Received: 21 September 2021 | Revised: 17 November 2021 | Accepted: 7 December 2021

DOI: 10.1002/cne.25291

RESEARCH ARTICLE

JCN RESEARCH IN SYSTEMS NEUROSCIENCE THE JOURNAL OF COMPARATIVE NEUROLOGY WILEY

Immunoreactive distribution of gonadotropin-inhibitory hormone precursor, RFRP, in a temperate bat species (*Eptesicus fuscus*)

Mattina M. Alonge[1] | **Lucas J. S. Greville**[2] | **Paul A. Faure**[2] | **George E. Bentley**[1]

[1] Department of Integrative Biology and Helen Wills Neuroscience Institute, University of California Berkeley, Berkeley, California, USA

[2] Department of Psychology, Neuroscience & Behavior, McMaster University, Hamilton, Ontario, Canada

Correspondence
Mattina M. Alonge, Department of **Integrative** Biology, University of California, Berkeley, 16 Barker Hall Mailcode #3204, Berkeley, CA 94720, USA
Email: mattina.alonge@berkeley.edu

Funding information
Natural Sciences and Engineering Research Council of Canada (NSERC); Discovery Grant RGPIN-2020-06906 awarded to P.A.F.; Post-Graduate Scholarship–Doctoral NSERC award to L.S.J.G. and NSF IOS 1856750 to G.E.B.

Abstract

Gonadotropin-inhibitory hormone (GnIH, also known RFRP-3 in mammals) is an important regulator of the hypothalamic–pituitary–gonadal axis and downstream reproductive physiology. Substantial species differences exist in the localization of cell bodies producing RFRP-3 and patterns of fiber immunoreactivity in the brain, raising the question of functional differences. Many temperate bat species exhibit unusual annual reproductive patterns. Male bats upregulate spermatogenesis in late spring which is asynchronous with periods of mating in the fall, while females have the physiological capacity to delay their reproductive investment over winter via sperm storage or delayed ovulation/fertilization. Neuroendocrine mechanisms regulating reproductive timing in male and female bats are not well-studied. We provide the first description of RFRP—precursor peptide of GnIH —expression and localization in the brain of any bat using a widespread temperate species (*Eptesicus fuscus*, big brown bat) as a model. RFRP mRNA expression was detected in the hypothalamus, testes, and ovaries of big brown bats. Cellular RFRP-immunoreactivity was observed within the periventricular nuclei, dorsomedial nucleus of the hypothalamus, arcuate nucleus (Arc), and median eminence (ME). As in other vertebrates, RFRP fiber immunoreactivity was widespread, with the greatest density observed in the hypothalamus, preoptic area, Arc, ME, midbrain, and thalamic nuclei. Putative interactions between RFRP-ir fibers and gonadotropin-releasing hormone (GnRH) cell bodies were observed in 16% of GnRH-immunoreactive cells, suggesting direct regulation of GnRH via RFRP signaling. This characterization of RFRP distribution contributes to a deeper understanding of bat neuroendocrinology, which serves as foundation for manipulative approaches examining changes in reproductive neuropeptide signaling in response to environmental and physiological challenges within, and among, bat species.

KEYWORDS
bats, brain, Chiroptera, hormones, hypothalamic–pituitary–gonadal, neuroendocrinology, reproduction

1 | INTRODUCTION

Many bat species breed seasonally when reproductive timing is correlated with optimal resource supply and environmental conditions (Racey, 1982). Temperate bat species, however, often exhibit annual reproductive life history patterns that—while not uncommon in reptiles, amphibians, and fish—are atypical for mammals. Hibernation is a common overwintering strategy among small mammals, but the timing of reproductive investment surrounding this period is unique in bats. Unlike many hibernating rodents, in which both males and females are reproductively quiescent in the fall and then upregulate reproduction in the spring, temperate-zone bats mate in the autumn prior to overwintering. This results in differing temporal periods of active reproductive investment between males and females. Often, male bats exhibit peaks of spermatogenesis in early summer and then engage in mating behavior in the fall. Once fall mating has occurred, female bats delay major reproductive investment (gestation/lactation) over winter, and they have the capacity to store sperm, delay ovulation, or postpone fertilization depending on the species (Crichton, 2000; Gustafson, 1979; Oxberry, 1979; E. G. Richardson, 1977; Willis, 2017). While there is a body of literature highlighting the phenology of these events and some important factors (i.e., temperature and food availability) that may serve as cues for reproduction (Racey, 1973; Racey & Swift, 1981), study of the neuroendocrine mechanisms involved in regulating reproduction in bats has not progressed significantly since the 1980s and early 1990s. Intraspecific variation in reproductive phenology and physiological capabilities (e.g., delayed ovulation, delayed embryonic development), as well as diversity of the environments inhabited, invites questions surrounding the neuroendocrine control of bat reproduction.

Like most other vertebrates, bats have a classic hypothalamo–pituitary–gonad (HPG) axis that is regulated by gonadotropin-releasing hormone (GnRH). GnRH induces release of luteinizing hormone and follicle-stimulating hormone from the anterior pituitary gland, and these gonadotropins regulate downstream gonadal activity with, presumably, associated steroid feedback. Seasonal variation in pituitary gonadotropes was described in greater mouse-eared bats (*Myotis myotis*) as early as 1956 (Herlant, 1956) and later expanded to species including the California leaf-nosed bat (*Macrotus californicus*) (B. A. Richardson, 1980, 1981) and little brown bat (*Myotis lucifugus*) (Anthony & Gustafson, 1984). Female bats show greater change in pituitary luteinizing hormone immunoreactivity across the annual cycle, with significant decreases during pregnancy and lactation as well as significant gonadotrope hypertrophy during hibernation. King et al. (1984) first characterized the distribution of GnRH-producing cell bodies in the Chiropteran brain, demonstrating a similar distribution in *M. lucifugus* as in primates but differing from known cellular immunoreactivity in rodents (Douglas, 1976). Localization of GnRH cells was later characterized in big brown bats (*Eptesicus fuscus*) with immunoreactivity in the periventricular nuclei (PVN), arcuate (Arc), and dorsomedial nucleus of the hypothalamus (DMH) as well as in the preoptic area (POA), median eminence (ME), and olfactory region (Oelschlager & Northcutt, 1992). GnRH-immunoreactive (GnRH-ir) fibers were predominantly located in the hypothalamus, infundibular stalk, POA, suprachiasmatic nuclei (SCN), and bed nucleus of the stria terminalis. Cells immunoreactive for GnRH were also found within the habenular nuclei of the epithalamus, suggesting a role for this brain area in processing environmental stimuli within the context of bat reproduction (Oelschlager & Northcutt, 1992). Studies exploring the temporal patterns of GnRH expression in bats reveal that GnRH-ir cell bodies and projection of GnRH-ir fibers decrease in quantity in postovulatory animals, suggesting that this neuropeptide can play a key role in regulating ovulation, as observed in other taxa (Anthony, 2000; Anthony et al., 1989). GnRH immunoreactivity also shows seasonal fluctuations, where fewer GnRH-ir cells are present in the summer than during the winter hibernation period, with greater seasonal variation within the hypothalamus than the POA; a pattern consistent across both sexes (Kawamoto et al., 1998). In 1998, the coexistence of FMRFamide-related protein (a neuropeptide from a larger family all possessing an -Arg-Phe-NH_2 at their C-terminus) and GnRH-immunoreactivity in the ARC and ME of the big brown bat was described, with colocalization between these two neuropeptides also occurring in the nervus terminalis. The FMRFamide fibers projected into the brainstem (Oelschlager et al., 1998). This FMRFamide peptide has not been definitively identified.

While the specific identity of the immunoreactive FMRF-amide peptide from Oelschlager et al. (1998) is unclear, its close association with GnRH suggests the potential for functional interaction of the two peptides. Gonadotropin-inhibitory hormone (GnIH, mammalian ortholog RFRP-3), a member of the RFamide family of peptides, was discovered in 2000 (Tsutsui et al., 2000) and has a neuroanatomical and/or functional interaction with GnRH in many vertebrate species (Bentley et al., 2003, 2006). In addition to interacting directly with GnRH neurons via cell membrane receptors, GnIH/RFRP-3 also acts on gonadotrophs within the anterior pituitary (Ubuka et al., 2006). Further, GnIH/RFRP-3 and its cognate receptor are synthesized in the gonads of all vertebrates studied and appear to regulate gonadal physiology (Bentley et al., 2008; Dickens & Bentley, 2014; McGuire & Bentley, 2010; Zhao et al., 2010).

