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Seasonal differences in hypothalamic EGR-1 and GnIH expression following capture-handling stress in house sparrows (*Passer domesticus*)

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ABSTRACT

Stress is a known inhibitor of reproductive function. The mechanisms by which stress acts to influence the reproductive axis have been intensely studied and appear to be extremely varied. Gonadotropin-releasing hormone (GnRH) is a critical component of the vertebrate reproductive axis and directly causes pituitary gonadotropin synthesis and release. A second neuropeptide, gonadotropin-inhibitory hormone (GnIH), directly inhibits pituitary gonadotropin synthesis and release in birds. We hypothesized that stress effects upon reproduction are mediated via the hypothalamic GnIH system. We examined the effects of capture-handling stress in the hypothalamus of male and female adult house sparrows (*Passer domesticus*) at the start (spring) and end of the breeding season (fall). We quantified numbers of GnIH neurons to provide an estimate of hypothalamic GnIH content. In addition, we quantified the expression of the protein product of the immediate-early gene, EGR-1, using this as an indicator of neuronal activation. We saw an increase in EGR-1 positive cells in the paraventricular nuclei of stressed birds as opposed to controls at both collecting times, but this stress response was more apparent in the spring as opposed to the fall. There were more GnIH-positive neurons in fall birds versus those sampled in the spring, and a significant increase in GnIH positive neurons was seen in stressed birds only in spring. GnIH cells show little to no activation of EGR-1, suggesting that EGR-1 is not involved in GnIH transcription in response to capture-handling stress. These data imply an influence of stress upon the paraventricular nucleus and the GnIH system that changes over the annual cycle of reproduction.

1. Introduction

Stress can have negative impact upon the reproductive system (Siegel, 1980; Moberg, 1991; Moore and Jessop, 2003; Boonstra, 2004). Our definition of stress here is in line with that used by John Wingfield and colleagues: that which occurs in association with unpredictable and life-threatening perturbations in the environment and invokes an "emergency life-history stage" (Wingfield et al., 1998; Wingfield and Sapolsky, 2003). Demands associated with specific stages of the predictable life-history cycle (as per Jacobs and Wingfield, 2000) are also considered stressful by some, but it is perhaps more useful to consider these demands as more predictable physiological demands, and thus not "stressful" per se. The concepts of allostasis and allostatic load permit differentiation between physiological states in the contexts of predictable and unpredictable energetic and behavioral demands (Sterling and Eyer, 1988; McEwen, 2002; McEwen and Wingfield, 2003). For passerine birds, perturbations such as capture-handling can induce a massive stress response that results from apparent perception of a life-threatening situation (Wingfield et al., 1992). In this paper we provide evidence in birds that stress can activate gonadotropin-inhibitory hormone (GnIH), a neuropeptide system that inhibits reproductive physiology and behavior in birds and mammals. Thus, we propose that stress-induced inhibition of reproduction might, to some degree, be mediated via the GnIH system.

For all vertebrates studied, reproduction is regulated by gonadotropin releasing hormone (GnRH) of one form or another. GnRH is released from neurons in the preoptic area of the hypothalamus to the median eminence, where it causes the anterior pituitary gland to release the gonadotropic hormones luteinizing hormone (LH) and follicle-stimulating hormone (FSH) into the bloodstream. LH and FSH act on the gonads and cause them to develop and increase in activity.

Gonadotropin-inhibitory hormone (GnIH) is a neuropeptide that inhibits gonadotropin release from cultured quail anterior pituitary (Tsutsui et al., 2000). GnIH inhibits pituitary gonadotropin release in vitro (Tsutsui et al., 2000; Ciccone et al., 2004), and in vivo in birds (Osugi et al., 2004; Bentley et al., 2006a; Ubuka et al., 2006) and mammals (Kriegsfeld et al., 2006; Johnson et al., 2007). GnIH also inhibits gonadotropin synthesis in vivo in birds (Ubuka et al., 2006), and GnIH and its mammalian homolog inhibit reproductive behaviors in birds and mammals (Bentley et al., 2006b; Johnson et al., 2007).

