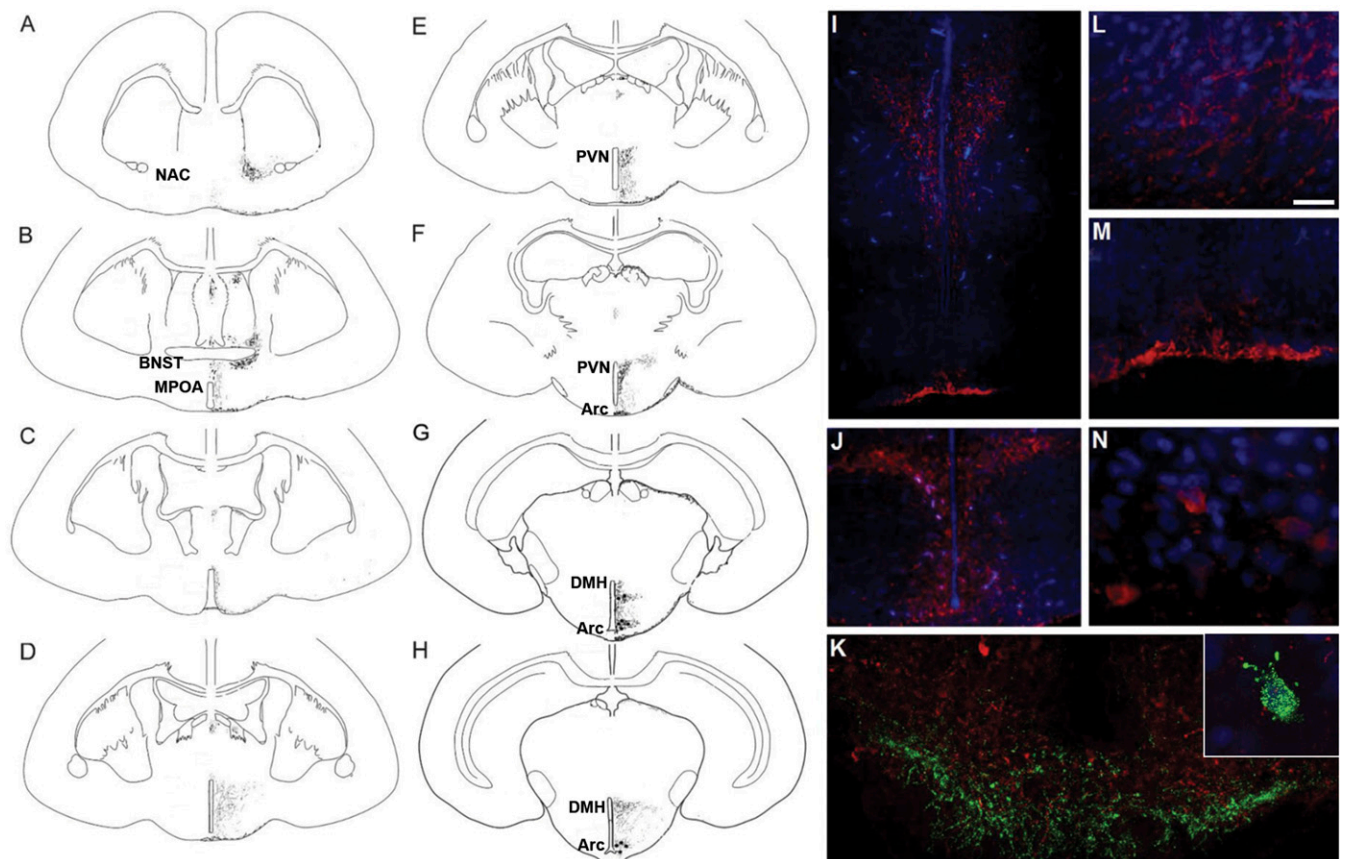

Fig. 1. RFRP-3 cell and fiber distribution in NMRs. (*A–H*) Tracing of the rostrocaudal extent of RFRP-3 fibers and cell bodies, which are clustered in the Arc and DMH and project throughout much of the NMR brain. (*I*) Low-power photomicrograph depicting RFRP-3 fibers (red) clustered in the PVN and OPT of a representative female subordinate NMR. (*L* and *M*) PVN (*L*) and OPT (*M*) projections at high power. (*J*) Low-power photomicrograph depicting dense RFRP-3 expression (red) in the DMH and Arc. (*N*) Arc cell bodies at high power. (*K*) RFRP-3 (red) and GnRH (green) coexpression in the NMR Arc and ME. (*Inset*) GnRH-ir cell body with RFRP-3 appositions in the DB. (*I–N*) Blue, DAPI. [Scale bar shown in *L*: 250 μm (*I* and *J*), 100 μm (*M*), 50 μm (*K* and *L*), and 25 μm (*N*).]
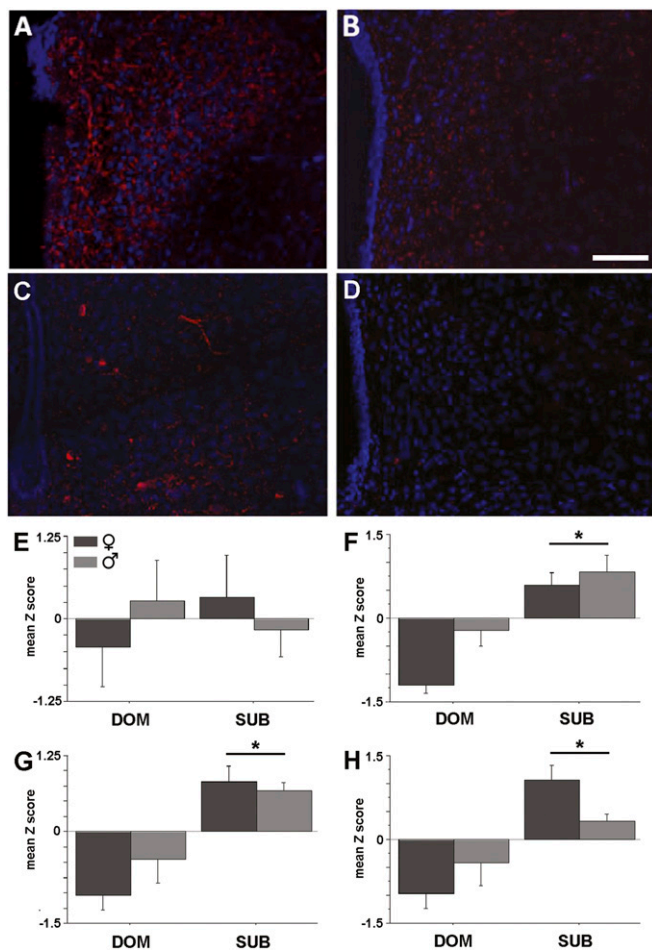
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Fig. 2. Neural RFRP-3 expression varies by social/reproductive status. (A–D) Representative photomicrographs of RFRP-3 immunoreactivity (red) in the PVN (A and B) and Arc (C and D) of subordinate (SUB; A and C) and dominant (DOM; B and D) NMR females (blue, DAPI). (E–H) Quantification of immunofluorescence in the NACs (E), PVN (F), DMH (G), and Arc (H). Light gray bars represent males and dark gray bars represent females. Values are given as normalized mean ± SEM (n = 3 per group). (Scale bar shown in B: 50 μm.) *P < 0.05 versus DOM group.

(dominant, DOM) and suppressed (SUB) colony members. As shown in Fig. 2, status × sex ANOVAs revealed a significant status effect in the PVN ($F_{1,8} = 35.62$, $P < 0.001$), DMH ($F_{1,8} = 31.18$, $P < 0.001$), and Arc ($F_{1,8} = 23.024$, $P = 0.001$), with SUBs exhibiting greater RFRP-ir than DOM subjects. A significant sex effect favoring males was observed in the PVN ($F_{1,8} = 6.44$, $P = 0.035$), though neither sex nor interaction effects were detected in other regions (all Ps > 0.05).