In the present study, we aimed to map the neural distribution of the precursor peptide for GnIH, RFRP (interchangeably referred to as neuropeptide-VF, NPVF), immunoreactivity in the big brown bat (*E. fuscus*), one of the most common and widespread temperate insectivorous bats in North America (Kurta & Baker, 1990). In temperate zones, *E. fuscus* mate in the fall and females arrest ovulation during winter hibernation (Wimsatt, 1960, 1969). Male reproductive physiology (i.e., spermatogenesis and androgen production) occurs in late spring and early summer and corresponds to periods of peak food availability and optimal climatic conditions (McWilliam, 1987). During winter, circulating estrogen in female pallid bats (*Antrozous pallidus)* is low but rises rapidly in the spring upon arousal from hibernation (Oxberry, 1979). Our goal was to examine the distribution of RFRP and determine a potential role for this peptide in regulating reproductive activity. The prior description of FMRFamide immunoreactivity in close proximity to GnRH in the big brown bat brain (Oelschlager et al., 1998) guided us to also explore putative neuroanatomical interactions between RFRP-ir fibers

and GnRH-ir cell bodies. A basic characterization of RFRP distribution is required before we begin exploring the complex subtleties involved in reproductive neuroendocrine signaling in response to environmental and physiological challenges within, and among, bat species. Fundamental knowledge of how the HPG axis is regulated in a temperate bat species provides the groundwork for how reproductive physiology might respond, and be well-adapted to the unique challenges that bat populations face in terms of seasonal environmental change and increased urbanization. This will ultimately move us toward greater understanding not only of species-level differences but also the subtle variation that exists at the individual level including degree of plasticity within rapidly changing environments.

2 | MATERIALS AND METHODS

2.1 | Animals and tissue sampling

Big brown bats (*E. fuscus*) were captured from the wild in southern Ontario, Canada, and housed in a free flight colony at McMaster University where the temperature and lighting varied with ambient conditions (Skrinyer et al., 2017). Animals freely progressed through annual cycles of breeding and hibernation. Bats were given ad libitum access to food (mealworms) and water. Four male bats and four (nonreproductive) female bats were captured during fall 2017 and spring 2018 respectively, heavily anesthetized with isoflurane, and perfused with phosphate buffered saline (PBS) followed by 4% paraformaldehyde. Two additional male bats and two female bats, from which fresh gonadal tissue was collected, were euthanized by barbiturate overdose with 0.4 or 0.5 mL sodium pentobarbital (pentobarbital sodique, 54.7 mg/mL; Ceva Santé Animale, Libourne, France) via intraperitoneal injection. Gonadal tissues were subsequently collected, immediately frozen on dry ice, and stored at −80°C. For all individuals, whole brains were removed and fixed in 4% paraformaldehyde in PBS for 24-48 h before they were transferred to a 30% sucrose in PBS cryoprotectant solution for 5 days at 4°C. Brains were then frozen on dry ice and stored at −80°C until further processing.

2.2 | Presence of RFRP mRNA expression in brain and gonads

One hypothalamus, ovary, and testis were used for the identification of cDNA encoding big brown bat RFRP. Total RNA was isolated from tissues using TriZOL (Invitrogen, Carlsbad, CA), and 1 μg of RNA was reverse transcribed into cDNA (iScript cDNA synthesis kit, Bio-Rad, Hercules, California, USA). Gene-specific primers (Table 1) were designed to amplify target regions corresponding to RFRP, as well as beta-actin as a positive control in each of the tissues. Polymerase chain reaction (PCR) products were visualized using ethidium bromide on a 1.5% agarose gel (Figure 1) where known target amplicon size was confirmed.

2.3 | Identification of RFRP (NPVF) sequence

Amplification products from endpoint PCR for RFRP were submitted to the UC Berkeley Functional Genomic Laboratory for Sanger sequencing. The returned sequence was used as a query for nucleotide BLAST (https://blast.ncbi.nlm.nih.gov) whereby the resulting search reported a 91% identical match (Figure 2a) for the predicted *E. fuscus* RFRP mRNA sequence (accession number XM_008150846.1). A multiple sequence alignment was generated using predicted big brown bat RFRP mRNA sequence and known sequences in quail, mouse, rhesus macaque, and human (Figure 2b).

2.4 | Immunohistochemistry: RFRP and GnRH

Fixed and frozen male ($n = 4$) and female brains ($n = 4$) were cryostat-sectioned (40 μm) into antifreeze prior to labeling via floating immunohistochemistry. Each brain was organized into four parallel series during sectioning with one brain of each sex cut on the sagittal plane and the remaining three cut on the coronal plane. Brain sections were stored at −20°C until further immunohistochemical processing.

Sections were washed in 1× PBS (0.1 M) before incubating in 0.05% hydrogen peroxide diluted in 0.1 M PBS (pH for 15 min at room temperature. After washing again in 0.1 M PBS, a blocking solution of PBS/0.2% TritonX-100/2% normal goat serum (NGS) was added, and sections were gently agitated for 2 h at room temperature. For RFRP single labeling, tissue was incubated in anti-white crowned sparrow GnIH primary antibody (PAC123/124 1:5000, generated by George Bentley, in PBS/0.2% TritonX-100/1% NGS) for 48 h at 4°C. Following incubation in primary antibody, sections were washed in 0.2% PBS-T and incubated in goat anti-rabbit biotinylated secondary antibody (1:200 in PBS-T; Vector Laboratories, #BA-1000) for 1 h, followed by Vectastain Elite ABC as described by the manufacturer (Vector Laboratories, #PK-6100) for 1 h. Sections were washed in 0.2% PBS-T, and color development for RFRP immunoreactivity was performed using VIP substrate according to manufacturer's instructions (Vector Laboratories, #SK-4600).

For double-labeling RFRP alongside GnRH, sections were first incubated in anti-GnRH primary antibody (gift from H. Urbanski, at 1:10,000 in 0.2% PBS-T/1% NGS) for 48 h at 4°C. Sections were incubated in goat anti-rabbit biotinylated secondary antibody as above before Vectastain Elite ABC, and color development of GnRH-immunoreactive material was performed according to manufacturer's instructions using Vector DAB substrate (Vector Laboratories, #SK-4100). Following incubation with the DAB solution, sections were washed 5× in 1× PBS before applying antibody for RFRP after which tissue was washed thoroughly 5× with 0.2% PBS-T and then incubated in anti-RFRP primary antibody (PAC123124 1:5000 in 0.2% PBS-T/1% NGS) for 48 h at 4°C replicating the protocol as described above.

GnRH/RFRP double-labeled sections were used to determine putative interactions between RFRP-immunoreactive fibers and GnRH-immunoreactive cell bodies. Oil immersion microscopy (Zeiss Imager M.1, Zeiss Plan-NEOFLUAR oil 1018-595 100× objective) was used to

TABLE 1 List of PCR primer sequences

Gene	Forward primer	Reverse primer
Beta Actin (ref)	TCCCTGGAGAAGAGCTACGA	ACAGGTCCTTACGGATGTCG
GAPDH (ref)	GGAGCGAGATCCCGCCAACAT	GGGAGTTGTCATACTTGTCATGG
RFRP/NPVF	ATGAGCACACCTGCAGTCAA	GCTGTTGTTGTCCCAAACCT

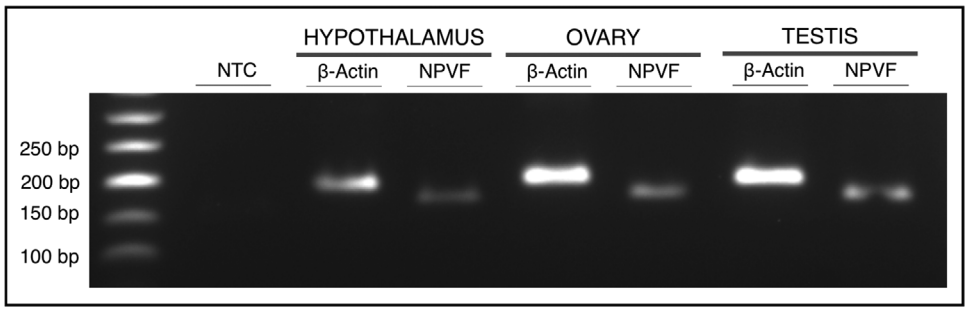

FIGURE 1 RFRP mRNA expression was observed in isolated hypothalamus, ovary, and testis of big brown bats. Amplification products matched the predicted size for both RFRP (155 bp) and actin (178 bp) controls