GnIH producing neurons are located in the paraventricular nucleus (PVN) of birds and dorsomedial hypothalamus (DMH) of

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rodents and appear to make direct contact with GnRH-I producing neurons in birds and mammals (Bentley et al., 2003, 2006a,b; Kriegsfeld et al., 2006; Ubuka et al., 2008). Furthermore, GnIH receptor mRNA is expressed in GnRH-I and -II neurons (Ubuka et al., 2008). Thus there is potential for GnIH to influence the activity of GnRH neurons directly, in addition to the action of GnIH upon pituitary gonadotropin release. Significantly for the study of stress and its role in regulating reproduction, GnIH-producing neurons in rats also express glucocorticoid receptors (Kirby et al., 2007). This suggests that the glucocorticoid stress response could be directly communicated to GnIH cells and from there to the reproductive axis, given the neural architecture of the GnIH/GnRH systems.

Physiologically, a stress response generally involves an increase in adrenal glucocorticoid secretion, which can reduce activity of the hypothalamo–pituitary–gonadal (HPG) axis (Moberg, 1985; Sapolsky, 1987, 1992; Romero and Sapolsky, 1996; Wingfield and Romero, 2000; Wingfield and Sapolsky, 2003). The action of corticotropin releasing hormone (CRH) and glucocorticoid feedback on the hypothalamus may thus inhibit GnRH-I release (Williams et al., 1990).

The glucocorticoid stress response varies seasonally in many species (birds: Wingfield, 1994; Breuner and Orchinik, 2001; Romero, 2002; Romero et al., 2006; mammals: Kenagy and Place, 2000; herpetofauna: Licht et al., 1983; Smith and John-Adler, 1999). We used a seasonally breeding songbird species, house sparrow (*Passer domesticus*), to examine GnIH at both the beginning (spring) and end (fall) of the breeding season in association with a capture-handling stress response. A capture-handling stress paradigm is known to elevate glucocorticoid levels in many species, including house sparrows (Wingfield et al., 1992; Wingfield, 1994; Breuner et al., 1999; Wingfield and Romero, 2000). Therefore we further assessed the physiological effects of this manipulation by evaluating the neuronal response within the PVN by measuring the expression of the protein product of the immediate early gene EGR-1. Activation of immediate-early genes, such as EGR-1, is thought to be one of the first measurable neurobiological responses to external stimuli. As such, immediate-early genes are excellent markers of neuronal activity in response to environmental input (Morgan and Curran, 1989, 1995). Therefore we measured EGR-1 positive cells in the PVN, the only area in the avian brain known to contain GnIH positive neurons, as a measure of that area's response of stress. Given our hypothesis that GnIH is involved in mediation of stress-induced reproductive dysfunction, we predicted that GnIH-producing neurons would be more abundant in stressed animals. Due to seasonal differences in the stress response as well as possible seasonal differences in the presence and role of GnIH (as per Bentley et al., 2003), we predicted a difference in the response of GnIH-producing neurons to stress at the beginning versus the end of the breeding season.

2. Materials and methods

2.1. Animals

Adult male and female house sparrows (*Passer domesticus*) were collected in 2007 in Lodi, California using Japanese mist nets. Birds were sampled during the second week of February (9 males, 6 females) and the last week of July though the first week of August, just prior to molting (10 males, 7 females), which are the beginning and end of the northern California house sparrow breeding season, respectively (Keck, 1934; Calisi and Bentley, pers. obs.). Birds were transferred to aviaries and kept under ambient photoperiod for 1 week before sampling. Using a common capture-handling stress paradigm known to elevate glucocorticoid levels (Wingfield, 1994; Breuner et al., 1999; Wingfield and Romero, 2000), birds were held in small ventilated bags for 1 h prior to killing by decapitation (spring: 4 males, 2 females; fall: 4 males, 3 females). Controls were killed within 10 min of researchers entering the aviary in which they were housed (spring: 5 males, 4 females; fall: 6 males, 4 females). After decapitation, brains were fixed in 4% paraformaldehyde and sucrose solutions and later stored in −80 °C until sectioning. Although house sparrows generally do not show sex differences in the glucocorticoid response (Breuner and Orchinik, 2001), we tested for sex differences in both EGR-1 and GnIH cell abundance in control and stressed animals. All procedures were approved by and in compliance with the University of California Office of Lab Animal Care and federal regulations.

