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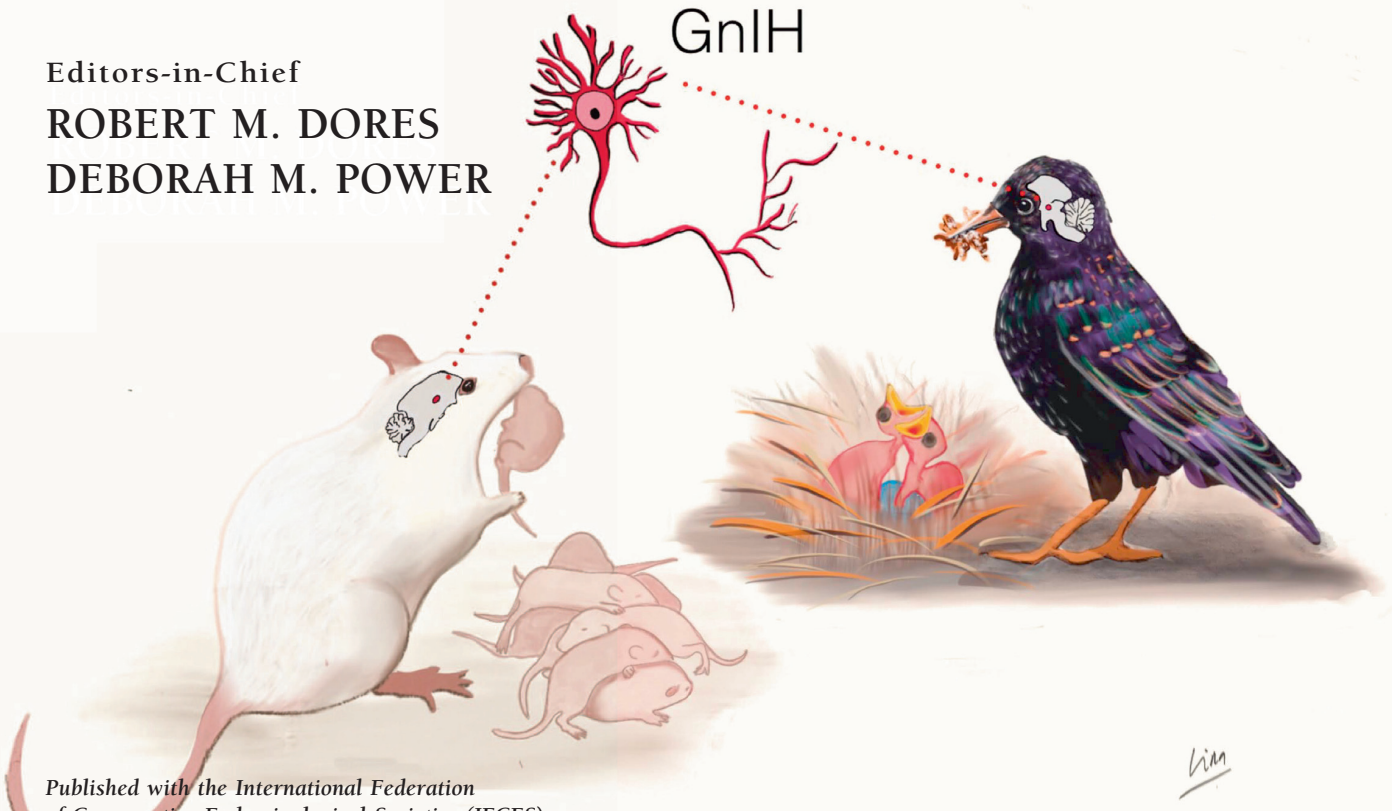

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Research paper

Patterns of hypothalamic GnIH change over the reproductive period in starlings and rats

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ABSTRACT

Gonadotropin inhibitory hormone (GnIH) exerts powerful inhibitory effects on various levels of the vertebrate hypothalamic–pituitary–gonadal (reproductive) axis, yet little is known of how it might change naturally over the course of reproduction. We characterized patterns of hypothalamic GnIH cell abundance over the reproductive period in two popular models used for the study of reproductive endocrinology: European starlings (*Sturnus vulgaris*) and Sprague-Dawley rats (*Rattus norvegicus*). We also examined the effects on an unpredictable change in the environment on GnIH cell abundance during the reproductive period, specifically during the period of parental care, by simulating a nest predation event and removing eggs/pups. In both species, we report changes in GnIH cell abundance are occurring at similar reproductive time points but are not always directionally parallel; this may be due to a difference in life histories and physiology mediating parental care. We discovered that cells immunoreactive for the GnIH peptide in male and female starlings are most highly abundant on the first day of incubation and the first day after the first chick hatches. Conversely in rats, GnIH cell abundance decreases in dams on the first day after pups are born. In both male and female starlings and female rats, GnIH cell abundance increases in response to egg/pup loss, indicating that GnIH responds to an unpredictable change in the environment in a potentially conserved fashion. These changes in GnIH cell abundance during the reproductive period inspire further investigation of its adaptive role in reproductive physiological events and behaviors, especially parental care.

