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Lipopolysaccharide injection induces rapid decrease of hypothalamic GnRH mRNA and peptide, but does not affect GnIH in zebra finches

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ABSTRACT

Lipopolysaccharide (LPS) is frequently used experimentally to mimic acute infection. Through activation of the host's immune response, an LPS injection has profound effects on the adrenocortical response to stress and on behaviors including reduction in activity, water and food intake, and libido. These behavioral changes occurring during infection are collectively called "sickness behavior." It is thought that adoption of sickness behavior reallocates energy from other fitness-enhancing activities, such as reproduction, for use in the immune response. Although the behavioral effects of LPS treatment are well-known, less information is available regarding the effects of LPS on the brain in terms of controlling reproductive behavior, specifically concerning a newly discovered neuropeptide, gonadotropin-inhibitory hormone (GnIH). This study investigated the effects of an LPS injection on the behavior and the hypothalamic neuropeptides controlling reproduction [GnIH and gonadotropin-releasing hormone (GnRH)] of zebra finches (*Taeniopygia guttata*). Overall, there was a decrease in activity in birds injected with LPS. The number of GnRH-immunoreactive neurons was significantly reduced in birds injected with LPS when compared to controls, while the number of GnIH-releasing neurons remained unchanged. At the level of gene expression, a similar pattern was found: there was reduced expression of GnRH mRNA in LPS-injected animals, whereas GnIH expression remained unchanged. Plasma testosterone did not change significantly in LPS-injected animals, nor did plasma corticosterone. Taken together, these results indicate a rapid (within 3 h) inhibition of the reproductive axis during an immune challenge mimicking an infection, specifically acting on the GnRH system. The present study expands our knowledge on the interaction between the immune system and the reproductive system.

Introduction

Animals possess a finite amount of energy to distribute to their daily activities (such as foraging, growing, reproducing, and immune function). Thus, if any given activity must be prioritized energetically, there will be less energy available for all others. For example, it has been hypothesized that animals experiencing an infection will save energy for the immune response by reducing their overall activity. These "sickness behaviors" include lethargy, apathy, anorexia (decreased food consumption), and adipsia (decreased fluid intake) (Hart 1988). Such responses often occur simultaneously with the suppression of other functions, e.g. reproduction, that are not essential for immediate individual survival (Ashley and Wingfield, 2012; Bonneaud et al., 2003; Owen-Ashley and Wingfield, 2006; Yirmiya et al., 1995).

In vertebrates, reproduction is regulated by the hypothalamic pituitary gonadal (HPG) axis. In males, the pulsatile release of the hypothalamic peptide gonadotropin-releasing hormone-I (GnRH-I) stimulates the pituitary gland, causing synthesis and release of the gonadotropins (luteinizing hormone (LH) and follicle stimulating hormone (FSH)). LH travels through the blood stream, ultimately causing testosterone synthesis and secretion from the testis. Testosterone has a multitude of behavioral effects in adult birds, including activation of sexual behavior, mate attraction and mate guarding (Hau 2007; Wingfield et al., 1990). Another neuropeptide, gonadotropin-inhibitory hormone (GnIH), acts within the hypothalamus, pituitary gland and gonads and has an overall suppressive effect on the HPG axis (for a review see Tsutsui et al., 2012). In addition to its neuroendocrine effects, GnIH has been linked to inhibition of reproductive behavior (birds: Bentley et al., 2006; mammals: Johnson et al., 2007). GnIH is also a potential mediator of energy balance (Clarke et al., 2009, Johnson et al., 2007, Qi et al., 2009, Tachibana et al., 2005). To our knowledge, GnIH has never been explored in the context of sickness behavior.

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Lipopolysaccharide (LPS), a component of Gram-negative bacterial cell wall, is routinely administered experimentally to induce sickness behavior in animals, and it appears to disrupt gonadotropic functions in several mammals (for a review, see: Tomaszewska-Zaremba and Herman 2009). Most of the effects of endotoxin challenge on the reproductive system have been explored in females. For example, in ewes, exposure to an endotoxin causes: suppression of the pulsatile release of GnRH and LH secretion (Harris et al., 2000), inhibition of pituitary responsiveness to GnRH (Williams et al., 2001), disruption of the follicular phase (Battaglia et al., 2000), and disruption of cyclicity and induction of preterm labor (Schlafer et al., 1994). Also in female rats and monkeys, LH release is suppressed by endotoxin injection (rats: He et al., 2003; Iwasa et al., 2008; Watanobe and Hayakawa, 2003; and monkeys: Xiao et al., 2000). The work by He et al. (2003) suggests that the inhibitory effect of LPS on the HPG axis occurs upstream of the pituitary, because an intravenous injection of GnRH still induces LH release in LPS-injected female rats. Males are not well studied in the context of the effects of an LPS injection on the HPG axis. Nonetheless, castrate rats exhibit reduced levels of LH after receiving an injection or an implant of LPS (Ebisui et al., 1992, Refojo et al., 1998, Rivest and Rivier, 1993; Rivier, 1990). Only two studies report the effect of an LPS injection on LH levels in birds, but the pattern appears to be similar to what is observed in mammals. Twenty-four hours after exposure to an endotoxin challenge, male and female white crowned-sparrows, *Zonotrichia leucophrys*, demonstrated low circulating LH levels (Owen-Ashley et al., 2006). Song sparrows (*Melospiza melodia*) have their LH levels reduced at 6 h after an LPS injection, but LH levels are no different from control-injected birds at 22 h post-injection (Adelman et al., 2010). To our knowledge, the brain neuropeptides controlling reproduction have never been explored in birds subjected to an endotoxin challenge.