2.2. Immunocytochemistry

2.2.1. EGR-1

Brains were sectioned coronally at 40 μm using a cryostat and collected into phosphate buffered saline (PBS). Every fourth section throughout the hypothalamus was collected for immunocytochemistry. Sections were washed three times in PBS and treated with 0.01% hydrogen peroxide in methanol for 10 min to reduce background immunoreactivity. Sections were again washed three times with PBS and then submerged in 5% normal goat serum (NGS) in 0.2% PBS-T (PBS + Triton X-100) for 1 h to block background immunoreactivity. EGR-1 primary antibody (code sc189, Santa Cruz Biotechnology Inc.) was used to incubate sections for 48 h at a concentration of 1:1000 in 0.2% PBS-T. Three subsequent washes in 0.2% PBS-T were followed by an hour of 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 0.03% 3,3-diaminobenzidine (DAB) intensified with 0.15% nickel chloride for 5 min.

2.2.2. GnIH

Directly following EGR-1 visualization, sections were washed five times in 0.2% PBS-T. Goat anti-rabbit affinity-purified GnIH primary antibody (code PAC 123/124) was used to incubate sections for 24 h at a concentration of 1:5000 in 0.2% PBS-T. Three subsequent washes in 0.2% PBS-T were followed by an hour of incubation in Alexa Fluor 568 (Invitrogen Labs) at a concentration of 1:500 in 0.2% PBS-T for visualization.

2.3. Quantification of EGR-1 and GnIH Immunoreactivity

Using a Zeiss Axio Imager A1 microscope and AxioVision 4.5 software, photographs were taken of the PVN, which was defined by the presence of GnIH-immunoreactive (GnIH-ir) neurons. We counted the total number of EGR-1-ir nuclei within the field of vision of the GnIH-ir cell bodies and the total number of GnIH-ir cell bodies throughout the PVN. We noted any colocalization between EGR-1 nuclei and GnIH neurons. The average number of cells per section was used for analysis.

2.4. Statistical analysis

An analysis of variance (ANOVA) was performed on the response variables of EGR-1 and GnIH positive cells, with sex, breeding season and stress response as explanatory variables. Significance was determined based on sequential Bonferroni adjustments using the Dunn–Sidák method. Independent *t*-tests were then preformed to examine breeding season inter- and intra-differences in the EGR-1 and GnIH stress response. Because there was no effect of sex, male and female data were combined for analysis. Significance, after Bonferroni adjustment, was determined at $P<0.0125$.

3. Results

3.1. EGR-1 response to stress

The ANOVA on EGR-1 showed a significant effect of breeding season ($F_{1,24}=12.96$, $P=0.001$) and stress ($F_{1,24}=24.83$, $P<0.001$). The individual *t*-tests showed an increase in EGR-1 positive cells in stressed birds as opposed to controls (spring: $P=0.002$; fall: $P=0.002$), and stressed birds had higher EGR-1 positive neuron numbers in the spring compared to the fall ($P<0.001$). EGR-1 showed minimal co-localization with GnIH positive neurons.

3.2. GnIH response to stress

The ANOVA on GnIH showed a significant effect of breeding season ($F_{1,24}=10.04$, $P=0.004$), but not stress, most likely due to a difference in the GnIH stress response seen during the spring versus the fall. A significant increase in GnIH positive neurons was seen in stressed birds only in the spring ($P=0.007$) as compared to the fall, and GnIH positive neuron numbers in controls were higher in birds in the fall as compared to the spring ($P=0.001$). See Table 1 for complete statistical results, Figs. 1 and 2 for EGR-1 and GnIH cell number means and standard errors, respectively, and Fig. 3 for representative photographs.

