Hormones and Behavior 61 (2012) 725–733

Contents lists available at SciVerse ScienceDirect

Hormones and Behavior

journal homepage: www.elsevier.com/locate/yhbeh

Isotocin regulates paternal care in a monogamous cichlid fish ☆

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ARTICLE INFO

Article history:
Received 1 February 2012
Revised 22 March 2012
Accepted 24 March 2012
Available online 1 April 2012

Keywords:
Paternal care
Testosterone
11-Ketotestosterone
Isotocin
Lateral septum
Preoptic area

ABSTRACT

While the survival value of paternal care is well understood, little is known about its physiological basis. Here we investigate the neuroendocrine contributions to paternal care in the monogamous cichlid, *Amatitlania nigrofasciata*. We first explored the dynamic range of paternal care in three experimental groups: biparental males (control fathers housed with their mate), single fathers (mate removed), or lone males (mate and offspring removed). We found that control males gradually increase paternal care over time, whereas single fathers increased care immediately after mate removal. Males with offspring present had lower levels of circulating 11-ketotestosterone (11-KT) yet still maintained aggressive displays toward brood predators. To determine what brain regions may contribute to paternal care, we quantified induction of the immediate early gene c-Fos, and found that single fathers have more c-Fos induction in the forebrain area Vv (putative lateral septum homologue), but not in the central pallium (area Dc). While overall preoptic area c-Fos induction was similar between groups, we found that parvocellular preoptic isotocin (IST) neurons in single fathers showed increased c-Fos induction, suggesting IST may facilitate the increase of paternal care after mate removal. To functionally test the role of IST in regulating paternal care, we treated biparental males with an IST receptor antagonist, which blocked paternal care. Our results indicate that isotocin plays a significant role in promoting paternal care, and more broadly suggest that the convergent evolution of paternal care across vertebrates may have recruited similar neuroendocrine mechanisms.

Introduction

Care for offspring can represent an evolutionary trade-off between current and future reproduction, especially for males (Huxley, 1938). Although behavioral ecologists have shown *why* males care for their young (Gonzalez-Voyer et al., 2008), we know relatively little about *how* the male brain transforms to promote parental behavior. The hormonal basis of paternal care has been examined in several vertebrates (Ketterson and Nolan, 1994; Knapp et al., 1999; Wynne-Edwards and Timonin, 2007), and points to a role for sex steroid hormones, prolactin, and nonapeptides (Gubernick and Nelson, 1989; Knapp et al., 1999; Wynne-Edwards and Timonin, 2007). Specifically, both oxytocin (mammalian homologue of isotocin, IST) and vasopressin (the mammalian homologue of vasotocin, AVT) have been implicated in paternal care in mammals (Gordon et al., 2010; Parker and Lee, 2001) and teleost fish (Ripley and Foran, 2010). However, where these hormones exert their effects in the brain to promote paternal care has received considerably less attention. The induction of immediate early genes (IEGs) by behavioral stimulation has been utilized to identify brain regions that may regulate paternal behavior in mammals, such as the preoptic area, thalamus, and bed nucleus of the stria terminalis (BNST) (de Jong et al., 2009; Kirkpatrick et al., 1994). Additionally, the lateral septum has also been associated with both paternal care and pair bond formation in monogamous rodents (Liu et al., 2001; Wang et al., 1994). However, it is difficult to disentangle other confounding social factors in such studies, such as sexual or parental experience and pair-bonding.

The monogamous and biparental Central American convict cichlid, *Amatitlania nigrofasciata*, is a nonmammalian model species for the study of both pair bonding and parental care (Itzkowitz et al., 2001; Oldfield and Hofmann, 2011), although the neural basis of these behaviors are currently unknown. Despite the unusual development of the teleost telencephalon (Mueller and Wullimann, 2009), much progress has been made in determining homology relationships between mammalian and teleost brains (O'Connell and Hofmann, 2011), allowing us to ask whether similar neural substrates underlie a behavioral phenotype that has clearly evolved independently multiple times. Putative homologues for regions that appear to regulate paternal care in mammals can be identified in the teleost brain, including the putative lateral septum homologue (Vv, ventral

☆ Financial disclosure: this work was supported by an NSF DDIG 1011253 to LAO, a University Co-Op Undergraduate Research Fellowship to BJM, and an NSF grant (0843712) to HAH.

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doi:10.1016/j.yhbeh.2012.03.009

part of the ventral telencephalon) and the preoptic area (POA). However, the teleost homologue of the mammalian BNST is ambiguous. A region similar to the extended amygdala has been identified in the teleost telencephalon, although this region is neurochemically more similar to the medial amygdala than the BNST (O'Connell and Hofmann, 2011). Additionally, area Dc (the central part of the dorsal telencephalon, for which no mammalian homologue is known; but see Mueller et al., 2011) in the teleost forebrain has been implicated in paternal care in the bluegill sunfish (*Lepomis macrochirus*), a species with male-only parental care (Demski and Knigge, 1971).

In order to better understand the neuroendocrine basis of paternal care, we analyzed the behavioral, hormonal, and neural responses of convict cichlid males in different family structures. We then tested the hypothesis that IST mediates paternal care in this species. Our results suggest that similar neural pathways and neurochemicals may have been recruited independently in the convergent evolution of paternal care across vertebrates.

