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Glucocorticoid receptor exhibits sexually dimorphic expression in the medaka brain

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ABSTRACT

The differential impact of stress on brain functions of males and females has been widely observed in vertebrates. Recent evidence suggests that stress-induced glucocorticoid signaling affects sexual differentiation and sex changes in teleost fish. These facts led us to postulate that there were sex differences in glucocorticoid signaling in the teleost brain that underlie some sex differences in their physiological and behavioral traits. Here we found sexually dimorphic expression of a glucocorticoid receptor gene (*gr1*) in the brain of medaka fish (*Oryzias latipes*), with females having greater expression in several preoptic and thalamic nuclei. Further, *gr1* exhibits female-biased expression in neurons of the anterior parvocellular preoptic nucleus that produce the neuropeptides vasotocin and gonadotropin-releasing hormone 1 (these neuropeptides have been implicated in the regulation of neuroendocrine and behavioral functions). These findings suggest that glucocorticoids have a greater influence on physiology and behavior mediated by these neuropeptides in females than in males, which may contribute to sex differences in the brain's response to stress.

1. Introduction

Glucocorticoids, also known as stress hormones, are a class of steroid hormones secreted by the adrenal cortex (interrenal gland in teleost fish) in response to various stresses in vertebrates. One of the major target organs for glucocorticoids is the brain, where they induce a wide range of behavioral and neuroendocrine responses to stress (Whirledge and Cidlowski, 2013; Myers et al., 2014). Glucocorticoids exert their action by binding to and activating the intracellular glucocorticoid receptor (GR), a ligand-dependent transcription factor that activates or represses the transcription of target genes. However, the information about neural genes and cells that are direct targets for glucocorticoids is limited, and the downstream cascade of glucocorticoid signaling within the brain is not fully understood.

The differential impact of stress on brain functions between males and females has been extensively observed in vertebrates (Kudielka and Kirschbaum, 2005; Bourke et al., 2012; Handa and Weiser, 2014). It is thus reasonable to assume that there are sex differences in the effectiveness of glucocorticoids in the brain. Indeed, in rodents, sex steroid hormones secreted from the gonads in a sex-dependent manner have been shown to have modulatory effects on GR action (Bourke et al., 2012). More direct evidence comes from the finding that GR is differentially expressed between the sexes in the mouse hippocampus (Goel and Bale, 2010). However, there have been no studies showing sex differences in GR expression in other brain regions and in the brains of other species.

Recent evidence suggests that glucocorticoids play a key role in the process of sexual differentiation and sex change in teleost fish. For example, high temperature stress reportedly increases the level of cortisol, the primary glucocorticoid in teleosts, which in turn causes the undifferentiated gonad to develop into testes instead of ovaries in flounders (*Paralichthys olivaceus*), medaka (*Oryzias latipes*), and pejerreys (*Odontesthes bonariensis*) (Hattori et al., 2009; Yamaguchi et al., 2010; Yamaguchi and Kitano, 2012; Hayashi et al., 2010; Fernandino et al., 2012). In addition, plasma cortisol levels are elevated during the process of sex change in the anemonefish (*Amphiprion ocellaris*) (Iwata et al., 2012). Although no evidence indicates that glucocorticoid signaling affects sexual differentiation and sex change at the level of the brain, these previous findings led us to expect sex differences in glucocorticoid signaling in the teleost brain, which may underlie some sex differences in brain functions.

In the present study, we found sex differences in GR expression in several preoptic and thalamic nuclei of the medaka brain.

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Further, one of these nuclei exhibited differential GR expression between the sexes in neurons producing neuropeptides that have been implicated in the regulation of behavioral and neuroendocrine functions. Our data suggest that there are sex differences in glucocorticoid efficacy in these brain functions.

2. Materials and methods

2.1. Animals

All animal procedures were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee of the University of Tokyo. Medaka (d-rR strain) were maintained at 28 °C with a 14-h light/10-h dark photoperiod and were fed 3 times per day with live brine shrimp and commercial fish food pellets. Sexually mature adult fish (3–5 months of age) not subjected to any particular stress were sampled at 1–3 h after the onset of light and used for all analyses.

2.2. Examination of sex differences in GR expression in the brain

Teleost species, including medaka, generally possess two paralogous GR genes, *gr1* and *gr2* (Alsop and Vijayan, 2009; Trayer et al., 2013; Miyagawa et al., 2015). The medaka expressed sequence tag (EST) clones for *gr1* (clone ID, oleb52f22; GenBank accession numbers, XM_004076127) and *gr2* (clone ID, olgi51k04; GenBank accession numbers, NM_001163133) were obtained from National BioResource Project (NBRP) Medaka (http://www.shigen.nig.ac.jp/medaka/). Partial fragments of the *gr1* (1451 bp) and *gr2* (1416 bp) cDNAs were PCR-amplified from these EST clones, using the following primer sets: 5′-AGTTGTTGACGGATCCGCGAGA-3′/5′-CCTGGCAAGCCTGAGGCAGA-3′ for *gr1* and 5′-CTAATGGCCAGTTG CACAGCGT-3′/5′-GAGAGCTTGAGAAAGCCTCACT-3′ for *gr2*. The resulting DNA fragments were subcloned into the pGEM-Teasy vector (Promega, Madison, WI, USA) and transcribed *in vitro* to generate digoxigenin (DIG)-labeled *gr1* and *gr2* cRNA probes using DIG RNA Labeling Mix and T7/SP6 RNA polymerases (Roche Diagnostics, Basel, Switzerland). To avoid cross-hybridization, each of the *gr1* and *gr2* probes was designed in the 5′-untranslated region and 5′-half of the open reading frame that had minimal sequence homology to each other.