Table 1
Comparisons of EGR-1 and GnIH cell numbers between sexes, stress and control groups, and seasons

	EGR-1			GnIH		
	F	df	*P*	*F*	df	*P*
ANOVA						
Sex	0.10	1,24	0.76	0.48	1,24	0.83
Season	12.96	1,24	0.001*	10.04	1,24	0.004*
Stress	24.83	1,24	<0.001*	2.18	1,24	0.15
Sex × Season	0.03	1,24	0.87	0.03	1,24	0.86
Sex × Stress	0.41	1,24	0.53	0.05	1,24	0.82
Season × Stress	2.56	1,24	0.12	2.45	1,24	0.13
Sex × Stress × Season	0.00	1,24	0.99	0.20	1,24	0.66
t-Test						
Stressed vs. Control						
Spring	−3.85-	13	0.002*	−3.178	13	0.007*
Fall	−3.739	15	0.002*	−0.025	15	0.98
Spring vs. Fall						
Stressed	−5.035	11	<0.001*	1.227	11	0.25
Control	−1.519	17	0.15	3.913	17	0.001*

Because there were no sex differences, male and female data were pooled to examine differences between the fall and the spring. Asterisks denote statistical significance.

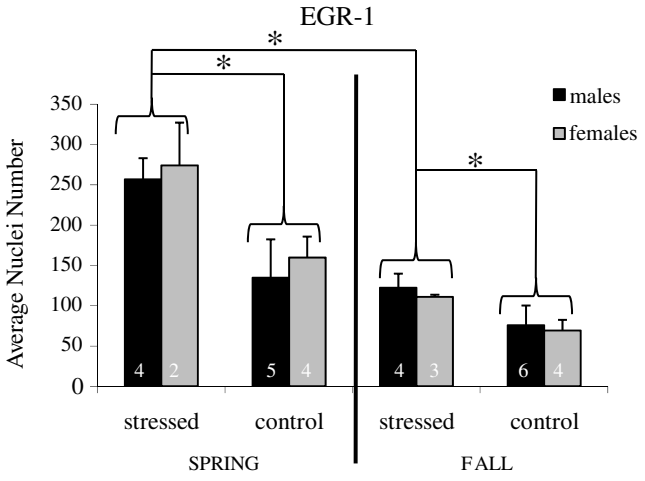

Fig. 1. EGR-1 is expressed in more cells of stressed birds at start and end of breeding season compared to control group. However, EGR-1 stress response is attenuated at end of breeding season, as opposed to start. There is no difference between males and females within groups. Numbers within bars indicate sample sizes. Asterisks denote statistical significance.

4. Discussion

There is strong evidence that GnIH acts in direct opposition to GnRH and it is thus a key regulator of the reproductive axis and associated behaviors. We hypothesized that the inhibitory effects of stress on reproduction may be mediated via the GnIH system. Because physiological responses to stress can vary seasonally (Wingfield et al., 1982, 1992, 1994; Astheimer et al., 1994; Romero et al., 1997; Romero and Remage-Healey, 2000), we examined the stress response both at the start and termination of breeding in a seasonally breeding songbird. Elevated levels of glucocorticoids often occur in stressed and control animals during breeding as compared to non-breeding time-periods (Dawson and Howe, 1983; Romero et al., 1998a,b,c; Breuner and Orchinik, 2001; Romero, 2002; but see Breuner et al., 1999; Romero and Remage-Healey, 2000; Romero, 2002). We localized and quantified neurons expressing the protein product of EGR-1 to assess the cellular response of the PVN to capture-handling stress. In addition, we measured the numbers of GnIH-ir neurons in control and stressed birds in spring and fall.