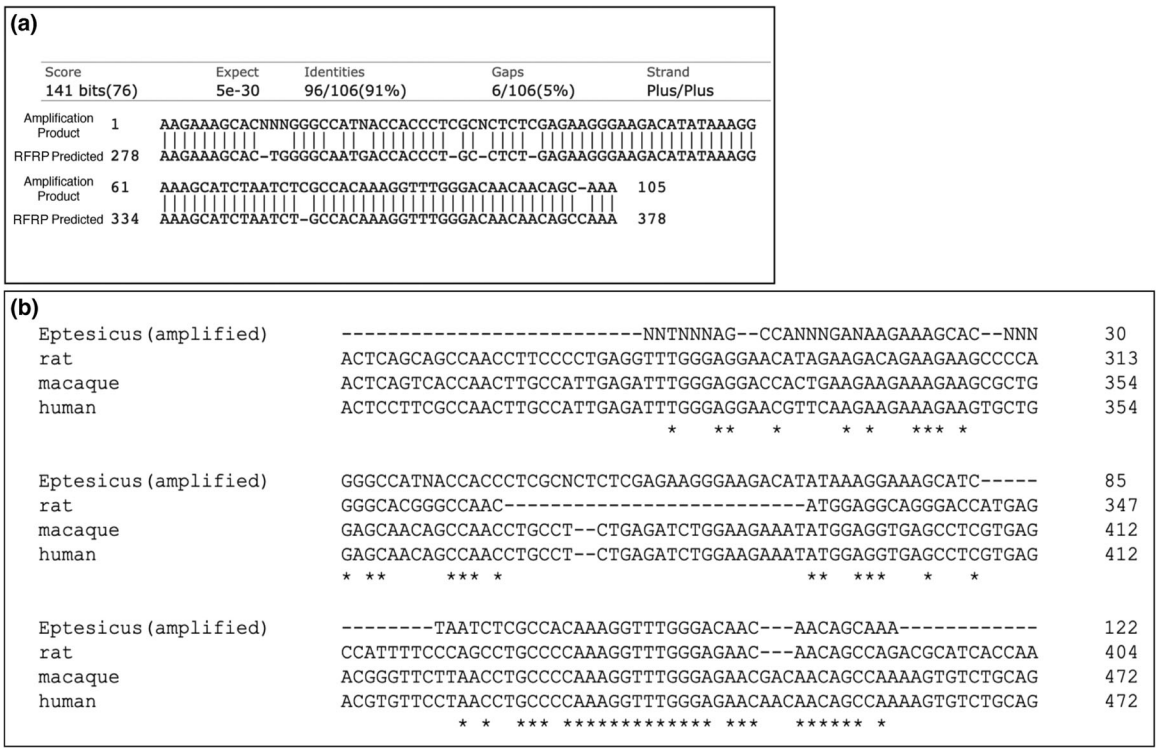

FIGURE 2 (a) RFRP amplification product showed 91% match to the big brown bat (*Eptesicus fuscus*) predicted sequence (NPVF, NCBI Ref: XM_008150846.1). (b) Multiple mRNA sequence alignment including partial big brown bat transcript, where * indicates regions most conserved across species

visualize GnRH-immunoreactive cells at high magnification throughout each the brain of each animal. For each bat, the total number of GnRH-ir cells was counted as well as the number of these cells that possessed RFRP-ir fibers touching or crossing over the soma in the same plane of focus, from which we calculated a percentage.

2.5 | RFRP cell location and fiber density analysis

Cell locations were documented by brain region using an unpublished *E. fuscus* brain atlas (atlas researched and produced by Dr. Matt Carter with the guidance of Dr. John Casseday and Dr. Ellen Covey,

Department of Psychology, University of Washington) in conjunction with a cytoarchitectural brain atlas of the common vampire bat (Bhatnagar, 2008). Two brains from nonreproductive female *E. fuscus* (collected in spring) were used to generate fiber distribution data. Color images were converted in Adobe Photoshop to 8-bit black and white with a resolution of 360 pixels/inch. Fiber density was analyzed using ImageJ. The immunoreactivity threshold in these images was standardized across all representative tissue sections to ensure that any background coloration was not included in fiber density calculations. The density of RFRP immunoreactive fibers was quantified by determining the percent area of immunoreactivity within 200 μm × 200 μm regions of interest (ROI) placed as a grid over each brain section (ImageJ). Based on minimum (3%) and maximum (79%) immunoreactivity output for all tissue sections analyzed, ranges of fiber density were set where LOW = 5–19%, MED = 20–30%, and HIGH = >30%.

2.6 | Antibody characterization

One series of *E. fuscus* brain sections (coronal) was used to perform an anti-RFRP antibody (PAC123124) preadsorption using chicken-derived GnIH peptide (as performed in Bentley et al., 2003) to confirm specificity of this antibody in this particular species. No immunoreactivity was observed in these sections via microscopy. This antibody has been shown to be highly specific for RFRP across several mammalian and avian species.

3 | RESULTS

3.1 | Bats exhibit RFRP-3 precursor (RFRP) mRNA expression in the hypothalamus and gonads

We first aimed to determine whether big brown bats express RFRP within the HPG axis. RFRP mRNA was detected in hypothalamic and gonadal tissue isolated from both male and female *E. fuscus* (Figure 1). A comparison of the amplified partial mRNA sequence for RFRP in the big brown bat to known RFamide-related peptide mRNA sequences for rat, human, and macaque (Figure 2b) indicated that bats show the greatest degree of homology with non-human primates with respective similarities of 58.33%, 60.83%, and 63.33% (via ClustalOmega2.1 multiple alignments).

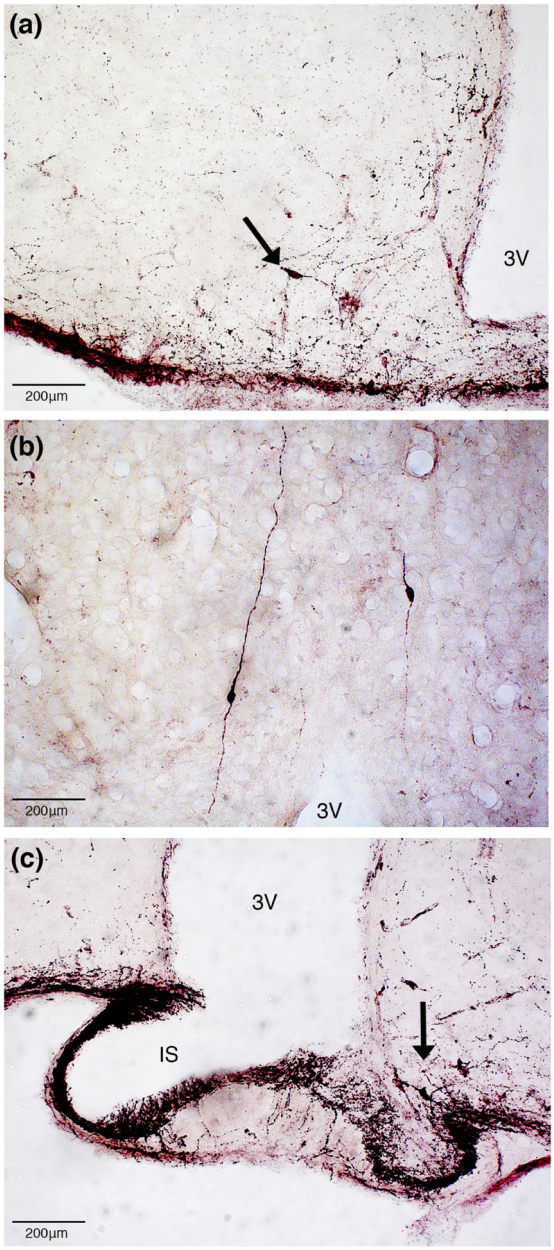

FIGURE 3 RFRP immunoreactive cell bodies (highlighted by arrows) were observed in the brain with predominant localization within the hypothalamus. (a, c) Distribution was most abundant within the arcuate nucleus (Arc) and median eminence with RFRP fibers surrounding the infundibular stalk (IS), however, (b) cells were also seen surrounding the third ventricle (3V) within the DMH and PVN.

