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EXPRESSION PATTERNS OF *MINERALOCORTICOID* AND *GLUCOCORTICOID RECEPTORS* IN BENGALESE FINCH (*LONCHURA STRIATA* VAR. *DOMESTICA*) BRAIN SUGGEST A RELATIONSHIP BETWEEN STRESS HORMONES AND SONG-SYSTEM DEVELOPMENT

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Abstract—Much evidence suggests that song traits function as an honest signal of male quality during mate choice in songbirds. Because songbirds learn vocalizations during the juvenile stage, development of the song system and song traits is affected by stressful conditions. However, it remains unknown how stressful conditions affect later song traits during development. To explore the relationship between glucocorticoids and song-system development, we performed *in situ* hybridization analysis of the *glucocorticoid* and *mineralocorticoid receptors* in juvenile and adult brains. The *glucocorticoid receptor* showed weak expression in song nuclei and strong expression in the hypothalamus, whereas the *mineralocorticoid receptor* showed strong song-nuclei-related expression. Thus, it appears that glucocorticoids are involved in song development directly by binding to receptors in song nuclei or indirectly by regulating sex hormones through hypothalamic hormones.

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Abbreviations: AP, alkaline phosphatase; cDNA, complementary DNA; CMM, caudomedial mesopallium; DIG, digoxigenin; DLM, dorsal lateral nucleus of the medial thalamus; DM, dorsal medial nucleus of the midbrain; GC, glucocorticoid; GR, glucocorticoid receptor; Hp, hippocampus; LMAN, lateral magnocellular nucleus of the anterior nidopallium; MPOA, medial preoptic area of the hypothalamus; MR, mineralocorticoid receptor; NCM, caudal medial nidopallium; nXIIts, nucleus XII, tracheosyringeal part; PBS, phosphate buffered saline; PFA, paraformaldehyde; RA, robust nucleus of the arcopallium; RT-PCR, reverse transcription-polymerase chain reaction; SSC, standard sodium citrate; TnA, nucleus taeniae.

Key words: Bengalese finch, *glucocorticoid receptor*, *mineralocorticoid receptor*, songbird, stress hormone, vocal development.

Birds use various vocalizations for courtship or territory defense. Female birds often select male birds by evaluating their song attributes (Catchpole and Slater, 1995; Searcy and Yasukawa, 1996). Female birds tend to prefer more complex songs (the larger song- or syllable-type repertories, or more syntactically complex songs) (Kroodsma, 1976; Catchpole and Slater, 1995; Lampe and Saetre, 1995; Hasselquist et al., 1996; Okanoya, 2004a,b). Mounting evidence suggests that birdsongs are used as an indicator of male quality during mate selection. In songbirds, song traits are very dependent on the nestling and fledgling conditions (e.g. food limitations, parasitic infections, corticosterone administration, or sibling competition) during the developmental stage. Indeed, many studies have shown that developmental conditions influence later song traits of songbirds (Nowicki et al., 2002; Buchanan et al., 2003; Spencer et al., 2003, 2004, 2005a; Soma et al., 2006; Zann and Cash, 2008). Songs by males with stressful development are less successful for mate attraction (Spencer et al., 2005b). Because song traits represent the developmental conditions during the growth period, it is reasonable to use birdsongs as an honest indicator of male quality (Nowicki et al., 1998, 2000, 2002; Nowicki and Searcy, 2004). However, the molecular mechanisms by which developmental conditions affect song traits remain unknown.