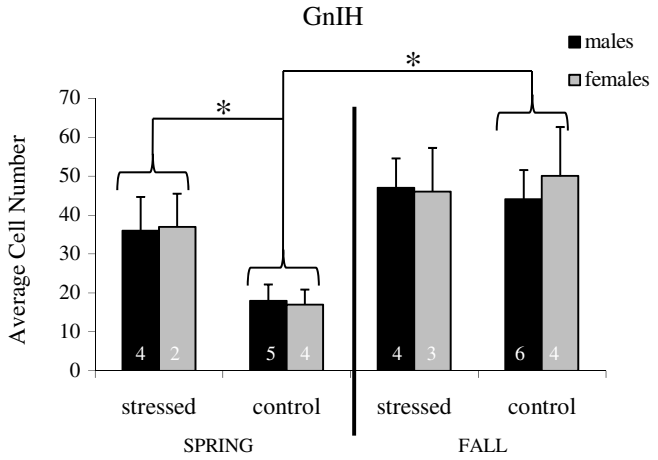

Fig. 2. More GnIH cells were observed in stressed birds at start of breeding season compared to control group. More GnIH cells were present overall at the end of the breeding season, as opposed to the start. There is no difference in number of cells expressing GnIH at the end of breeding season between stressed and control birds. There is no difference between males and females within groups. Numbers within bars indicate sample sizes. Asterisks denote statistical significance.

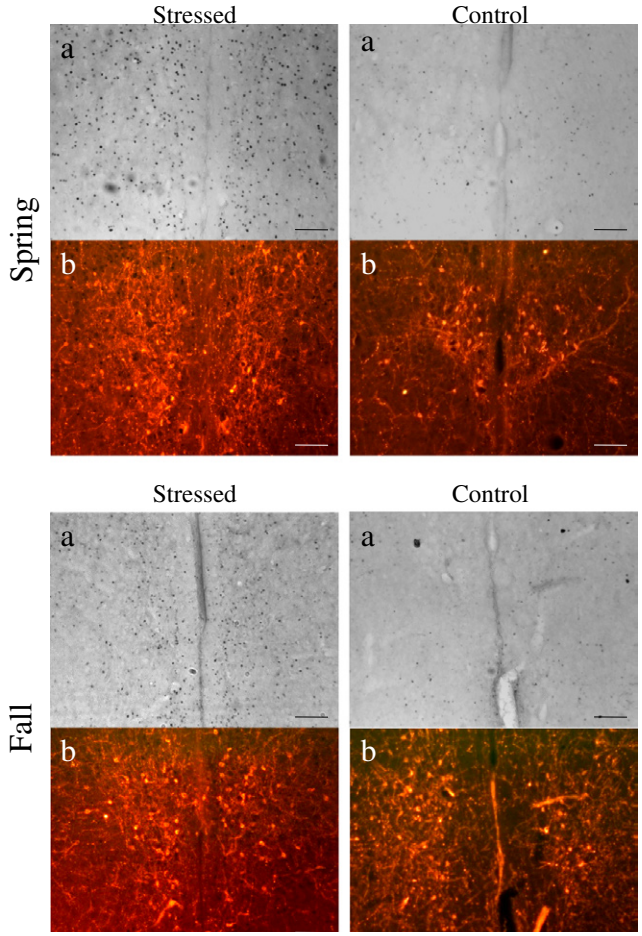

Fig. 3. Representative photographs of EGR-1 (a) and GnIH (b) positive cells in the PVN of the hypothalamus in stressed vs. control animals at the start and end of the breeding season. Scale bars are 100 μm.

4.1. EGR-1 response to stress

EGR-1 positive cells were more abundant in stressed birds compared to controls, independent of sampling time. Cell numbers

were not significantly different in control animals at the start or at the end of the breeding season, but stressed animals at the start of the season possessed a significantly higher number of EGR-1-positive cells as opposed to stressed animals at the end of the breeding season. A capture-handling stress paradigm is known to elevate glucocorticoid levels in house sparrows and other species (Wingfield, 1994; Breuner et al., 1999; Wingfield and Romero, 2000), many-fold, with baseline and stress response glucocorticoid levels being significantly higher in breeding versus non-breeding animals (Dawson and Howe, 1983; Romero et al., 1998a,b,c; Breuner and Orchinik, 2001; Romero, 2002). Our EGR-1 data confirm this stress response pattern and extend it to a cellular response in the PVN.