1. Introduction

GnIH has reshaped the way reproductive endocrinology is understood because of the active inhibitory role it plays in reproductive physiology and sexual behaviors. However, we still know very little of how its actions influence behavior and are shaped by the environment. Vertebrate reproduction is regulated by the hypothalamic neurohormone gonadotropin-releasing hormone (GnRH; Schally et al., 1971). Release of GnRH causes the pituitary gland to secrete the gonadotropins luteinizing hormone (LH) and follicle-stimulating hormone (FSH) into the bloodstream. LH travels to the gonads, where it stimulates the production of reproductive steroids such as androgens and estrogens, whereas FSH guides gamete production. The sex steroids provide feedback to the brain and pituitary, creating a regulatory feedback system necessary for reproduction and its associated behaviors. The framework used to compartmentalize and discuss such physiological function is referred to as the hypothalamic–pituitary–gonadal axis, or more colloquially as the reproductive axis. In birds and mammals, neural inhibition of gonadotropins was thought to be solely the result of increased feedback into the brain from the pituitary and gonads. The discovery of GnIH and its active inhibitory effects of the reproductive axis altered this view (Tsutsui et al., 2000).

GnIH decreases the activity of GnRH neurons in addition to reducing synthesis and release of the gonadotropins LH and, in some cases, FSH from the pituitary gland (Ubuka et al., 2006; Bentley et al., 2009; Calisi, 2014; Ubuka et al., 2008b). GnIH also reduces testosterone release from the gonads. Central administration of GnIH can decrease sexual behaviors in birds and rodents (Bentley et al., 2006; Johnson et al., 2007), but little is understood concerning how GnIH fluctuates in response to the environment (reviewed: (Calisi, 2014)). Calisi et al. (2008) reported that stressful stimuli perceived from the environment can affect GnIH. We found that by restraining wild-caught house sparrows during their

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breeding season, cells producing the GnIH peptide (hereafter termed "GnIH cells") increased in abundance in the paraventricular nucleus of the hypothalamus. Kirby et al. (2009) reported a similar phenomenon in rodents, suggesting a conserved mechanism for inhibiting reproduction in a stressful environment. These studies were important because they revealed that the external environment could affect reproduction and its associated behaviors via GnIH.

Here, we continue our investigations of environmental influence on GnIH and examine the relationship of reproductive stage on GnIH cell abundance in two popular models commonly used for the study of reproductive endocrinology: European starlings (*Sturnus vulgaris*) and Sprague-Dawley rats (*Rattus norvegicus*). European starlings are obligate cavity-nesters, and both sexes participate in nest building, incubation and offspring provisioning. We examined GnIH cell abundance in male and female European starlings prior to nesting, prior to incubation, at the beginning and end of incubation and at the start of chick care. We examined GnIH cell abundance only in female rats, as males of this species do not offer care and are typically separated from females post-copulation to prevent aggressive confrontations. Time points for rat sampling were prior to copulation, early gestation and late gestation, one day and four days postpartum. Previously, we found that the number of cells producing the peptide for hypothalamic GnIH increased when male and female European starlings began to incubate their eggs (Calisi et al., 2011). This finding inspired our further investigation of the changes in the patterns of GnIH cell abundance over the reproductive period and how conserved they might be across species.

In regards to our previous finding (Calisi et al., 2011) and the parallel response of GnIH in starlings and rats to external stimuli, i.e. a stress test (Calisi et al., 2008; Kirby et al., 2009), we predicted that GnIH cell abundance would increase at the beginning of incubation and chick care (birds) and gestation and pup care (rats) as compared to prior time points (nesting and late incubation in birds, and prior to copulation and parturition in rats). In addition to characterizing GnIH cell abundance over the course of the parental care period, we manipulated the environment during this time to investigate how an unpredictable disturbance could affect GnIH cell abundance. To do this, we removed eggs at the end of incubation and pups soon after parturition to simulate a predation event. GnIH cell abundance was then compared to that of starlings/rats at a similar time point to those whose eggs/offspring were left undisturbed. The patterns we report of GnIH cell abundance over the course of the reproductive period in starlings and rats, accompanied by results from experimental manipulations, reveal a relationship between GnIH and major transition points in the reproductive period, particularly surrounding the time when important changes in parental care behaviors occur.