3.2 | RFRP cellular immunoreactivity is localized in the hypothalamus, arcuate nucleus, and ME while RFRP fiber-ir is widespread in the bat brain

Histological localization of neuron fibers and cell bodies containing mature RFRP peptides was determined using immunohistochemistry. In male and nonreproductive female big brown bat brains, RFRP immunoreactive cell bodies were concentrated in the arcuate nucleus (Arc). Nearly all cells observed were bipolar, and the broad distribution did not differ between the sexes. As shown in Figure 3, cells were commonly seen closely surrounding the third ventricle (3V) including the Arc, paraventricular nucleus (PVN), dorsomedial nucleus (DMH), ventromedial nucleus (VMH), and ME. A single small population of RFRP-immunoreactive cells was observed caudally at the top of the spinal column (Figure 5d) and was surrounded by a network of dense RFRP-ir fibers. RFRP fiber immunoreactivity, as in other vertebrates, was observed in multiple areas of the bat brain, ranging from anterior regions such as the nucleus accumbens to posterior projections

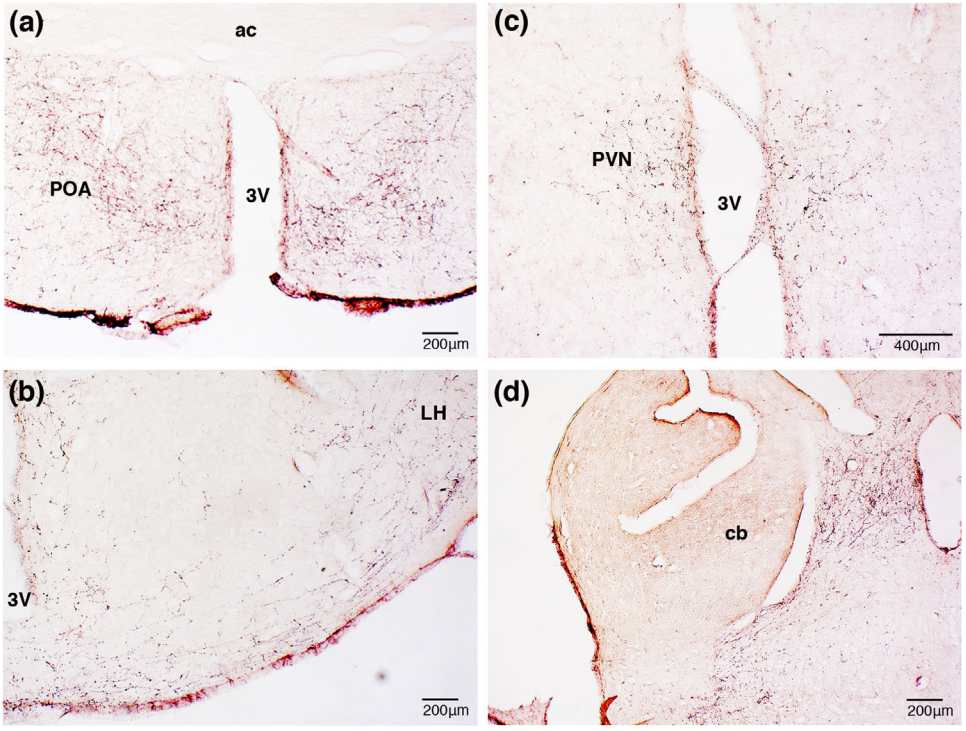

FIGURE 4 Coronal sections showing RFRP immunoreactive fiber labeling in the brain. Fiber-ir was greatest in the (a) preoptic area and (b) lateral hypothalamus (LH) below the anterior commisure (ac) , regions surrounding the third ventricle (3V) including (c) PVN, DMH, Arc, and median eminence, as well as (d) posteriorly toward the cerebellum (cb) within the spinal tract and surroudning the dorsal third ventricle.

extending down the spinal tract (Figures 4 and 5). Regions such as the POA, arcuate, ME, paraventricular and ventromedial thalamic nuclei, and cuneiform nuclei showed greatest density of RFRP immunoreactive fibers (Figure 6). Fibers were seen to project beyond these dense regions, extending bilaterally from the third ventricle toward the lateral hypothalamus (LH) and throughout the midbrain. These patterns of fiber density were consistent across individuals examined, with no noted differences between sexes.

3.3 | RFRP immunoreactive fibers project to a subset of GnRH cells

RFRP fibers were in high density within the POA, arcuate, and ME, so we explored potential interactions between RFRP-ir cell projections and GnRH-ir neuron cell bodies. Putative neuroanatomical connections between RFRP-ir fibers and GnRH-ir cells were observed in approximately 16% of GnRH-ir cells, with the majority of GnRH-ir cells lacking any detectable contact with RFRP-ir fibers and no difference between sexes (Figure 7).

4 | DISCUSSION

Over evolutionary time, species become well-adapted to the local and annual pattern of the environmental conditions they experienced. Energetic constraints on reproductive investment have led to the evolution of different reproductive strategies and variation in the flexibility within the reproductive (HPG) axis. The neural interaction of GnIH (mammalian ortholog RFRP-3) and GnRH is widely conserved across taxa (e.g., Bentley et al., 2003), suggesting a broadly conserved role in reproduction and thus lifetime fitness. There are, however, quite substantial species differences in the localization of cell bodies producing RFRP-3 as well as in patterns of fiber immunoreactivity in the brain. These differences raise the question of functional differences of GnIH in different species. In this study, we provide the first description of RFRP distribution in any bat species, characterizing sequence information as well as highlighting cellular localization in the brain. The latter varies in some ways from what we know in other, traditionally studied species in laboratory environments. This may not be surprising as the reproductive life-history strategies of many bat species are quite different from other mammals and may be a result of energetic constraints imposed by flight and, for females of temperate species, the somewhat unique need to pause reproductive investment during overwintering. Having said that, we still do not know the function of GnIH in bats.

Big brown bats express RFRP transcripts in the brain, ovaries, and testes. This agrees with what is seen in other mammals examined (Kriegsfeld et al.,2006; McGuire & Bentley, 2010; Singh et al., 2011; Zhao et al., 2010; reviewed in Bentley et al. (2017), including humans (Oishi et al., 2012), as first described in birds (Bentley et al., 2008). Like other hibernating mammals, temperate bats are seasonal breeders, upregulating their reproductive physiology at a specific time within the annual cycle. Bats offer a unique opportunity for future studies