In study 3, we examined intracerebroventricular (ICV) RFRP-3 effects on HPG activation following release from reproductive suppression, as indicated by plasma P and T concentrations 4 wk after removal from colony. A group × sex ANOVA revealed a significant effect of group on P ($F_{2,14} = 6.999$, $P = 0.008$), wherein heightened P was exhibited by SAL ($P = 0.001$), but not RFRP-3 animals ($P > 0.05$), relative to SUBs (Fig. 3). Sex and interaction effects on P failed to reach significance. No significant effects on T were detected across groups (all Ps > 0.05). We then examined sexual interest following release from reproductive suppression, as measured by opposite-sex genital investigation 4 wk after removal from colony. An effect of RFRP-3 treatment was detected (two-way ANOVA, effect of group, $F_{1,34} = 7.51$, $P = 0.01$), with SAL animals exhibiting more genital investigation of an unfamiliar,

opposite-sex conspecific than RFRP-3 animals (Fig. 3). This change was specific to sexual interest, as RFRP-3 treatment had no significant effect on antagonistic or nonsexual social behavior (all Ps > 0.05). Overall, females exhibited more sexual interest than males (effect of sex, $F_{1,34} = 4.97$, $P = 0.033$). No other sex or interaction effects were detected on any behavior (all Ps > 0.05). Finally, olfactory preference was also assessed after 4 wk. A three-way repeated-measures ANOVA revealed a significant main effect of bedding ($F_{2,68} = 12.301$, $P < 0.001$), with home-cage bedding preferred over bedding from natal/nonnatal colonies (both Ps < 0.05). No treatment, sex, or interaction effects were observed (all Ps > 0.05), demonstrating that RFRP-3 treatment did not alter olfactory discrimination (Fig. 3).

Discussion

We demonstrate here that RFRP-3 signaling is central to puberty delay in a continuous breeder exhibiting the most profound case of social reproductive suppression among mammals. Beyond clarifying that RFRP-3 expression fluctuates with reproductive status, with RFRP-ir enhanced in the PVN, DMH, and Arc of sexually immature adults, we show that exogenous RFRP-3 is effective at suppressing endocrine and behavioral puberty in NMRs given the opportunity to undergo sexual maturation. Specifically, in NMRs removed from colony and infused with central RFRP-3, inhibition of gonadal steroidogenesis (i.e., P production) is evident at 4 wk postcolony removal. Furthermore, RFRP-3 suppresses mating interest as indexed by opposite-sex genital investigation. Although RFRP-3 localization and areas of RFRP-3/GnRH cross-talk are largely conserved in this species, our findings diverge from reports on RFRP-3 and pubertal timing in conventional laboratory rodents,
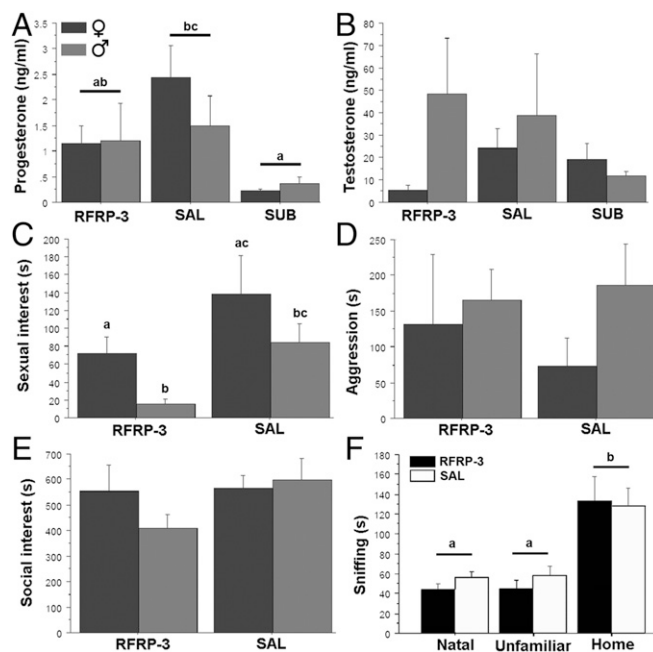

Fig. 3. RFRP-3 treatment suppresses hormonal and behavioral reproductive activation. (A and B) Gonadal hormones (nanograms per milliliter) in colony-housed subordinates (SUB), and SUBs separated from colony for 4 wk and treated with either saline (SAL) or RFRP-3. (C and E) Duration of (C) sexual interest, (D) antagonistic behavior, and (E) social interest in RFRP-3 and SAL-treated SUBs exposed to an unfamiliar opposite-sex adult following 4 wk of colony separation. (F) Duration of sniffing in olfactory preference paradigm. (C–E) Light gray bars represent males and dark gray bars represent females; in F, white bars represent SAL and black bars represent RFRP-3 animals. Values are given as mean ± SEM (n = 3–4 per group for A and B; n = 9–10 per group for C–F); groups labeled without the same letter are significantly different from each other.