GnIH cells show little to no activation of EGR-1, suggesting that EGR-1 is not involved in transcribing the resulting GnIH protein. Another immediate early gene, FOS, co-localizes with GnRH cells in mammals in response to various stimuli but may not be directly responsible for its release (Moenter et al., 1993; Witkin et al., 1994). Even after stimulation by long days or *N*-methyl-D-aspartate (NMDA) and a resulting release of LH (presumably as a result of increased GnRH release), co-localization of FOS and GnRH has not been observed in birds, although FOS activation in the hypothalamus and GnRH release were positively correlated (Meddle and Follett, 1997; Meddle et al., 1999). Mechanisms regulating GnIH secretion within this experimental paradigm are not yet known and require further study.

4.2. GnIH response to stress

Baseline (control) GnIH cell abundance was greater in the fall than the spring, and a response of the GnIH system to stress was only observed in the spring. Perhaps the lack of observable response of the GnIH system to stress was a result of maximal GnIH production during the fall, so no further increase could be induced. In the absence of data to the contrary and in light of Bentley et al. (2003), we assume that GnIH is maximal at this time of year. A similar increase in GnIH content at the termination of breeding occurs in song sparrows (*Melospiza melodia*; Bentley et al., 2003). It is possible that this increase in GnIH facilitates the fine-tuning of termination of reproduction in these species. Another possible explanation for the lack of change in GnIH in the fall is that animals have an attenuated stress response at the end of the breeding season (Romero, 2002; Breuner and Orchinik, 2001), and the GnIH stress response may also follow this pattern.

The GnIH stress response seen at the beginning of the breeding season may reflect a mechanism by which reproduction is slowed or halted in stressful conditions. This response could prove adaptive because animals experiencing a stressor may immediately and transiently be able to shunt physiological resources away from reproductive effort and towards metabolic demands associated with stress. The involvement of GnIH would allow for rapid changes in behavior without the need for long-term inactivation of the reproductive axis (GnRH system), as occurs at the end of the breeding season. Along these lines, GnIH and its receptor are present in avian gonads and reproductive tract (Bentley et al., 2008; McGuire et al., 2008), and rapid activation of the gonadal GnIH system during the breeding season might influence gonadal steroid release and reproductive behaviors.

By using the method of immunocytochemistry to measure EGR-1 and GnIH cell abundance, we are unable to elucidate the temporal dynamics of GnIH synthesis and release in response to our stimulus. An increase in cell number could signify the increased production of the EGR-1 and GnIH protein, but it may also be indicative of the decreased release of stored protein. However, data on GnIH mRNA in rats suggest the former (Kirby et al., 2007). Recently, GnIH was cloned in house sparrow (McGuire et al., 2008), which will permit us further study of this matter. By performing *in situ* hybridization, we will be able to examine GnIH mRNA and thus GnIH production.

In sum, our data show an effect of stress at the cellular level in the PVN of house sparrows. EGR-1 neuron number is elevated in stressed birds relative to respective controls in both the spring and fall seasons. The EGR-1 response to acute stress also shows seasonal variation, with higher cell number in stressed birds in the spring than in fall. EGR-1 showed little to no co-localization with GnIH cells, suggesting that EGR-1 most likely does not directly transcribe the GnIH peptide in this paradigm. GnIH neuron number exhibited a differential seasonal response to stress, with GnIH being more abundant in the fall, but only showing a significant increase in stressed birds in the spring. These data imply that GnIH might act as a gating system for the effects of stress on the reproductive axis at different times of year. Before we can conclude this, we must use this seasonal approach to examine GnIH mRNA in stressed birds as well as differential affects of stress on gonadotropin synthesis and secretion.

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