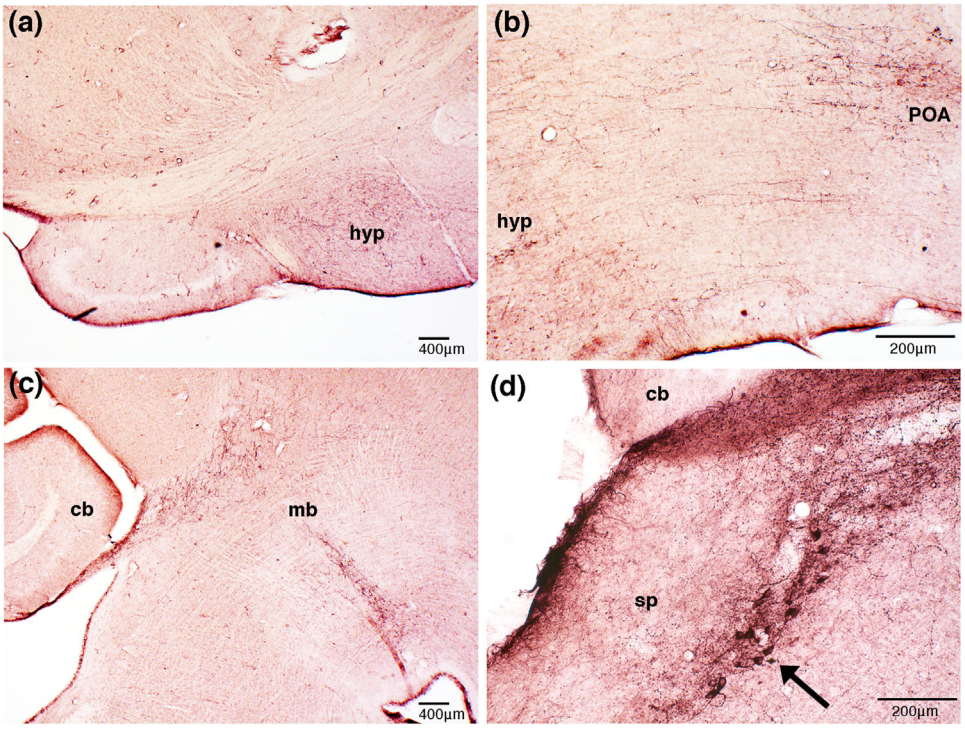

FIGURE 5 Sagittal sections showing RFRP immunoreactive fiber labeling in the brain. (a) Fiber-ir was most prominent in the hypothalamus (hyp), with (b) projections connecting the hypothalamus and preoptic area (POA), two dense regions of RFRP immunoreactivity. (c) Fibers could also be seen extending across the midbrain (mb) toward the cerebellum. (d) RFRP fibers were also present along the spinal tract (sp) where a single population of RFRP-ir cells (indicated by arrow) was observed near the base of the cerebellum (cb).

examining the pattern of reproductive neuropeptide expression within the context of male versus female reproductive physiology, including mechanisms involved in regulating extended reproductive suppression in overwintering female animals and HPG axis reactivation in the spring. While extended torpor and hibernation are not uncommon in small mammals, temperate-zone bats present an opportunity to examine the potential role of an inhibitory reproductive neuropeptide, RFRP-3, in the lengthy period of reproductive quiescence exhibited by hibernating females.

We found that big brown bats show cellular RFRP immunoreactivity in the dorsomedial hypothalamus (DMH), paraventricular nuclei of the hypothalamus (PVN), ventromedial nuclei of the hypothalamus (VMH), as well as lateral regions of the hypothalamus and the arcuate (Arc). This is a wider distribution than has been observed in rodent model systems (mouse, rat, hamster), where RFRP is confined primarily to cells of the DMH with few in the region of the VMH (Hinuma et al., 2000; Kriegsfeld et al., 2018; Legagneux et al., 2009; Tsutsui & Ubuka, 2018). Primates (macaque) and naked mole rats possess RFRP-ir cells in the PVN, but naked mole rats are the only species reported to exhibit RFRP-ir cells in the arcuate, as seen in the present study on big brown bats (Peragine et al., 2017). The arcuate is known to have abundant beta-endorphin neurons across taxa. Within the macaque brain, GnIH-immunoreactive fibers were seen to have putative interactions with beta-endorphin expressing cells, suggesting that there may be an interactive inhibitory effect on reproduction (Ubuka et al., 2009). While we did not explore it in the present study, RFRP presence in the Arc suggests that this is a possibility for big brown bats as well.

Big brown bats used in this study possessed abundant RFRP fiber immunoreactivity in multiple brain areas. Regions with the greatest density of RFRP-ir fibers were hypothalamic regions surrounding the third ventricle, the lateral hypothalamus, POA, ME, midbrain, and ventral posteromedial nucleus of the thalamus (VPM). However, we consistently observed sparse RFRP-3 fibers in the olfactory bulb as well as apparent connectivity between the POA and hypothalamus, and lengthwise down the spinal tract across all individuals examined. GnIH-ir fiber distribution has been well-characterized in other taxa. Japanese quail exhibit GnIH fibers mainly in the hypothalamus, POA, ME, telencephalon, optic tectum, dorsal motor nucleus in the medulla oblongata (Ukena et al., 2003). In rats, the lateral septal nucleus in the telencephalon, PVN, and other nearby hypothalamic nuclei, periaqueductal region of the midbrain, and pons were the areas with the greatest RFRP immunoreactive fiber labeling (Yano et al., 2003). Primates—with which we show that bats share the greatest RFPR genetic similarity—have been found to have RFRP-ir fibers in the nucleus of the stria terminalis, habenula, PVN, POA, arcuate, ME, dorsal hypothalamus (diencephalon area), medial region of the superior colliculus, midbrain, and pons (Ubuka et al., 2009). In seasonally reproducing mammals, reproductive physiology is activated or inhibited in synchrony with environmental factors, most notably photoperiod. Directional change in day length is integrated into the mammalian neuroendocrine system via the

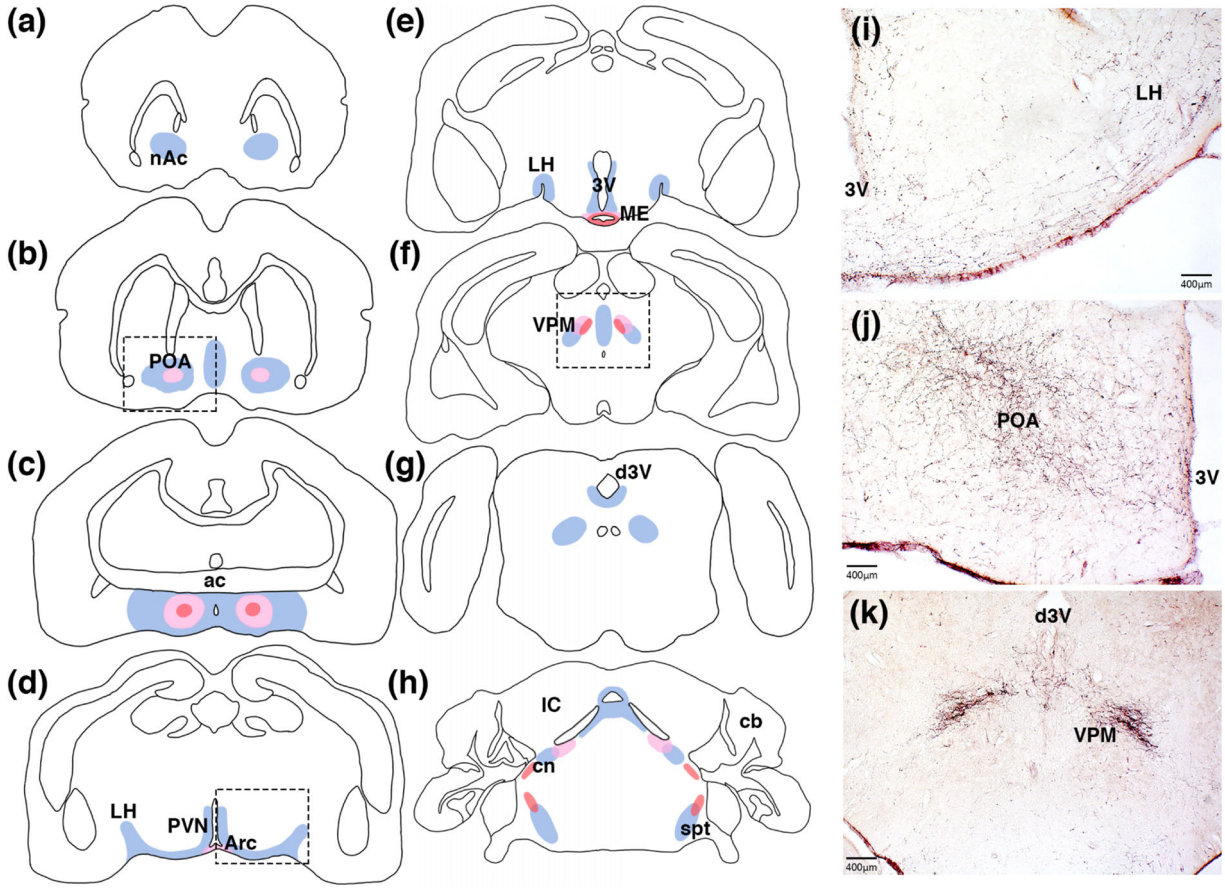

FIGURE 6 Immunoreactive RFRP fibers were quantified to map regions of low (blue), medium (pink), and high (red) density across the entire brain. Fibers were observed consistently from anterior to posterior (a–h) with POA, ME, VPM, and cn being areas of highest density. Representative microscopy images highlighting (i) low, (j) medium, and (k) high fiber density in the brain. (nAc = nucleus accumbens, ac = anterior commisure, LH = lateral hypothalamus, PVN = paraventricular nucleus of the hypothalamus, Arc = arcuate nucleus, 3V = third ventricle, ME = median eminence, VPM = ventral posteromedial nucleus of the thalamus, d3V = dorsal third ventricle, IC = inferior colliculus, cb = cerebellum, cn = caudate nucleus, spt = spinal tract.)