Glucocorticoid hormones (GCs; cortisol in fish and many mammals, and corticosterone in birds, reptiles, amphibians, and many rodents) are steroid hormones released when the hypothalamic–pituitary–adrenal axis is activated in response to stressful stimuli (Romero, 2004; Cockrem, 2007). Two GC receptors have so far been identified: *mineralocorticoid receptor* (*MR*) and *glucocorticoid receptor* (*GR*). *MR* is a high-affinity receptor for corticosterone and responds to basal levels of corticosterone under normal conditions, whereas *GR* is a low-affinity receptor and is activated only by high levels of corticosterone during stressful conditions (Reul and de Kloet, 1985; de Kloet and Reul, 1987; Arriza et al., 1988; de Kloet et al., 1990; de Kloet, 1991; Oitzl et al., 2010). GCs transactivate downstream gene expressions by binding to these nuclear hormone receptors, and this transcriptional regulation may lead to adaptive responses to stress, although detailed molecular mechanisms remain unknown. Many studies in

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rodents have suggested that corticosterone and its receptors are involved not only in the regulation of the stress response (de Kloet et al., 1998) but also in various neural developmental processes such as neurogenesis (Gould et al., 1992; Karishma and Herbert, 2002; Wong and Herbert, 2004, 2005, 2006), neuronal plasticity (Pavlides et al., 1995, 1996; de Kloet et al., 1999), learning, memory, and emotion (Oitzl and de Kloet, 1992; Sandi and Rose, 1994; Brinks et al., 2007).

It has been suggested that corticosterone is also involved in vocal development of songbirds. Developmental stress or chronic corticosterone administration in juvenile birds decreases the size of song nuclei, with a reduction in song complexity (Nowicki et al., 2002; Spencer et al., 2003, 2004; Buchanan et al., 2004; MacDonald et al., 2006). Furthermore, corticosterone levels are related to song traits (Wada et al., 2008; MacDougall-Shackleton et al., 2009). In contrast, although the expression of the corticosterone receptors *GR* and *MR* has been analyzed in the songbird brain (Breuner and Orchinik, 2001; Hodgson et al., 2007; Katz et al., 2008; Dickens et al., 2009; Wada and Breuner, 2010; Schmidt et al., 2010), these gene expression analyses did not focus on the song-system areas, and *in situ* hybridization or immunohistochemical *GR* and *MR* studies have never been reported for the song system. Thus, the bridge between corticosterone signaling and vocal development is still obscure.

Here, to examine corticosterone action on vocal learning and production in the developing and matured songbird brain, we analyzed detailed *GR* and *MR* expression patterns in juvenile and adult brains of Bengalese finch (*Lonchura striata* var. *domestica*), particularly focusing on the song nuclei.

EXPERIMENTAL PROCEDURES

Animals and sample preparation

The research protocols were approved by the Animal Care and Use Committee of RIKEN (#H20-2-231) and conformed to National Institutes of Health Guidelines. We used six 14-day postnatal (P14), P30, and adult (1–3-year-old) male Bengalese finches (*Lonchura striata* var. *domestica*) and four P14, P30, and adult (1–3-year-old) female Bengalese finches, bred at our laboratory facilities. Each bird was housed in family groups and given finch seed mixture, shell grit, and vitamin-enhanced water *ad libitum*. The light:dark period was 13:11; lights on from 8:00 to 21:00h. Room temperature and humidity were maintained at approximately 25 °C and 60%, respectively. All birds were deeply anesthetized with an i.m. injection of sodium pentobarbital (50 mg/kg) immediately after capture to avoid any effects of stress, and then rapidly sacrificed. After decapitation, their brains were embedded in a Tissue-Tek OCT compound (Sakura Fine Technical, Tokyo, Japan) and frozen on dry ice for cryosectioning. Frozen sections for *in situ* hybridization or thionine staining for neuroanatomical reference were cut serially in 20-μm thicknesses using a cryostat (Leica, Bannockburn, IL, USA). To extract total RNA, brain tissues were dissected and placed in Qiazol Lysis reagent (Qiagen, Valencia, CA, USA), and the RNA was purified using an RNeasy Lipid Tissue Mini kit (Qiagen). The sex of the birds was determined by extracting genomic DNA from a portion of a digit with a DNeasy Tissue kit (Qiagen) and performing a polymerase chain reaction (PCR) analysis with primers that amplify the chromo–helicase–DNA binding gene (Ellegren, 1996) or verified by inspection of sex organs.