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suggesting that unique areas of expression (i.e., Arc) might facilitate RFRP-3 regulation of puberty onset.

In common with other mammals (14), RFRP-3 cell bodies and fibers are distributed throughout the tel-, di-, and mesencephalon of the adult NMR brain. Dense fiber networks were present in regions known to regulate sexual motivation (NACs), copulatory behavior (BNST and PVN), and gonadotropin release (DMH and Arc), which is consistent with expression differences between reproductively activated versus quiescent individuals (study 2), and RFRP-3–induced alterations in sexual interest (but not agonistic or general investigative behavior; study 3). It is worthy of note that, although RFRP-3 manipulation altered one-on-one social processing as reflected by the genital investigation effect, it does not appear to influence olfactory discrimination or social processing overall as reflected by preference for colony-level odors. Moreover, locomotor activity was preserved in RFRP-3–infused NMRs, suggesting observed reductions in sexual behavior were not due to general debilitation or somnolence. Effects on reproductive function were further corroborated by clear colocalization of RFRP-3 fibers and GnRH cell bodies in the DB and MPOA. The greatest extent of RFRP-3/GnRH fiber colocalization was observed in the ME, where dense GnRH fiber networks were present in the external layer. Although heavy RFRP-ir was seen in the internal layer, projections to the external zone were scarce, suggesting limited hypophysiotrophism as in other mammalian species (14, 16, 27, 48–51). Rather than acting on both the pituitary and GnRH neurons as its avian ortholog does (11, 13, 52, 53), it is likely that RFRP-3 acts directly only on GnRH neurons to inhibit NMR reproductive function.

Whereas the pattern of RFRP-3 expression did not differ among sexually active and quiescent NMRs, reproductive activation was associated with decreased label intensity in the PVN and cell body regions such as the Arc and DMH. Given the suppressive effect of centrally delivered RFRP-3 on reproductive behavior and endocrinology (study 3), it is likely that greater RFRP-ir in reproductively quiescent colony members corresponds to increased RFRP-3 synthesis and release. However, we acknowledge that elevated RFRP-ir within a cell may reflect either increased production and release, or decreased release due to somatic buildup. We also note that, though no significant relationship between sex and RFRP-ir was detected, visual inspection of the graphs (Fig. 2) suggests females are driving status effects in some regions, most notably the Arc. Similarly, RFRP-3 administration appears to decrease T in females but not males (Fig. 3), though these effects do not reach statistical significance. These observations are consistent with the more complete gonadal suppression exhibited by non-reproductive female NMRs compared with males (37, 38, 54), and with a growing body of research indicating that status, rather than sex, has a predominant role in determining neural organization in this species (41–43). Further investigating how mechanisms of reproductive suppression might differ between males and females is an important area of future research.

Our results mirror changes in inhibitory tone in other mammals, where RFRP-3 neurons are confined to the DMH, and stress and breeding season-related decreases in cell numbers have been described (27–31). In these and avian species, the DMH is both the sole site of RFRP-3 synthesis and a chief regulator of neuroendocrine, autonomic, and behavioral responses to stress (for review, see ref. 55), including stress-related reproductive dysfunction and infertility. Indeed, a subset of RFRP-3 cells in the rat DMH express receptors for glucocorticoids, and stress has been shown to upregulate RFRP while down-regulating gonadotropin release (31, 56). In line with stimulation of the RFRP system, stress has been linked to reduced proceptive sexual behavior (57), and impregnation rates in conventional laboratory rodents (58), along with increased embryo resorption (56). This signaling pathway may extend to NMRs, with stress-induced up-regulation of RFRP-3 preserving HPG quiescence in subordinates, yet previous examinations of

stress and social rank in this species have yielded inconsistent results. Though reproductively active NMRs are socially dominant, and express more aggression than subordinates (59), most reports have revealed no difference in circulating glucocorticoids between dominants and subordinates (59–61). Future work will clarify whether the subordinate bias in hypothalamic RFRP-3 can be attributed to status-dependent differences in stress.

