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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