In situ hybridization was carried out following the previously reported procedure (Hiraki et al., 2012). In brief, whole brains ($n = 5$ for each sex) were fixed in 4% paraformaldehyde (PFA) and embedded in paraffin. Serial coronal sections (10 μm in thickness) of the entire brain were cut and hybridized with the DIG-labeled *gr1* and *gr2* probes described above. Hybridization signals were visualized using 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT) substrate (Roche Diagnostics). The color was allowed to develop for 7 days in the dark.

For semi-quantitative analysis, all brain sections were photographed and converted to black and white binary images by thresholding using Adobe Photoshop (Adobe Systems, San Jose, CA, USA). The total area of *gr1* and *gr2* expression was calculated for each nucleus using ImageJ (http://rsbweb.nih.gov/ij/). The subdivisions and nomenclature of brain nuclei were taken from the medaka brain atlases (Anken and Bourrat, 1998; Ishikawa et al., 1999) and extensively supplemented with information obtained from our Nissl-stained sections. The data were not subjected to any normalization process, because fish of approximately the same age and body size were selected for analysis, all serial sections throughout the brain were analyzed in each fish, and all sections were processed simultaneously under the same conditions.

2.3. Evaluation of sex differences in gr1 and vt/it/gnrh1 coexpression in the preoptic nuclei

The above analysis allowed us to observe sex differences in *gr1* expression in several brain regions, including two preoptic nuclei: the anterior parvocellular preoptic nucleus (PPa), containing neurons expressing vasotocin (*vt*; the teleost ortholog of mammalian arginine vasopressin (*Avp*)), isotocin (*it*; the teleost ortholog of mammalian oxytocin (*Oxt*)), and gonadotropin-releasing hormone 1 (*gnrh1*); and the basal lateral preoptic nucleus (Pbl), containing *gnrh1* neurons (Kawabata et al., 2012). Accordingly, we tested the possibility that *gr1* was coexpressed with *vt*/*it*/*gnrh1* in a sex-biased manner in these preoptic nuclei.

Partial fragments of the medaka *vt* (845 bp), *it* (738 bp), and *gnrh1* (428 bp) cDNAs were PCR-amplified and subcloned into the pGEM-Teasy vector (Promega) as described elsewhere (Kawabata et al., 2012). The fragments were *in vitro*-transcribed to generate fluorescein-labeled *vt*, *it*, and *gnrh1* cRNA probes using Fluorescein RNA Labeling Mix and T7 RNA polymerase (Roche Diagnostics). Double *in situ* hybridization was carried out as previously described (Takeuchi and Okubo, 2013). Briefly, whole brains ($n = 4$–8 for both sexes and for each pair of genes) were fixed in 4% PFA and embedded in 5% agarose (Type IX-A; Sigma–Aldrich, St. Louis, MO, USA) supplemented with 20% sucrose. The brains were frozen and sectioned into 20 μm coronal slices, and hybridized with the fluorescein-labeled *vt, it,* and *gnrh1* probes and the DIG-labeled *gr1* probe described above. The hybridized fluorescein-labeled probes were visualized using the TSA Plus Fluorescein System (PerkinElmer, Waltham, MA, USA) and the DIG-labeled probe was visualized using Fast Red (Roche Diagnostics). Fluorescent images were acquired using a confocal laser-scanning microscope (Leica TCS SP8; Leica Microsystems, Wetzlar, Germany). The excitation and emission wavelengths for detection were as follows: 4′,6-diamidino-2-phenylindole (DAPI) for nuclear staining, 405 nm and 410–480 nm; fluorescein, 488 nm and 495–545 nm; Fast Red, 552 nm and 620–700 nm.

For quantitative analysis, all sections of the PPa and Pbl were photographed and the number of neurons showing *vt*, *it*, and *gnrh1* expression and neurons showing both *gr1* and *vt*/*it*/*gnrh1* expression were counted manually. The proportion of *gr1* and *vt*/*it*/*gnrh1* coexpression was calculated as the percentage of the total number of *vt*/*it*/*gnrh1* neurons and compared between sexes.

2.4. Evaluation of sex differences in whole-body cortisol levels

Sex differences in basal whole-body cortisol levels were also examined. Whole bodies of fish ($n = 5$ pools of 2 individuals for each sex) were homogenized in phosphate-buffered saline. Steroids were extracted with chloroform/methanol (2:1 v/v) and purified on a Sep-Pak C18 cartridge (Waters Corporation, Milford, MA, USA). Cortisol levels were determined using the Cortisol EIA Kit (Cayman Chemical Company, Ann Arbor, MI, USA) according to the manufacturer's instructions.

2.5. Statistical analysis

Quantitative data are presented as the mean and standard error of the mean. Statistical analyses were performed using Microsoft Excel (Microsoft, Redmond, WA, USA) and GraphPad Prism (GraphPad Software, San Diego, CA, USA). Sex differences in the total area of *gr1* expression in each nucleus and in whole-body cortisol levels were evaluated for statistical significance by the unpaired two-tailed Student's *t*-test. Sex differences in the percentage of *gr1*-expressing *vt*/*it*/*gnrh1* neurons were evaluated by the unpaired two-tailed Mann–Whitney *U*-test.