2. Materials and methods

2.1. Experimental setup

2.1.1. European starlings

During the years of 2009 and 2011, 68 juveniles (33 male, 35 female), as identified by their distinct juvenile plumage, were caught and randomly assigned to large adjacent naturalistic outdoor aviaries at the University of California, Berkeley, Field Station for the Study of Behavior, Ecology, and Reproduction. Following their year of capture, these obligate cavity-nesters were provided with nest boxes during their first breeding season of reproductive maturity. Birds were exposed to natural light, climate, and conand hetero-specific interactions both within and through the wire of their enclosures. In addition to avian pellet feed and water given *ad libitum*, birds also foraged for and ingested food sources (most likely small invertebrates) from natural ground. As a result of this semi-natural setup, birds exhibited a range of natural breeding behaviors, including singing, nest site defense, aggressive interactions, copulation solicitations, nest construction, mate guarding, egg laying, egg incubation and chick care. This semi-natural setup has been a powerful way to study reproductive neuroendocrinology and behavior in wild-caught birds of this species (Amorin and Calisi, 2015; Bentley et al., 2013; Calisi, 2014; Calisi et al., 2011; Calisi and Bentley, 2009).

Brains were collected over the course of a 3-week period in the spring when birds were undergoing various stages of reproduction (Fig. 1). Birds without a nest box were randomly sampled over the course of this 3-week period to serve as a reference point ("No nest"; N = 13: 9M, 4F). We sampled birds that had paired and constructed a nest but had not yet laid eggs ("Nest, no eggs"; N = 17: 5M, 12F) and birds that were 1–3 days post laying of their first egg and observed spending long spans of time in their nest boxes, presumably incubating eggs ("Early incubation"; N = 15: 7M, 8F). Other sampling times included birds that were in Day 10 of their incubation period, as chicks generally hatch 11–12 days after the first egg is laid ("Late incubation"; N = 6: 3M, 3F); and birds on the first day after the first chick hatching ("Chicks"; N = 7: 4M, 3F). In addition, on Day 8 of incubation, eggs were removed from some nests to simulate a natural predation event ("Egg removal"; N = 10: 5M, 5F). Birds occupying these nests were then sampled on Day 10 and their level of GnIH cell abundance was compared to those sampled on Day 10 that were incubating eggs.

To confirm the occupants of each nest box, both visual observations as well as radio frequency identification tags (Cyntag, Inc., Cynthiana, KY) attached to leg bands were used. Birds were monitored daily, and nests were checked every morning to establish the timing of egg lay, incubation and chicks hatching. Because newly hatched chicks cannot survive without their parents, they were euthanized immediately and humanely after parents were removed. All animal care and procedures were approved by the University of California–Berkeley Animal Care and Use Committee (Protocol R297C).

2.1.2. Sprague-Dawley rodents

Adult female Sprague-Dawley rats (62 in total) were triple-housed on a 12/12 h light-dark cycle with lights on at 07:00 h and *ad libitum* food and water. For all studies, rats were acclimated for a week and then vaginal smears were obtained daily to verify normal cyclicity for 12 days before studies commenced. Rats that did not cycle normally were removed from the study. For pregnancy studies, females were housed individually with a male conspecific for one night, and then monitored daily by weight for pregnancy. Non-mated, virgin animals were left undisturbed in their home cages ("Virgin"; N = 15). Once mated, animals were sampled across a 4-week period to cover the different time points across pregnancy (Fig. 1). Post-copulation, animals were sampled the morning after mating with a male ("Early gestation"; N = 6). Only females exhibiting a sperm plug were tested, as verification of successful mating, and the vast majority of females exhibiting sperm plugs in this study did become pregnant. Animals were sampled at 20 days post-mating to capture the time point immediately prior to parturition ("Late gestation"; N = 14). Animals were sampled 1 day post the birth of their pups ("Day 1 Post-Birth"; N = 14) and 4 days post-birth ("Day 4 Pups"; N = 7). Pups were removed from their nest on postnatal day 1 and mothers were sampled 24 h after pup removal ("Day 1 Pups Removed"; N = 6). Because newly born pups have a difficult time surviving without their parents, they were euthanized immediately and humanely after parents were removed. All animal care and procedures were