DNA isolation and probe preparation

We isolated Bengalese finch *GR* and *MR* cDNAs by RT-PCR: *GR* (GenBank ID: AB604314) and *MR* (GenBank ID: AB604315). The primers we used are as follows: for *GR*, 5′-CCAAGCCCCTGT-GTCTACAT-3′ and 5′-ATGCCGGGAGTACACAGTTC-3′; for *MR*, 5′-AATTACCTGTGTGCGGGAAG-3′ and 5′-CCTGAGACT-CTCGGAAGGTG-3′. The nucleotide sequence similarities of Bengalese finch *GR* and *MR* cDNAs to zebra finch and chicken cDNAs are as follows: *GR* (99% and 88% for zebra finch and chicken *GR*, respectively); *MR* (99% and 94% for zebra finch and chicken *MR*, respectively), respectively. Each cDNA fragment was inserted in the pGEM-T Easy Vectors (Promega, Madison, WI, USA). To make sense or antisense probes, the plasmids were linearized with SpeI or ApaI enzyme to release the fragment, and probes were synthesized using T7 or SP6 RNA polymerase (Roche Diagnostics, Rotkreuz, Switzerland) with the digoxigenin (DIG)-labeling mix (Roche Diagnostics), respectively.

In situ hybridization

In situ hybridization of all tissue sections was performed using the method described by Matsunaga and Okanoya (2008). The sections were postfixed for 10 min with phosphate buffered saline (PBS; pH 7.1) solution and 4% paraformaldehyde (PFA) and then washed three times in PBS for 3 min. The slides were delipidated with acetone, acetylated, and washed in PBS with 1% Triton-X100 (Wako Pure Chemical, Osaka, Japan). The slides were then incubated at room temperature with hybridization buffer containing 50% formamide (Wako Pure Chemical), 5× standard sodium citrate (SSC), 5× Denhardt's solution (Sigma, St. Louis, MO, USA), 250 μg/ml yeast transfer RNA (Roche Diagnostics), and 500 μg/ml DNA (Roche Diagnostics). Sections were hybridized at 72 °C overnight in hybridization buffer with RNA probes. They were then rinsed in 0.2× SSC for 2 h and blocked for 2 h in a solution of 0.1 M Tris (pH 7.5) and 0.15 M NaCl with 10% sheep serum. The slides were incubated overnight with alkaline phosphatase (AP)-conjugated anti-DIG antibody (Roche Diagnostics). After washing, AP activity was detected by adding 337.5 mg/ml nitroblue tetrazolium chloride and 175 mg/ml 5-bromo-4-chloro-3-indolyl phosphate (Roche Diagnostics). All images were captured using an ORCA-Flash2.8 digital camera (Hamamatsu Photonics, Hamamatsu, Japan) under a BX-50 microscope (Olympus, Tokyo, Japan). The Photoshop software (ver. CS2; Adobe Systems, Mountain View, CA, USA) was used to crop unnecessary areas, juxtapose panels, and enhance contrast and brightness, as required. For quantification of expressing cells, we cut out images in 62,500 $\mu m^2$1 or 31,250 μm^2 dimension, changed brightness and contrast, and counted the number of cells by Image J software (NIH, Bethesda, MD, USA). Data are presented as the mean±SEM number of *GR*- or *MR*-expressing cells per 1 mm^2. Statistical analyses were conducted using Stat View software (SAS Institute, Berkeley, CA, USA). The density of *GR*- or *MR*-expressing cell was analyzed by three-way ANOVA (Age×Receptor types×Sex), and Bonferroni/Dunn test was applied for multiple comparison testing.

RESULTS

To explore *GR* and *MR* expressions, we first isolated *GR* and *MR* cDNA fragments by RT-PCR. To check specificity of probes, we performed *in situ* hybridization of the hippocampal region with sense and antisense probes, because it has been shown that both receptors are expressed in this region (Hodgson et al., 2007; Dickens et al.,