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.

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.

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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 reproducitively 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...
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, 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**

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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 hypothalamic distribution in other mammalian species (15–17, 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 nonreproductive 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 up-regulate RFRP while down-regulating gonadotropin release (31, 56). In line with stimulation of the RFRP system, stress has been linked to reduced preoptic 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 reproducitively 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 allogential 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 hypogonadotropic 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.
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 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, 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 decapitated with Avertin (2,2,2-trichloroethanol, 40% [vol/vol], Sigma-Aldrich) and rapidly decapitated during the light phase of their LD cycle. Brains were extracted and immersion fixed in 4% (vol/vol) paraformaldehyde (4 h) before transfer to 20% (vol/vol) sucrose in PBS (pH 7.4, 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% (w/vol) H2O2 in PBS (pH 7.4, 30 min at room temperature (RT)), then blocked in PBS+ (4% w/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) and 1/250 donkey anti-GnRH (mAb 1B1; Millipore). 0.25% Triton X-100 in PBS; 48 h at 4 °C. Following incubation in secondary antibody solution [1/125 Alexa Fluor 568/1:200 Alexa Fluor 488 (Molecular Probes)/4% (w/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. PAR-123/124’s specificity for RFRP-3 has been demonstrated previously (9). To confirm the specificity of RFRP antisem in NMR tissue, we used a preadsorption control. As done for this antibody in sheep (29) we preincubated antisem (diluted 1:250) with guil GnRH peptide (1 μg/mL; Phoenix Pharmaceuticals) overnight and processed the tissue as above (Fig. 5).

Digital Microscopy and Histological Quantification. Images were acquired for characterization/distribution analysis using an Olympus BUX5 fluorescence microscope, whereas a Zeiss AxiosObserver 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×/0.8) 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, NIH). Each Z stack was separated into individual acquisition channels.

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Fig. S1. Preadsorption test of RFRP-3 immunoreactivity in a representative female subordinate. Immunolabeling of RFRP-3 with (A and C) and without antiserum preadsorption (B and D). Prominent labeling in the Arc (B) and PVN (D) contrasts with reduced labeling following peptide preadsorption (A and C, respectively). No RFRP-3 immunoreactive cell bodies were visible in the Arc following preadsorption control. All tissue was from the same female subordinate. (Scale bar shown in B, 50 μm.)