Testosterone can have a suppressive effect on sickness behavior in male birds (Ashley et al., 2009). Hence, at least one component of the HPG axis interferes with the avian sickness response. Due to this intriguing relationship between testosterone and sickness behavior and our limited knowledge of how the immune system affects the reproductive system, especially in non-mammalian species, the present work was aimed at studying the effect of LPS administration on neuroendocrine components of the reproductive axis in male birds.

We predicted that males experiencing infections and exhibiting sickness behaviors would down-regulate their reproductive axis by increasing levels of GnIH mRNA and peptide, indicating an important role of GnIH in mediating the communication between immune and reproductive status. Additionally, we predicted that GnRH mRNA and peptide levels would be reduced, in similarity to what is found in mammalian females. We also predicted a decrease in circulating testosterone levels after LPS injection as a consequence. Because activation of the hypothalamic–pituitary–adrenal (HPA) axis is frequently seen upon an endotoxin challenge, an increase in plasma corticosterone was expected.

This study explores for the first time the effects of an endotoxin challenge on the main hypothalamic sites controlling reproductive function in male birds and specifically tries to generate new insights into the role GnIH might play during the course of an infection.

Methods

Animals and experimental design

The experiment was carried out in two phases (Phase I — April 2009 and Phase II — May 2011) at the University of California, Berkeley Field Station for the Study of Behavior, Ecology and Reproduction. All procedures were approved by and in compliance with the University of California Office of Lab Animal Care and federal regulations.

A colony of zebra finches (*Taeniopygia guttata*), including adult males, females and juveniles was housed in a 2.7 m by 2.5 m by 2.1 m indoor flight aviary. They were exposed to natural changes in day length, supplemented by artificial lighting at a light/dark schedule of 12 L:12 D. Food and water were provided ad libitum and consisted of German millet mixed with canary seed. All birds in our colony are uniquely color banded.

To facilitate individual identification within the colony, on the day prior to the experiment, twelve male zebra finches received randomized color markings on their chests using marker pens and were returned to the colony. The color used was the same for all (blue), with a different number of dots on the chest.

On the day of the experiment, the 12 male zebra finches were injected in the pectoral muscle with a sterile solution of either 100 μL of LPS 0.3 mg mL^{-1} (Sigma-Aldrich #L4005, Serotype 055: B5) or 100 μL of 10 $mmol^{-1}$ phosphate buffer saline (pH 7.2). The region to be injected was sterilized with ethanol, which was allowed to dry before injecting the animal. The dose of LPS was ca. 2 mg/kg of body weight. This dose is higher to what has been used previously in experiments with passerines. For example, Owen-Ashley et al. (2006) and Burness et al. (2010) used a dose of 1 mg/kg of body weight in white-crowned sparrows and zebra finches, respectively. When we tested both this dose and 2 mg/kg, the latter dose seemed to induce greater behavioral response (personal observation). Animals were randomly assigned to the injection treatment.

Behavior

Behavior was recorded 2 h after the injection, by direct observation. Two observers that were naïve to the treatment stood outside of the aviary. Observers were instructed to count the number of hops, flights, preening events, calls and songs, within a five-minute period. After the initial scoring, another 5 min were dedicated to observing the time the birds spent resting. Then, the observers moved on to the next bird. Thus, each bird was observed for a total of 10 min and all birds were observed within the same sixty-minute period.

Approximately 3 h after the injection, the birds were captured using butterfly nets, deeply anesthetized via isoflurane inhalation and decapitated. The brain was immediately removed and placed on dry ice and trunk blood was stored on regular ice. The blood was then centrifuged at 1500 *g* for 10 min and the plasma portion was placed into separate tubes. All tissues were maintained at −80 °C until further analysis. Time from entering the aviary until euthanizing birds was on average 11.7 min (±S.E.M.: 2.6 min) for control-injected and 12.9 min (±S.E.M.: 2.8 min) for LPS-injected birds. This time is not significantly different between treatments ($t(10) = 0.702$, $P = 0.499$).

Immunohistochemistry (IHC)

Using a cryostat, 20 μm coronal sections of the brains collected in Phase I were cut and every fifth slice was placed directly onto slides. A hydrophobic barrier was created around the slices on the slide, by the use of a PAP pen (Sigma-Aldrich # Z377821). The brain sections were then fixed using a 4% paraformaldehyde solution for 1 h. The slides were then washed three times in phosphate buffered saline (PBS, 10 mM, pH 7.2) and exposed to a 1% solution of hydrogen peroxide in PBS for 30 min. A new wash in PBS for 5 min was repeated three times, after which 2% normal goat serum (NGS) in 0.2% PBS-Triton (PBS-T) was added for 1 h. Subsequently, GnRH primary antibody (HU60, gift from Dr. Henryk Urbanski, Portland, OR, USA) at a concentration of 1:5000 in 0.2% PBS-T was added and allowed to incubate at room temperature (r.t.) for 1 h and subsequently for 48 h at 4 °C. The slides were then washed three times in 0.2% PBS-T, followed by incubation in 1:250 biotinylated goat anti-rabbit IgG (Vector Labs #BA-1000) in 0.2% PBS-T for 1 h and an additional three washes in