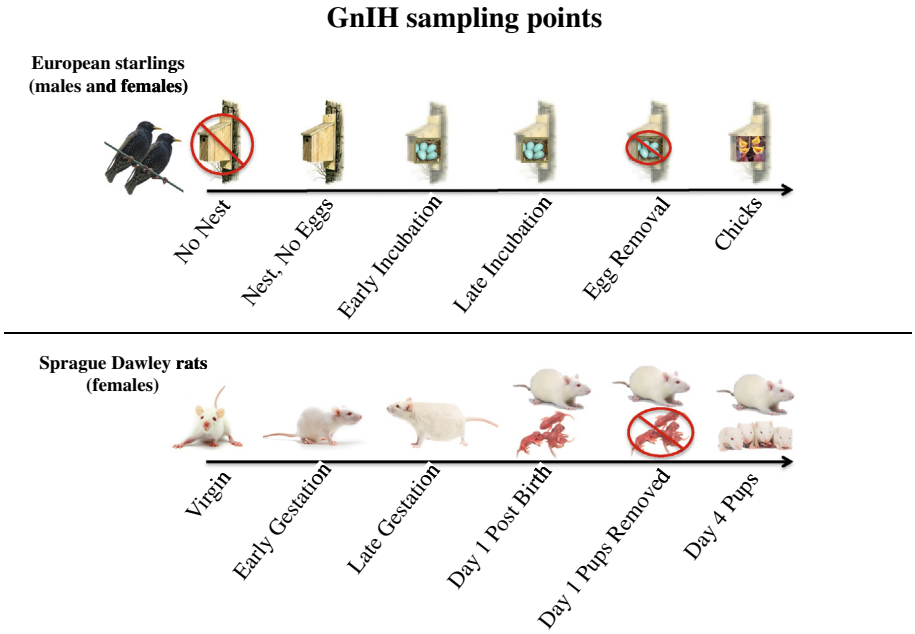

Fig. 1. Brain sampling time points for starlings (top) and rats (bottom).

approved by the University of California–Berkeley Animal Care and Use Committee (Protocol R303-0313BC).

2.2. Brain sampling and immunohistochemistry

2.2.1. European starlings

The following protocol has been previously validated and thoroughly documented in this and other avian species (Amorin and Calisi, 2015; Bentley et al., 2003; Calisi et al., 2011, 2008; Lopes et al., 2012). In brief, targeted birds were collected between 10:00 and 10:30 h by hand net in less than 5 min of entering the aviary. Birds were euthanized immediately by decapitation following rapid terminal anesthesia using isoflurane. Immediately following decapitation, brains were extracted and frozen on dry ice and then stored at −80 °C until sectioning. Brains were sectioned coronally at 40 μm using a cryostat and mounted directly onto silane-coated slides. Every fourth section throughout the hypothalamus was collected to assay for GnIH peptide.

Cells producing the peptide for GnIH in the periventricular nucleus of the hypothalamus were visualized via immunohistochemistry (IHC). Briefly, sections were fixed in 4% paraformaldehyde for 1hr. After this incubation period, sections were washed three times in phosphate buffered saline (PBS, 0.1 M) and treated with a 0.01% hydrogen peroxide solution for 10 min to reduce background immunoreactivity. Sections were washed three more times with PBS and then incubated in 2% normal goat serum in 0.2% PSB with Triton X-100 (PBS-T). This incubation (1 h) helped to block background immunoreactivity. Goat anti-rabbit affinity-purified GnIH primary antibody (PAC 123,124, antigen sequence SIKPFSNLPLRF, Bentley, Berkeley, CA, USA, used at dilution 1:5000 in 0.2% PBS-T) was used to incubate sections at a concentration of 1:5000 in 0.2% PBS-T for 48 h at 4 °C. The specificity of the antibody has been previously confirmed via preabsorbtion studies (Kriegsfeld et al., 2006; Ferris et al., 2015); rat and avian GnIH peptides share a common C terminus, and thus the same antibody can be used for both species (Kriegsfeld et al., 2006). Three subsequent washes in 0.2% PBS-T were followed by incubation in biotinylated goat anti-rabbit IgG (1:250 in 0.2% PBS-T), followed by three more washes of 0.2% PBS-T. Sections were incubated in avidin-biotin complex (ABC; Vectastain Elite kit, Vector Labs) for 1 h and visualized in Vector VIP Peroxidase (Vector Labs). Cells immunoreactive for the GnIH peptide (GnIH-ir) were counted using a Zeiss Axio Imager A1 microscope in a blind fashion in which an arbitrary number was assigned to each sample.