Methods

Animals

Adult *A. nigrofasciata* were obtained through the pet trade or bred in the laboratory from animals purchased in the pet trade, and housed in single-sex groups on a 12:12 h photoperiod, immediately preceded and followed by a 10-min period of dim incandescent lights to simulate dawn and dusk. Mean body mass and standard length for focal males was 3.71 g (range: 2.11 g–5.72 g) and 44 mm (range: 37 mm–51 mm). All male and female pairs were of the "barred" color morph. Juveniles that served as a social stimulus were of either the barred or leucistic color morphs and less than 20 mm in length. All procedures were approved by the University of Texas at Austin Institutional Animal Care and Use Committee.

Behavior

Tanks (110 l) were separated into two compartments using a clear perforated plastic divider. Each side contained a terracotta pot that served as a shelter and substrate for egg laying. Size- and color-matched adult pairs were placed in one compartment and observed every other day for baseline behavior and every day for the presence of a brood for up to 7 days. If the pair did not spawn within this time period, a new adult pair was established. The compartment opposite the adult pair contained five (unrelated) juveniles to provide a non-reproductive social stimulus that mimicked potential brood predation by conspecifics (Alonzo et al., 1999), yet did not threaten the stability of the pair bond.

On the day of spawning, the pair was allowed to remain intact (biparental control group; n = 11) or the female (single fathers; n = 12), or both the eggs and female (lone group; n = 11) were removed. A "sham" removal was performed on the intact family group to control for the introduction of the hand net into the focal male's environment. Following the day of spawning (Day 0), focal males were observed between 10 AM and 11 AM for 10 min each morning for four consecutive days (Days 1–4) using an ethogram of convict behavior established previously (Oldfield and Hofmann, 2011). We grouped the observed displays of social behavior into three categories: aggressive, affiliative and parental. Individuals showed aggression by charging and biting at perceived threats, or by chasing others away from the nest. Affiliative behavior by the males included frontal and lateral displays directed at the mate, glide-bys, circling of females, tail beating, and affiliative bites. Parental behavior was directed specifically towards the young or nest site and included digging out the burrow or a new nesting site, nipping or cleaning young, skimming over eggs, fanning oxygen-rich water over the brood, or transporting offspring via their mouths in what we referred to as "bus stop".

On the final day (Day 4), 1 h after behavioral observations, we recorded the body mass and standard length of each focal male and harvested blood from the dorsal aorta using heparinized 26G butterfly infusion sets (Becton Dickson). Plasma was stored at −80 °C for subsequent hormone assays. Males were killed by rapid cervical transsection; brains were rapidly dissected and fixed overnight at 4 °C in 4% paraformaldehyde for immunohistochemistry. Not all individuals used in the behavioral analyses were represented in the hormone analysis due to insufficient plasma volumes in some cases, and similarly in the immunohistochemical analysis due to tissue loss in brain dissection or sectioning.

Pharmacology

Adult pairs were established as described above and observed daily for the presence of a brood. We conducted pharmacological experiments only in males housed with their mate (biparental males). An intraperitoneal injection of an IST receptor antagonist or vehicle control was administered on the day of spawn (Day 0) and each day for two consecutive days. We used the oxytocin receptor antagonist desGly-NH_2-d$(CH_2)_5$[D-Tyr^2,Thr^4]OVT, a kind gift from Dr. Maurice Manning (Manning et al., 2008), and conducted a pilot experiment to determine a dose response relationship for the IST receptor antagonist with three different doses plus a saline control and observed behavior 5 min, 30 min, and 1 h after injections (data not shown). In the main experiment we used 0.5 μg/g b.w. desGly-NH_2-d$(CH_2)_5$[D-Tyr^2,Thr^4]OVT and 1 h between injection and observations, which elicited the largest change in aggressive or affiliative behavior in paired males, or 20 μl/g b.w. 1× PBS as vehicle control. Each male (n = 8 per treatment) received the same drug throughout the treatment period. Ten minute observations were conducted 1 h after injection (injection time was typically between 9:00 and 10:30 h) on Days 1 and 2 after spawning by an observer blind to treatment.

Hormone assays

We measured circulating levels of free androgens, including testosterone (Enzo Life Sciences, Cat no. ADI-900-065) and the teleost androgen 11-KT (Cayman Chemicals, Cat. no. 582751), using ELISA as previously described (Kidd et al., 2010). Sample sizes for testosterone and 11-KT ELISA were as follows: biparental males (n = 10 and 8), single fathers (n = 9 and 9), and lone males (n = 10 and 7), respectively. Plasma samples were diluted 1:30 in assay buffer provided in the ELISA kits. Intra-assay variation was 5.50% for testosterone and 5.15% for 11-KT ELISA plates.

c-Fos immunohistochemistry (IHC)

After being fixed overnight at 4 °C in 4% paraformaldehyde, brains were rinsed in 1× PBS and cryoprotected in 30% sucrose overnight at 4 °C before embedding in O.C.T. compound (Tissue-Tek) and storage at −80 °C. Brains were then sectioned on a cryostat at 30 μm and thaw-mounted onto Super-Frost Plus slides (Fisher Scientific) in four series that were stored at −80 °C for 4–8 weeks until processing for IHC as previously described (Munchrath and Hofmann, 2010) using 1:500 rabbit anti-c-Fos primary antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA; catalog # sc-253). For control sections, all procedures were the same except that primary antibody was omitted (Fig. S1). Specificity of the c-Fos antibody to *A. nigrofasciata* antigens was confirmed using a Western blot (see below). For immunohistochemical analysis, we used n = 7 biparental males, n = 9 single fathers, and n = 8 lone males.