Beyond reducing HPG activation in the face of stressful encounters, RFRP-3 may function to combat the stress of antagonism in NMR subordinates. In particular, RFRP-3 innervation of the PVN has been shown to trigger oxytocin (OT) release in other rodents (62) and may contribute to status differences in local OT production. Consistent with present RFRP-3 expression patterns (study 2), more OT-producing cells are seen in the PVN of subordinates than NMR breeders, and this difference dissipates following colony removal and reproductive activation (43). Among subordinate colony members, RFRP-3–induced OT secretion may not only produce anxiolytic effects, but also preserve prosocial tendencies, including alloparental pup care and colony defense (63, 64).

RFRP-3's impact on behavioral and endocrine markers of NMR puberty challenges current assumptions about RFRP-3's developmental relevance. Although our data appear to contradict reports that RFRP-3's involvement in puberty regulation is dispensable, and subordinate to that of kisspeptin, a role for kisspeptin in the current results cannot be ruled out entirely. In tandem with an unprecedented level of RFRP-3 control over pubertal activation, we also document the presence of RFRP-3–producing neurons in the Arc, a chief site of kisspeptin synthesis in other mammalian species (65). In contrast to the small proportion of Kiss1/GPR147 coexpressing cells in other areas of the rodent brain, 25% of kisspeptin somata in the mouse Arc express GPR147 or GPR74, whereas 35% receive contact from RFRP fibers (66). Further, RFRP KO mice show higher numbers of Kiss1 positive neurons and increased total Kiss1 gene expression, specifically in the Arc (35). Hence, although kisspeptin neurons do not appear to signal to RFRP neurons (66), RFRP-3 may directly modulate a subset of hypothalamic kisspeptin cells in conventional laboratory rodents. Should this pathway extend to NMRs, in whom Arc synthesis of kisspeptin is conserved, but Kiss1 cell numbers do not vary with reproductive state in both sexes (44), it may be that RFRP suppresses HPG function via downstream kisspeptin-dependent mechanisms. Specifically, RFRP signaling on kisspeptin neurons of the Arc may restrain kisspeptin activation, thereby contributing to central suppression of the GnRH system via indirect mechanisms.

Collectively, results implicate RFRP-3 in preserving reproductive quiescence in adult subordinates until environmental conditions are permissive for breeding. Our data bolster reports that the RFRP system is active developmentally and so challenge conclusions of congenital knockdown studies. Indeed, although disruption of RFRP-3 signaling fails to alter puberty onset in mice (34, 35), it is presumptuous to rule out a role for RFRP-3 in reproductive development entirely as knockdown triggers a degree of prepubertal hyperactivity (35). Cross-species comparisons will determine whether exceptional features of the reproductive system underlie RFRP-3–mediated puberty delay in NMR subordinates and other hypogonadotrophic adults (e.g., subordinate marmosets), and so advance research on neural correlates of environmentally induced HPG dysfunction, which has thus far relied heavily on seasonally breeding species. This research is crucial, not only because a possible developmental role for RFRP-3 has been neglected, but because the human reproductive axis is profoundly sensitive to environmental influence, with puberty delay (1), lowered sperm motility (2), and infertility frequently attributed to social factors (3). These findings provide important insight into the mechanisms preserving mammalian reproductive quiescence before puberty and underscore the value of nontraditional animal models to advancing reproductive science and human and mammalian health.