2.2.2. Sprague-Dawley rodents

The following protocol has been previously validated and published in this species (Kirby et al., 2009; Geraghty et al., 2015). In brief, rats were collected between 9:00 and 12:00 h by hand in less than 5 min post disturbance. Rats were anesthetized with Euthasol euthanasia solution and transcardially perfused with ice cold 0.1 M PBS followed by 4% paraformaldehyde in 0.1 M PBS. Brains were post-fixed for 24 h at 4 °C, equilibrated in 30% sucrose in 0.1 M PBS and then stored at −80 °C. Immunostaining was performed on a 1 in 4 series of free-floating 40 μm cryostat sections. Sections were rinsed in 0.1 M PBS and then incubated in 0.3% H_20_2 in PBS for 10 min to reduce background immunoreactivity. After rinsing, tissue was blocked with 2% normal donkey serum (0.3% Triton X-100 in PBS) and then transferred into a solution containing primary antibody against GnIH (PAC123,124, antigen sequence SIKPFSNLPLRF, Bentley, Berkeley, CA, USA, used at dilution 1:5000 in 0.3% PBS-T), and sections were incubated in antibody overnight, on rotation, at 4 °C. The next day, sections were rinsed in PBS and incubated in a secondary antibody for 1 h at room temperature (Biotin donkey anti-rabbit 1:500, Jackson ImmunoResearch). Following rinsing, sections were incubated in ABC reagent (Vector) and then amplified by incubating in biotinylated tyramide for 30 min. Tertiary incubation for 1 h at room temperature was followed with streptavidin-Alexa594 (1:1000 in PBS, Jackson Immunoresearch). After rinsing in PBS-T, slides were coverslipped using DABCO antifading medium and stored in the dark at 4 °C.

2.2.3. GnIH cell quantification

Cells immunoreactive for the GnIH peptide (GnIH-ir) were counted by researchers blinded to experimental groups on an Axioimager A1 (Zeiss) at 40x magnification. In starlings, GnIH-ir cells were live counted in every 4th section throughout the PVN.

In rats, GnIH-ir cells were live counted in every 6th section throughout the DMH. The total number of GnIH-ir cells in starlings and rats were multiplied by 4 and 6, respectively, to get total cell number.

2.3. Statistical analyses

Statistical analyses were performed using IBM SPSS Statistics V23 (Chicago, IL, USA). Data were tested for deviations from normality using a Shapiro–Wilk test and were found to be non-normally distributed. A square root transformation achieved normality for all data sets ($P > 0.05$), and these values were used to conduct the following analyses. In the starling dataset, an analysis of variance was performed on the response variable of GnIH cells, with sex (male, female), year (2009, 2011), and group (Reference, Nest no eggs, Early incubation, Late incubation, Chicks) as explanatory variables. In the rat dataset, only females were tested, and an analysis of variance was performed on the response variable of GnIH-ir cells, with group (Reference, Early gestation, Late gestation, Day 1 pups born, Day 4 pups born) as potential explanatory variables. Pending a significant result yielded from each model ($P < 0.05$), Tukey's Honest Significant Difference test (Tukey's HSD) was used post hoc to assess pairwise comparisons, with significance determined at $P < 0.05$ and a non-significant statistical trend determined at $P < 0.10$. Two Student's T-tests were used to assess differences in the means of our removal manipulations as compared to their un-manipulated counter sampling points: (1) the Egg removal group compared to the Late incubation group, and (2) the Day 1 pups removed group compared to the Day 1 pups born group. Mean values were deemed significantly different at $P < 0.05$, with a non-significant statistical trend determined at $P < 0.10$.

3. Results

3.1. European starlings

There was a significant effect of reproductive period on GnIH cell abundance in starlings ($F_{14,43} = 8.351$, $P < 0.001$; Table 1). Similar to previous findings in European starlings during the reproductive period (Amorin and Calisi, 2015; Calisi et al., 2011), there was no significant effect of sex on GnIH cell abundance ($F_{1,43} = 1.243$, $P = 0.271$). There was also no effect of year data were collected ($F_{1,43} = 2.613$, $P = 0.113$). Thus, data from both sexes and both years were combined for post hoc analyses.

Birds without a nest (the reference group) had more GnIH cells than those with a nest but no eggs ($P < 0.001$). Birds beginning their incubation phase had a greater abundance of GnIH cells than

Table 1
Statistical results depict a significant effect of sampling period ("Group") on GnIH cell abundance in starlings (a) and rats (b). Asterisks next to bolded numbers denote statistical significance ($P < 0.05$).