retinohypothalamic tract which generates a signal to the SCN and regulates melatonin synthesis and secretion from the pineal gland (Elliott and Tamarkin 1994, Moore 1995). The duration of melatonin secretion is transduced in the hypothalamus to regulate downstream reproductive physiology (Carter & Goldman, 1983; Bittman & Karsch, 1984; Nakane & Yoshimura, 2019). No study to date has examined how RFRP expression, synthesis, or secretion is impacted by environmental cues in a bat species. Our map of RFRP fiber distribution suggests that it may be acting on cell populations across the brain via an extensive network, including the potential for signaling within the SCN. Given the annual reproductive patterns of temperate bat species, future controlled experiments should be designed to explore potential interactions between photoperiodic machinery and regulation of the HPG axis in bats.

We found that approximately 16% of GnRH-immunoreactive cell bodies receive putative contacts from RFRP-ir fibers, with GnRH neurons located primarily in the ME, arcuate, PVN, with some scattered within the DMH/VMH and POA. The localization of GnRH cell bodies we see in the big brown bat differs from what is well-characterized in rodents, where GnRH cell bodies are localized primarily in the anterior hypothalamus and POA (Silverman et al., 1979). Our findings are consistent with the early immunohistochemical characterization of GnRH in the little brown bat (King et al., 1984), big brown bat (Oelschläger & Northcutt, 1992), Japanese long-fingered bat (Mikami et al., 1988), and that which is seen in primates/humans (Parker et al., 1980; Silverman et al., 1982). Our findings also support the overlap in a distribution described in 1998 between GnRH (previously named LHRH) and FMRFamide-like immunoreactivity in the brains of *E. fuscus* (Oelschläger and Northcutt, 1992), the same study species used here. The putative neuroanatomical interaction we describe between RFRP-ir fibers and GnRH cells provides some evidence that RFRP may have the potential to regulate GnRH secretion directly at some level; however, RFRP-ir fibers also projected to regions beyond those containing GnRH cells suggesting functions that may extend beyond upstream HPG regulation. Only one study has described a reproductive neuropeptide during hibernation in bats, finding a greater number of GnRH-immunoreactive neurons within the arcuate and noting that these cells were also larger in size than that seen in nonhibernating bats (Mikami et al. 1988). It has been suggested that low ambient temperature and declines in food availability may provide cues to

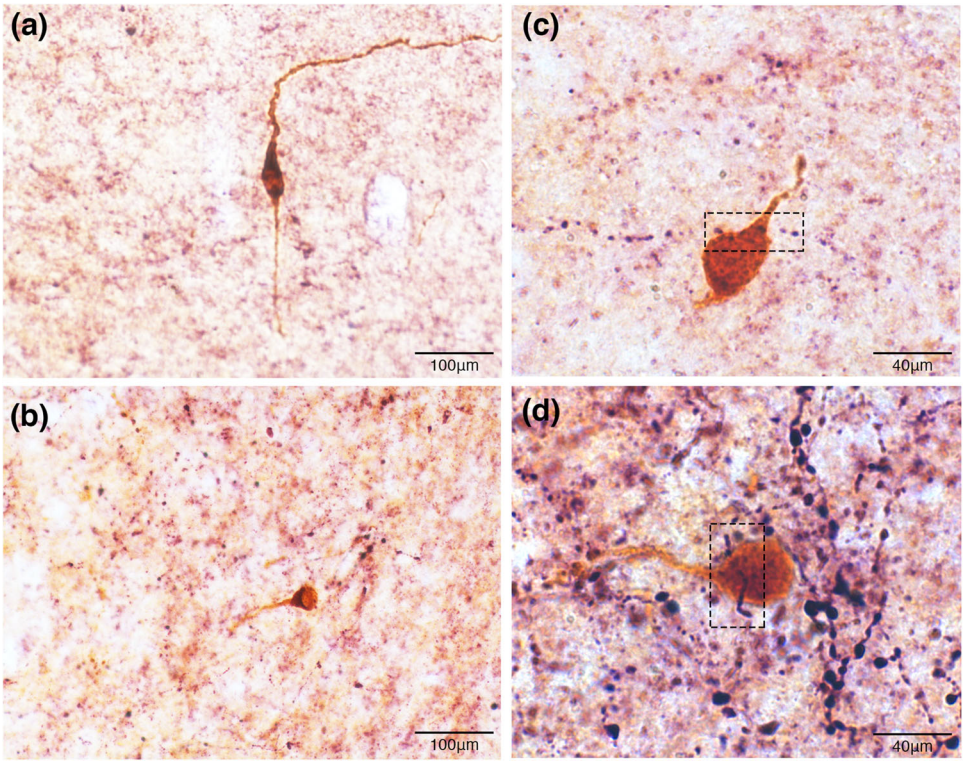

FIGURE 7 The majority of GnRH-ir cell bodies observed in the brain of big brown bats did not appear to interact with RFRP-ir cells (a, b), however, putative contacts (c, d) were observed between RFRP immunoreactive fibers and GnRH-immunoreactive cell bodies in approximately 16% of cells

downregulate reproductive biology in female bats entering hibernation (Racey, 1982). It is possible that RFRP is acting upstream of GnRH to provide an inhibitory signal that maintains the HPG axis in a quiescent state throughout the challenges of a harsh winter. If this is the case, it would then be important to understand what cues—stress, environmental temperature, resource availability, social stimuli, etc.—that may cause upregulation of RFRP in the brain of temperate bats.

The brain is a critical regulator of seasonal reproduction, translating environmental cues into molecular signals that dictate proper timing for physiological investment in reproduction. Natural selection has led to the use of photoperiod as the most invariant environmental cue (for any particular date of the year) to determine timing of reproduction (Prendergast et al., 2001), with a strong spring bias across mammals (Bronson, 1989). The anomalous temporal delay in reproductive events that hibernating bat species exhibit makes bats an interesting model for examining neuroendocrine patterns associated with fertility and reproductive behavior across the annual cycle. Next steps should aim to characterize the expression pattern and dynamics of key reproductive neuropeptides in response to seasonal and environmental variation and physiological stressors, as well as mechanistic studies that can assess the direct downstream effects of these neuropeptides. Acquisition of this knowledge will allow us not only to understand the mechanisms regulating reproductive physiology/fertility in bats but also help us make stronger predictions regarding population dynamics and inform decisions about their management in the wild. Furthermore, using bats as models within comparative studies advances the fields of reproductive neuroendocrinology and physiology through understanding the components of HPG axis regulation not only in a classic comparison between seasonal versus "opportunistic" breeders but also emphasizes the value of exploring the variation in reproductive phenology and physiology that exists within those broad classifications.

ACKNOWLEDGMENTS

We are grateful to, then undergraduate, Taylor Schobel for early laboratory assistance with immunohistochemistry as well as Devon Comito for detailed comments and feedback while developing the manuscript. We would also like to thank Dr. Kathleen Delaney, Dawn Graham, and the staff of the Central Animal Facility at McMaster University for veterinary and animal care support.

AUTHOR CONTRIBUTIONS

Mattina M. Alonge: conceptualization, data curation, formal analysis, validation, visualization, investigation, writing (original draft). Lucas J.S. Greville: methodology, resources, writing (review and editing). Paul A. Faure: project administration, supervision, funding acquisition, writing (review and editing). George E. Bentley: conceptualization, project administration, supervision, funding acquisition, writing (review and editing).