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Materials and Methods

Animals and Housing. NMR colonies were maintained at the University of Toronto Mississauga in polycarbonate cages connected by acrylic tubing. Animals were fed ad libitum on sweet potato and wet 19% protein mash (Harlan Laboratories) and housed under temperature- and humidity-controlled conditions (28–30 °C/50% relative humidity) on a 12:12 h light/dark (LD) cycle. As NMRs achieve adult body size within 1 y and do not show signs of aging until well into their third decade (67, 68), experimental animals were young-aged adults (13–65 mo of age; 34–58 g). For characterization, distribution, and quantification of RFRP-3 (studies 1 and 2), a total of 12 colony-housed animals were used: (*i*) nonreproductive subordinates (SUB; *n* = 6, 3 males, 3 females), and (*ii*) verified breeders (DOM; *n* = 6, 3 males, 3 females). For ICV manipulation of RFRP-3 (study 3), a total of 38 nonreproductive subordinates were used: subordinates isolated from their natal colonies and treated with (*i*) saline (SAL; *n* = 19, 10 males, 9 females), or (*ii*) RFRP-3 (*n* = 19, 10 males, 9 females). Following random treatment assignment, animals were transferred to individual polycarbonate tubs (46 cm length × 24 cm width), implanted with ICV minipumps, and maintained out-of-colony for 4 wk. A subset of ICV animals (*n* = 20, 3 RFRP-3 males, 4 RFRP-3 females, 3 SAL males, 3 SAL females), and subordinates housed in colony (SUB; *n* = 7, 4 males, 3 females) were used for hormone analysis. All animal care and use procedures were approved by the University of Toronto Animal Care Committee and conducted in accordance with Canadian federal standards and guidelines.

Histological Processing. Animals were overdosed with Avertin (2,2,2-tribromoethanol, 40 mg/100 g; Sigma-Aldrich), and rapidly decapitated during the light phase of their LD cycle. Brains were extracted and immersion fixed in 4% (wt/vol) paraformaldehyde (4 h) before transfer to 20% (wt/vol) sucrose in phosphate buffer (>24 h). Brains were then frozen sectioned into four series of 30-μm coronal sections using a sliding microtome and stored in cryoprotectant at −20 °C. For characterization and distribution analysis, double immunofluorescence labeling for RFRP-3/GnRH was performed on a one-in-four series. In brief, free-floating sections were washed 9 × 5 min with 0.1 M PBS and incubated in sodium citrate buffer (30 min at 80 °C). Tissue was quenched with 3% (vol/vol) H_2O_2 in PBS [pH = 7.4, 30 min at room temperature (RT)], then blocked in PBS+ [4% (vol/vol) donkey serum/0.5% Triton X-100/PBS; 18 h at 4 °C], and transferred to primary antibody solution [1:250 rabbit anti-RFRP (PAC 123/124, provided by George Bentley, University of California, Berkeley, CA)/1:2,500 monoclonal mouse anti-GnRH (Hu11B; Millipore)/0.25% Triton X-100 in PBS; 48 h at 4 °C]. Following incubation in secondary antibody solution [1:250 Alexa Fluor 568/1:200 Alexa Fluor 488 (Molecular Probes)/4% (vol/vol) donkey serum in PBS; 2 h at RT], sections were treated with Sudan black to reduce autofluorescence (0.3% Sudan black in 70% EtOH; 5 min at RT), and mounted onto gel-subbed slides using Prolong Antifade Mounting Medium with DAPI (Molecular Probes). Five × 5 min PBS washes were performed between steps. For quantitative comparisons, single immunofluorescence labeling for RFRP-3 was performed as above, excluding anti-GnRH, on two-in-four series. PAC 123/124's specificity for RFRP-3 has been demonstrated previously (9). To confirm the specificity of RFRP antiserum in NMR tissue, we used a preadsorption control. As done for this antibody in sheep (29) we preincubated antiserum (diluted 1:250) with quail GnIH peptide (1 μg/mL; Phoenix Pharmaceuticals) overnight and processed the tissue as above (Fig. S1).