Source	Sum of squares	df	F	Sig.
(a) European starling: Results				
Intercept	21186.545	1	1728.508	<0.001
Group	409.441	4	8.351	**<0.001***
Sex	15.236	1	1.243	0.271
Year	32.028	1	2.613	0.113
Group * Year	20.633	2	0.842	0.438
Group * Sex	17.494	4	0.357	0.838
Corrected model	746.610	14	4.351	**<0.001***
Error	527.056	43		
Total	26845.500	58		
	Sum of squares	df	F	Sig.
(b) Sprague-Dawley rat: Results				
Intercept	2000.351	1	262.397	<0.001
Group	112.583	4	3.692	**0.010***
Error	388.792	51		
Total	2672.071	56		

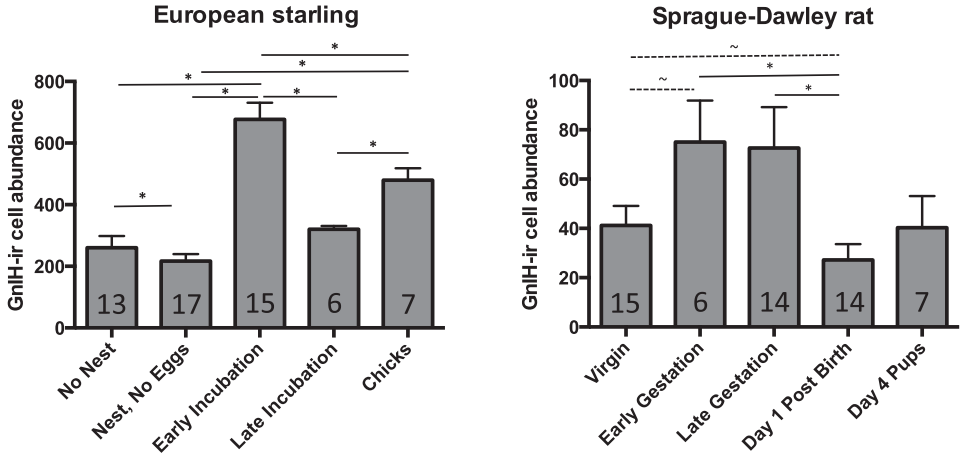

Fig. 2. Hypothalamic cells immunoreactive for the GnIH peptide significantly change in abundance during the transition to parental care behaviors in male and female starlings (left) and female rats (right). Similar to the findings of Calisi et al. (2011), male and female starlings did not differ in GnIH cell abundance, and data were combined for post hoc analyses and for depiction in this graph. Asterisks denote statistical significance ($P < 0.05$); a tilde denotes a non-significant trend ($P < 0.1$). The number within each bar indicates group sample size.

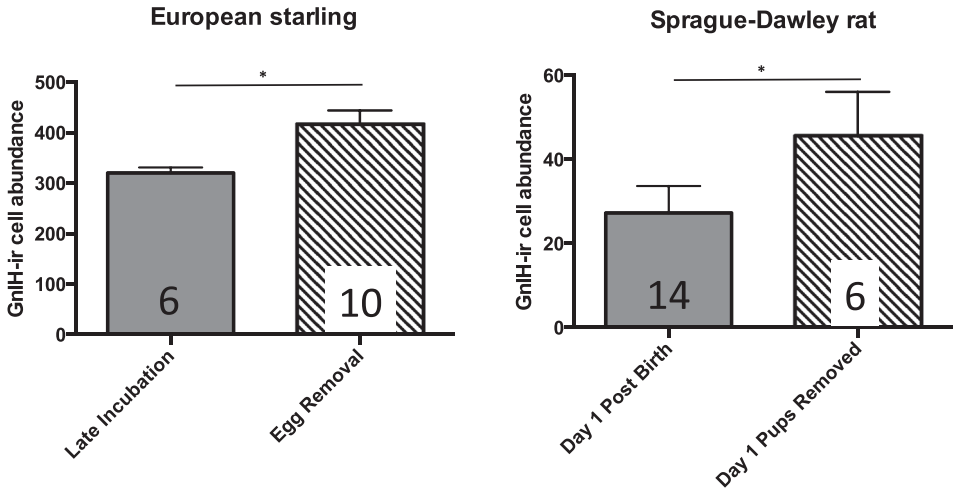

Fig. 3. Hypothalamic cells immunoreactive for the GnIH peptide significantly increase in parents when eggs (left) or pups (right) are removed from the nest as compared to cell abundance in parents with eggs/pups at the same time point. Asterisks denote statistical significance ($P < 0.05$). The number within each bar indicates group sample size.