ORCID

Mattina M. Alonge https://orcid.org/0000-0002-6598-1749

REFERENCES

Anthony, E. L. (2000). Endocrinology of reproduction in bats: Central control. In E. G. Crichton & P. H. Krutzsch (Eds.), *Reproductive biology of bats* (pp. 1-26). Academic Press.

Anthony, E. L., & Gustafson, A. W. (1984). Seasonal variations in pituitary LH-gonadotropes of the hibernating bat *Myotis lucifugus lucifugus*: An immunohistochemical study. *American Journal of Anatomy*, *170*(1), 101–115. https://doi.org/10.1002/aja.1001700108

Anthony, E. L., Weston, P. J., Montvilo, J. A., Bruhn, T. O., Neel, K., & King, J. C. (1989). Dynamic aspects of the LHRH system associated with ovulation in the little brown bat (*Myotis lucifugus*). *Reproduction (Cambridge, England)*, *87*(2), 671-686. https://doi.org/10.1530/jrf.0.0870671

Bentley, G. E., Perfito, N., Ukena, K., Tsutsui, K., & Wingfield, J. C. (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. *Journal of Neuroendocrinology*, *15*(8), 794-802. https://doi.org/10.1046/j.1365-2826.2003.01062.x

Bentley, G. E., Kriegsfeld, L. J., Osugi, T., Ukena, K., O'brien, S., Perfito, N., Moore, I. T., Tsutsui, K., & Wingfield, J. C. (2006). Interactions of gonadotropin-releasing hormone (GnRH) and gonadotropin-inhibitory hormone (GnIH) in birds and mammals. *Journal of Experimental Zoology, Part A: Comparative Experimental Biology*, *305*(9), 807-814. https://doi.org/10.1002/jez.a.306

Bentley, G. E., Ubuka, T., McGuire, N. L., Chowdhury, V. S., Morita, Y., Yano, T., Hasunuma, I., Binns, M., Wingfield, J. C., & Tsutsui, K. (2008). Gonadotropin-inhibitory hormone and its receptor in the avian reproductive system. *General and Comparative Endocrinology*, *156*(1), 34-43. https://doi.org/10.1016/j.ygcen.2007.10.003

Bentley, G. E., Wilsterman, K., Ernst, D. K., Lynn, S. E., Dickens, M. J., Calisi, R. M., Kriegsfeld, L. J., Kaufer, D., Geraghty, A. C., ViviD, D., & McGuire, N. L. (2017). Neural versus gonadal GnIH: Are they independent systems? A mini-review. *Integrative and Comparative Biology*, *57*(6), 1194-1203. https://doi.org/10.1093/icb/icx085

Bhatnagar, K. P. (2008). The brain of the common vampire bat, *Desmodus rotundus murinus* (Wagner, 1840): A cytoarchitectural atlas. *Brazilian Journal of Biology*, *68*(3), 583-599. https://doi.org/10.1590/S1519-69842008000300017

Bittman, E. L., & Karsch, F. J. (1984). Nightly duration of pineal melatonin secretion determines the reproductive response to inhibitory day length in the ewe. *Biology of Reproduction*, *30*(3), 585-593. https://doi.org/10.1095/biolreprod30.3.585

Bronson, F. H. (1989). *Mammalian reproductive biology*. University of Chicago Press.

Carter, D. S., & Goldman, B. D. (1983). Antigonadal effects of timed melatonin infusion in pinealectomized male Djungarian hamsters (*Phodopus sungorus sungorus*): Duration is the critical parameter. *Endocrinology*, *113*(4), 1261-1267. https://doi.org/10.1210/endo-113-4-1261

Crichton, E. G. (2000). Sperm storage and fertilization. In E. G. Crichton & P. H. Krutzsch (Eds.), *Reproductive biology of bats* (pp. 295-320). Academic Press.

Dickens, M. J., & Bentley, G. E. (2014). Stress, captivity, and reproduction in a wild bird species. *Hormones and Behavior*, *66*(4), 685-693. https://doi.org/10.1016/j.yhbeh.2014.09.011

Douglas, S. G. (1976). Distribution of gonadotropin-releasing hormone in the mouse brain as revealed by immunohistochemistry. *Endocrinology*, *98*(6), 1408-1417. https://doi.org/10.1210/endo-98-6-1408

Elliott, J. A., & Tamarkin, L. (1994). Complex circadian regulation of pineal melatonin and wheel-running in Syrian hamsters. *Journal of Comparative Physiology A*, *174*(4), 469-484.

Gustafson, A. W. (1979). Male reproductive patterns in hibernating bats. *Reproduction (Cambridge, England)*, *56*(1), 317-331. https://doi.org/10.1530/jrf.0.0560317

Herlant, M. (1956). Genito-pituitary correlations in the female flittermouse, *Myotis myotis*. *Archives de Biologie*, *67*(1), 89-180.

Hinuma, S., Shintani, Y., Fukusumi, S., Iijima, N., Matsumoto, Y., Hosoya, M., Fujii, R., Watanabe, T., Kikuchi, K., Terao, Y., & Yano, T. (2000). New neuropeptides containing carboxy-terminal RFamide and their receptor in mammals. *Nature Cell Biology*, *2*(10), 703-708. https://doi.org/10.1038/35036326

Kawamoto, K., Kurahashi, S.,Hayashi, T. (1998). Changes in the gonadotropin-releasing hormone (GnRH) neuronal system during the annual reproductive cycle of the horseshoe bat, *Rhinolophus ferrumequinum*. *Zoological Science*, *15*(5), 779-786. https://doi.org/10.2108/zsj.15.779

King, J. C., Anthony, E. L., Gustafson, A. W., & Damassa, D. A. (1984). Luteinizing hormone-releasing hormone (LH-RH) cells and their projections in the forebrain of the bat *Myotis lucifugus lucifugus*. *Brain Research*, *298*(2), 289-301. https://doi.org/10.1016/0006-8993(84)91428-8

Kriegsfeld, L. J., Mei, D. F., Bentley, G. E., Ubuka, T., Mason, A. O., Inoue, K., Ukena, K., Tsutsui, K., & Silver, R. (2006). Identification and characterization of a gonadotropin-inhibitory system in the brains of mammals. *Proceedings of the National Academy of Sciences*, *103*(7), 2410-2415. https://doi.org/10.1073/pnas.0511003103

Kriegsfeld, L. J., Jennings, K. J., Bentley, G. E., & Tsutsui, K. (2018). Gonadotrophin-inhibitory hormone and its mammalian orthologue RF amide-related peptide-3: Discovery and functional implications for reproduction and stress. *Journal of Neuroendocrinology*, *30*(7), 12597. https://doi.org/10.1111/jne.12597

Kurta, A., & Baker, R. H. (1990). Eptesicus fuscus. *Mammalian Species*, *356*, 1-10. https://doi.org/10.2307/3504258

Legagneux, K., Bernard-Franchi, G., Poncet, F., La Roche, A., Colard, C., Fellmann, D., Pralong, F., & Risold, P. Y. (2009). Distribution and genesis of the RFRP-producing neurons in the rat brain: Comparison with melanin-concentrating hormone-and hypocretin-containing neurons. *Neuropeptides*, *43*(1), 13-19. https://doi.org/10.1016/j.npep.2008.11.001

McGuire, N. L., & Bentley, G. E. (2010). Neuropeptides in the gonads: From evolution to pharmacology. *Frontiers in Pharmacology*, *1*, 114. https://doi.org/10.3389/fphar.2010.00114

McWilliam, A. N. (1987). The reproductive and social biology of Coleura afra in a seasonal environment. In M. B. Fenton, P. Racey, & J. M. V. Rayner (Eds.), *Recent advances in the study of bats* (pp. 324-350). Cambridge University Press.

Mikami, S. I., Chiba, S., Taniguchi, K., Kubokawa, K., & Ishii, S. (1988). Immunocytochemical localization of neuropeptides in the hypothalamus of the Japanese long-fingered bat, *Miniopterus schreibersii fuliginosus*. *Cell and Tissue Research*, *254*(1), 49-57. https://doi.org/10.1007/BF00220016

Moore, R. Y. (1995). Organization of the mammalian circadian system. *Circadian clocks and their adjustments*, *183*, 88-106.