Digital Microscopy and Histological Quantification. Images were acquired for characterization/distribution analysis using an Olympus BX51 fluorescence microscope, whereas a Zeiss Axio Observer Z1 inverted microscope equipped with a Laser Scanning Microscope 700 module and Zeiss LSM510 laser scanning confocal microscope (Carl Zeiss MicroImaging) was used for quantification. Confocal microscope images were obtained with a 20× objective (Plan-Apochromat 20×/0.8 M27 and EC Plan-Neofluar 20×/0.5) and a Z stack was acquired with 0.73-μm optical sections and seven optical slices. To allow comparisons between samples, confocal parameters (i.e., laser intensity, gain, pinhole size, scanning speed, and image averaging) were held constant on Z stacks of like magnification. Confocal microscope images were processed and analyzed with ImageJ (WS Rasband, ImageJ, NIH). Each Z stack was separated into individual acquisition channels

(568 nm or 488 nm for RFRP-3 and GnRH, respectively), restacked, and a maximum intensity projection of the entire Z stack was produced. For each section, fluorescence intensity values were obtained by measuring the grayscale intensity in a fixed rectangular region of interest (150 μm width × 200 μm height). Two to three sections were chosen per animal to calculate mean fluorescence intensity in the following fiber-rich brain regions: NACs, PVN, DMH, and Arc.

Surgeries. ICV cannulations were performed as described (69). Infusion samples of normal saline (0.9%) or RFRP-3 (H-5846, Bachem), were delivered over 4 wk using an automated infusion pump (model 2004 with brain infusion kit 3, Alzet). RFRP-3–treated animals received 200 ng/μL RFRP-3 released at 0.25 μL/h, resulting in 1,200 ng/d, for a total of 4 wk. Treatment duration corresponds with previous reports on the time course of gonadal activation following colony removal (46). Infusion volume and flow rate did not differ among SAL and RFRP-3 animals. All ICV animals were killed 4 wk after cannulation surgery. Brains were extracted as above and accurate cannula placement was confirmed for all animals.

Behavior Testing and Scoring. All testing was conducted on ICV animals 24 h before tissue collection. Behaviors were scored blind using Observer XT 10 (Noldus Information Technology), and total behavior durations were compared between RFRP-3 and SAL groups. For olfactory preference, animals were transferred to a fresh polycarbonate tub (48 cm × 27 cm × 16 cm) containing clean bedding and three ceramic bowls. Bowls were placed equidistant apart in random order and contained soiled bedding from the subject's (*i*) postsurgical individual home cage, (*ii*) natal colony, or (*iii*) an unfamiliar colony. Stimulus bedding was obtained from cages that had not been changed for at least 24 h. Total time spent sniffing each odorant across 10 min was tabulated. Next, animals were transferred to a fresh polycarbonate tub (48 cm × 27 cm × 16 cm) with clean bedding for 30 min and introduced to an opposite-sex subordinate adult from an unfamiliar colony. Social behaviors were scored according to a mutually exclusive coding scheme and characterized as indicating: (*i*) antagonism (i.e., shoving, batting, open-mouth gaping, muzzle/incisor fencing), (*ii*) social interest (i.e., face/flank sniffing), or (*iii*) sexual interest (i.e., genital investigation via sniffing/nuzzling).

Serum Collection and Hormone Assays. Trunk blood was collected at time of killing, and serum was isolated and stored at −20 °C. Circulating T and P were detected by ELISA at an absorbance of 405 nm, as per manufacturer's instructions. All samples were run in duplicate with a Synergy-HT-Bio-Tek 96-well microplate reader and measured as described (46, 47).

Statistical Analysis. For immunoquantitative comparisons, a two-way status (SUB, DOM) × sex ANOVA was conducted on average regional RFRP-ir in the NACs, PVN, DMH, and Arc. To control for immunofluorescence batch effects, analyses were performed on Z scores that were tabulated using batch-specific variance. Experimental groups were yoked across batches. Behavior scores for total duration of antagonism, social interest, and sexual interest displayed during opposite-sex pairing were examined separately using two-way group (RFRP-3, SAL) by sex ANOVAs. Olfactory preference values were analyzed using a three-way repeated-measures ANOVA with group (RFRP-3, SAL) and sex as between-subjects variables and odorant (home cage, natal colony, unfamiliar colony) as the within-subjects factor. Plasma concentrations of T and P were examined separately using two-way group (SUB, RFRP-3, SAL) by sex ANOVAs. Post hoc comparisons used Tukey's HSD. All analyses were conducted using SPSS (version 21), with α set at 0.05.

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