those with a nest but that had not yet laid eggs ($P < 0.001$). Birds with a nest but no eggs also had fewer GnIH cells as compared to birds caring for newly hatched chicks ($P < 0.001$). During their early incubation phase, birds had more GnIH cells than birds that had entered into their late incubation phase ($P < 0.001$). Birds with newly hatched chicks had more GnIH cells than those undergoing their late incubation phase ($P = 0.035$). In sum, GnIH cells were most highly abundant during two reproductive stages – when birds first began incubating their eggs and when newly-hatched chicks were present (Fig. 2). Our egg removal manipulation resulted in an increase in GnIH cell abundance as compared to birds with eggs that were in Day 10 of their incubation phase ($t(5) = -3.617$, $P = 0.015$).

3.2. Sprague-Dawley rodents

There was a significant effect of reproductive period on GnIH cell abundance in rodents ($F_{4,51} = 3.692$, $P = 0.010$; Table 1). Females experiencing both early and late stages of gestation had significantly more GnIH cells as compared to females caring for newborn pups (early gestation, $P = 0.003$; late gestation, $P = 0.002$; Fig. 2). Virgin animals (the reference group) showed a trend for having fewer GnIH cells than those experiencing their early gestation phase, but this relationship was not statistically significant ($P = 0.077$). Virgin animals also showed a trend for having more GnIH cells than those one day postpartum, but this relationship was not statistically significant ($P = 0.096$). Our experimental removal of pups on Day 1 post birth resulted in a significant increase in GnIH cell abundance as compared to females with pups at the same time point ($t(5) = -2.830$, $P = 0.037$; Fig. 3).

4. Discussion

We characterized the pattern of GnIH cell abundance over various reproductive stages in European starlings and rats. We discovered that important transition points during the parental care stage – the initiation of incubation behaviors and presence of hatchlings in starlings, and the presence of pups in rats – are associated with significant changes in the abundance of GnIH cells in the hypothalamus. Changes in GnIH cell abundance in starlings and rats happened during relatively similar reproductive time points, although the directionality of these changes differed between the species – namely changes in GnIH cell abundance between the species were not always positively related. We discuss possible reasons for this difference below. Further, we found that an unpredictable change in environment during a time of parental care – the removal of eggs/pups – affects GnIH cell abundance similarly in both starlings and rats.

Parental care is a suite of behaviors undertaken by a vast array of taxa to promote offspring survival, with clear and direct links to evolutionary fitness. Some of the most commonly studied hormones implicated in the activation and maintenance of parental care behavior in vertebrates include oxytocin and vasopressin (avian homologues: mesotocin and vasotocin), vasoactive intestinal peptide, and prolactin (e.g. (Goodson and Kingsbury, 2011; Ross et al., 2009; Vleck and Vleck, 2010; Young et al., 2011)). Since its discovery in 2000 (Tsutsui et al., 2000), GnIH has been found to affect vertebrate reproductive physiology in a number of ways, although no association of it to parental care has been investigated. Previously, we reported that the number of cells producing the peptide for GnIH increased when male and female European starlings began to incubate their eggs (Calisi et al., 2011). This study offered the first report of the effects of social environment on GnIH and inspired our current study examining patterns of GnIH cell abundance over the reproductive period in multiple taxa.

Changes in testosterone and prolactin often facilitate behaviors important for parental care. Testosterone can negatively affect parental care behaviors, and GnIH has been reported to have an inhibitory effect on circulating testosterone (Ubuka et al., 2006; Calisi, 2014). Testosterone in many birds, including European starlings (Calisi et al., 2011; Pinxten et al., 2007), can decrease during the parental care period to facilitate parental behaviors (Ketterson and Val Nolan, 1994; Magrath and Komdeur, 2003). European starling testosterone peaks during nest building, remains high during the fertile period, particularly if nesting sites are close together, and then gradually decreases during the period of parental care (Ball and Wingfield, 1987; Pinxten et al., 2007). The increase in GnIH activity at this time may be related to the inhibition of testosterone to facilitate parental care either by directly acting on the gonads and/or acting within the brain to facilitate the switch from aggressive and sexual behaviors to parental care behaviors (Bentley et al., 2009; Calisi, 2014). Receptors for GnIH are expressed on two avian hypothalamic populations of GnRH cells: GnRH-I and GnRH-II (Ubuka et al., 2008a). GnRH-II is thought to play a role in facilitating sexual behaviors (Kauffman and