Nakane, Y., & Yoshimura, T. (2019). Photoperiodic regulation of reproduction in vertebrates. *Annual Review of Animal Biosciences*, *7*, 173-194. https://doi.org/10.1146/annurev-animal-020518-115216

Oelschläger, H. A., & Northcutt, R. G. (1992). Immunocytochemical localization of luteinizing hormone-releasing hormone (LHRH) in the nervus terminalis and brain of the big brown bat, *Eptesicus fuscus*. *Journal of Comparative Neurology*, *315*(3), 344-363. https://doi.org/10.1002/cne.903150309

Oelschläger, H. A., Helpert, C., & Northcutt, R. G., (1998). Coexistence of FMRFAMIDE-like and LHRH-like immunoreactivity in the terminal nerve and forebrain of the big brown bat, *Eptesicus fuscus*. *Brain, Behavior and Evolution*, *52*(3), 139-147. https://doi.org/10.1159/000006558

Oishi, H., Klausen, C., Bentley, G. E., Osugi, T., Tsutsui, K., Gilks, C. B., Yano, T., & Leung, P. C. (2012). The human gonadotropin-inhibitory hormone ortholog RFamide-related peptide-3 suppresses gonadotropin-induced progesterone production in human granulosa cells. *Endocrinology*, *153*(7), 3435-3445. https://doi.org/10.1210/en.2012-1066

Oxberry, B. A. (1979). Female reproductive patterns in hibernating bats. *Reproduction (Cambridge, England)*, *56*(1), 359-367. https://doi.org/10.1530/jrf.0.0560359

Parker, C. R., Jr., Neaves, W. B., & Porter, J. C. (1980). Regional and subcellular localization of luteinizing hormone releasing hormone in the adult human brain. *Brain Research Bulletin*, *5*(3), 307-313. https://doi.org/10.1016/0361-9230(80)90174-4

Peragine, D. E., Pokarowski, M., Mendoza-Viveros, L., Swift-Gallant, A., Cheng, H. Y. M., Bentley, G. E., & Holmes, M. M. (2017). RFamide-related peptide-3 (RFRP-3) suppresses sexual maturation in a eusocial mammal. *Proceedings of the National Academy of Sciences*, *114*(5), 1207-1212. https://doi.org/10.1073/pnas.1616913114

Prendergast, B. J., Kriegsfeld, L. J., & Nelson, R. J. (2001). Photoperiodic polyphenisms in rodents: Neuroendocrine mechanisms, costs, and functions. *The Quarterly Review of Biology*, *76*(3), 293-325. https://doi.org/10.1086/393989

Racey, P. A. (1973). Environmental factors affecting the length of gestation in heterothermic bats. *Journal of Reproduction and Fertility*, *19*, 175-189.

Racey, P. A., & Swift, S. M. (1981). Variations in gestation length in a colony of pipistrelle bats (*Pipistrellus pipistrellus*) from year to year. *Reproduction (Cambridge, England)*, *61*(1), 123-129. https://doi.org/10.1530/jrf.0.0610123

Racey, P. A. (1982). Ecology of bat reproduction. In T. H. Kunz (Ed.), *Ecology of bats* (pp. 57–104). Springer.

Richardson, E. G. (1977). The biology and evolution of the reproductive cycle of *Miniopterus schreibersii* and *M. australis* (Chiroptera: Vespertilionidae). *Journal of Zoology*, *183*(3), 353-375. https://doi.org/10.1111/j.1469-7998.1977.tb04193.x

Richardson, B. A. (1980). *The pars distalis of the female California leaf-nosed hat, Macrotus californicus, and its possible role in delayed development* [Ph.D. Thesis]. University of Arizona.

Richardson, B. A. (1981) Localization of gonadotrophic hormones in the pituitary gland of the California leaf-nosed bat (*Macrotus californicus*). *Cell and Tissue Research*, *220*, 115-123. https://doi.org/10.1007/BF00209970

Silverman, A. J., Krey, L. C., & Zimmerman, E. A. (1979). A comparative study of the luteinizing hormone releasing hormone (LHRH) neuronal networks in mammals. *Biology of Reproduction*, *20*(1), 98-110. https://doi.org/10.1093/biolreprod/20.1.98

Silverman, A. J., Antunes, J. L., Abrams, G. M., Nilaver, G., Thau, R., Robinson, J. A., Ferin, M., & Krey, L. C. (1982). The luteinizing hormone-releasing hormone pathways in rhesus (*Macaca mulatta*) and pigtailed (*Macaca nemestrina*) monkeys: New observations on thick, unembedded sections. *Journal of Comparative Neurology*, *211*(3), 309-317. https://doi.org/10.1002/cne.902110309

Singh, P., Krishna, A., Sridaran, R., & Tsutsui, K. (2011). Immunohistochemical localization of GnRH and RFamide-related peptide-3 in the ovaries of mice during the estrous cycle. *Journal of Molecular Histology*, *42*(5), 371-381. https://doi.org/10.1007/s10735-011-9340-8

Skrinyer, A. J., Faure, P. A., Dannemiller, S., Ball, H. C., Delaney, K. H., Orman, R., Stewart, M., & Cooper, L. N. (2017). Care and husbandry of the world's only flying mammals. *Laboratory Animal Science Professional*, *June*, 24-27.

Tsutsui, K., Saigoh, E., Ukena, K., Teranishi, H., Fujisawa, Y., Kikuchi, M., Ishii, S., & Sharp, P. J. (2000). A novel avian hypothalamic peptide inhibiting gonadotropin release. *Biochemical and Biophysical Research Communications*, *275*(2), 661-667. https://doi.org/10.1006/bbrc.2000.3350

Tsutsui, K., & Ubuka, T. (2018). How to contribute to the progress of neuroendocrinology: Discovery of GnIH and progress of GnIH research. *Frontiers in Endocrinology*, *9*, 662. https://doi.org/10.3389/fendo.2018.00662

Ubuka, T., Ukena, K., Sharp, P. J., Bentley, G. E., & Tsutsui, K. (2006). Gonadotropin-inhibitory hormone inhibits gonadal development and maintenance by decreasing gonadotropin synthesis and release in male quail. *Endocrinology*, *147*(3), 1187-1194. https://doi.org/10.1210/en.2005-1178

Ubuka, T., Lai, H., Kitani, M., Suzuuchi, A., Pham, V., Cadigan, P. A., Wang, A., Chowdhury, V. S., Tsutsui, K., & Bentley, G. E. (2009). Gonadotropin-inhibitory hormone identification, cDNA cloning, and distribution in rhesus macaque brain. *Journal of Comparative Neurology*, *517*(6), 841-855. https://doi.org/10.1002/cne.22191

Ukena, K., Ubuka, T., & Tsutsui, K. (2003). Distribution of a novel avian gonadotropin-inhibitory hormone in the quail brain. *Cell and Tissue Research*, *312*(1), 73-79. https://doi.org/10.1007/s00441-003-0700-x

Willis, C. K., (2017). Trade-offs influencing the physiological ecology of hibernation in temperate-zone bats. *Integrative and Comparative Biology*, *57*(6), 1214-1224. https://doi.org/10.1093/icb/icx087

Wimsatt, W. A. (1960). Some problems of reproduction in relation to hibernation in bats. *Bulletin Museum Comparative Zoology Harvard*, *124*, 249-270.

Wimsatt, W. A. (1969). Some interrelations of reproduction and hibernation in mammals. *Symposium of Society for Experimental Biology*, *23*, 511-549.

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 Research*, *982*(2), 156-167. https://doi.org/10.1016/S0006-8993(03)02877-4

Zhao, S., Zhu, E., Yang, C., Bentley, G. E., Tsutsui, K., & Kriegsfeld, L. J. (2010). RFamide-related peptide and messenger ribonucleic acid expression in mammalian testis: Association with the spermatogenic cycle. *Endocrinology*, *151*(2), 617-627. https://doi.org/10.1210/en.2009-0978

How to cite this article: Alonge, M. M., Greville, L. J. S., Faure, P. A., Bentley, G. E. (2022). Immunoreactive distribution of gonadotropin-inhibitory hormone precursor, RFRP, in a temperate bat species (*Eptesicus fuscus*). *Journal of Comparative Neurology*, 1-11. https://doi.org/10.1002/cne.25291