Rissman, 2004; Maney et al., 1997), and thus GnIH may aid in the transition of behavior during this time by inhibiting the activity or release of GnRH-II. In addition, GnIH may not affect circulating prolactin, which often facilitates incubation (El Halawani et al., 1984; March et al., 1994) and chick care in birds (Angelier and Chastel, 2009; Miller et al., 2009; O'DWYER et al., 2006), and pregnancy and lactation in mammals (Andrews, 2005). In a study conducted using cultured ovine pituitary cells from both ewes and rams, administration of the GnIH homologue RFRP-3 decreased the expression of LHβ and FSHβ subunit genes, but there was no effect on the expression of prolactin genes (Sari et al., 2009). Thus, the increased number of GnIH cells at important transition points of parental care during the reproductive period may facilitate the inhibition of testosterone without interfering with prolactin production.

In view of the previous findings of (Calisi et al., 2011) and the parallel response of GnIH in starlings and rats to external stimuli (Calisi et al., 2008; Kirby et al., 2009), we originally predicted that GnIH cell abundance would increase at the beginning of incubation and chick presence in starlings and gestation and pup care in rat dams as compared to prior time points (nesting and late incubation in birds, and prior to copulation and parturition in rats). All predictions were supported except for the last: cell abundance in rat females decreased rather than increased at Day 1 post-parturition. We posit that this occurrence may be related to the postpartum estrus that these rats experience. Sprague-Dawley rats, similar to many mammals, experience a postpartum estrus in which they ovulate within 24 h of parturition (Connor and Davis, 1980a,b). If a female conceives during this period, she will be able to gestate while simultaneously lactating to feed her current offspring. We found that GnIH cell abundance is low at this time as compared to the last day of gestation, and this lessened inhibition to the reproductive axis could facilitate the reproductive physiology and sexual behaviors associated with postpartum estrus.

The female rats in our study were not exposed to males postpartum and thus did not conceive within 24 h of parturition. They entered into a lactational diestrus in which estrous cycles are delayed until pups are weaned (about 25–30 days). Prolactin is a well-known facilitator of lactation. Administration of hRFRP-1, a mammalian GnIH homologue, results in an increase in plasma prolactin levels in rats (Hinuma et al., 2000). GnIH expression is also high in lactating rats sampled at day 8 of lactation as compared to rats separated from their pups early on and sampled at day 8 post-parturition (Adavi et al., 2011). While we did not see a significant increase in GnIH cell abundance in lactating rats on day 4 post-parturition as compared to day 1, cell abundance on day 4 did not differ from the high abundance documented on the last day of gestation.

In addition to characterizing GnIH cell abundance over the predictable course of the reproductive period in starlings and rats, we experimentally removed eggs and pups to examine how unpredictable events during this time could affect GnIH. In both species, cell abundance increased as a result of egg/pup removal. Initially, we expected a decrease in GnIH cell abundance would be most likely in order to facilitate reproductive activity to replace lost offspring. However, GnIH can increase in response to acute stress in seasonally breeding birds (Calisi et al., 2008) and acute and chronic stress in rats (Kirby et al., 2009). Thus, in this case, perhaps an increase in reproductive inhibition may be important, because reproducing when there is a known predator afoot may be maladaptive. However, a later sampling point after offspring removal might indeed follow our originally predicted pattern of GnIH cell abundance, and further study is needed to gain a better picture of how GnIH responds to an unpredictable event over time.

In summary, we report changes in patterns of hypothalamic neuropeptide GnIH cell abundance over the reproductive period in starlings and rats (Fig. 4). The increase in the number of GnIH peptide cells in starlings laying and incubating eggs may help to mediate the neural inhibition of aggressive and sexual behaviors during incubation. The opposite pattern presented in female rodents, a decrease in GnIH cells 1 day post-parturition, may be necessary to promote female postpartum estrus. Further studies that involve the manipulation of GnIH and the measurement of consequent effects on parental care behaviors and physiology are necessary to test these hypotheses. Our removal manipulation of eggs and pups resulted in an increase in GnIH cell abundance in both species, revealing a conserved response of GnIH to an unpredictable change in the environment during the reproductive period. Taken together, our results suggest dynamic activity of GnIH over the reproductive period and inspire further investigation of the causes and consequences of such activity, especially surrounding the parental care stage.

Fig. 4. An artistic representation of our investigation of hypothamalic GnIH in rats and European starlings during the reproductive period.

Acknowledgments

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