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Isotocin regulates paternal care in a monogamous cichlid fish ☆

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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

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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.

Co-localization of c-Fos with IST

An alternate series was used for c-Fos and IST fluorescent double-labeling immunohistochemistry. Sections were removed from −80 °C and treated as described above with the following exceptions. Hydrogen peroxidase treatment was not done prior to antigen retrieval. Sections were incubated in a mix of 1:5000 guinea pig-anti-oxytocin polyclonal antibody (Millipore, AB15704) and 1:500 c-Fos primary antibody in 1× PBS with 2% normal goat serum (NGS) and 0.3% Triton X-100 at room temperature overnight. Slides were washed twice in 1× PBS and then incubated in a mix of 1:200 anti-rabbit Texas Red (Invitrogen, T2767) and 1:800 anti-guinea pig Alexa Fluor 488 (Invitrogen, A11073) in a 2% NGS and 0.3% Triton X-100 in 1× PBS solution. After rinsing twice in 1× PBS, slides were coverslipped using Vectashield hardset mounting media with DAPI (Vector Laboratories). Controls included slides where the primary antibody was omitted. To confirm the specificity of the anti-oxytocin antibody to isotocin, we included immunoprecipitation controls where the anti-oxytocin antibody was preabsorbed with IST (Fig. S2), which completely blocked all signal, or AVT, which did not block signal (Fig. S3).

Western blot

To determine whether the c-Fos antibody would bind specifically to the cichlid antigens, we extracted protein from *A. nigrofasciata* whole brain using a Mammalian Cell Lysis kit (Sigma) according to the manufacturer's instructions. Whole brain protein extract was run on an SDS-PAGE gel and then was transferred onto a nitrocellulose membrane overnight. The membrane was then blocked in 5% dry milk in wash buffer (0.5% Triton X-100, 0.1% Tween-20 in 1× Tris-buffered saline [TBS]) for 30 min and then incubated in primary antibody (1:5000 c-Fos in 1× TBS and 2% NaN_3) for 1 h. After incubation, the membrane was washed five times for 3 min each in wash buffer, and then incubated in goat-anti-rabbit HRP-conjugated antibody (Santa Cruz) in blocking solution for 30 min. After washing five times for 3 min each with wash buffer, the membrane was exposed to HRP substrate (Immobilon Western, Millipore) and exposed to film for 10 min. Using the c-Fos antibody, two bands were visualized at the predicted size of 52 and 68 kDa (Fig. S4), putatively representing c-Fos, as previously characterized in zebrafish (Hirayama et al., 2005). Two bands were expected, as there are two *c-fos*-like paralogs in zebrafish, likely due to the teleost-specific genome duplication.

Cell counting

Quantification of c-Fos induction

Coded slides were processed by an observer blinded to treatment. Cells labeled by c-Fos IHC were counted using the Fractionator routine of the Stereo Investigator software package (Microbrightfield, Williston, VT, USA). Three sections of each brain region were quantified and averaged per individual. A region of interest was defined under low power, and then, under higher magnification, positive cells were counted that fell within 75-μm-square counting frames. The computer placed counting frames systematically every 80 μm (i.e. with 5 μm between each counting frame) within the region, after a randomly chosen start-site. Cell nuclei containing c-Fos protein were clearly marked by dark brown staining and were counted using a 20× objective.

Fluorescent co-localization

Fluorescence signal was detected using a Zeiss AxioImager.A1 AX10 microscope equipped with DAPI, FITC, and TRITC filters to allow visualization of the DAPI counter-stain, immunoreactivity of IST with the FITC channel, and immunoreactivity of c-Fos with the TRITC channel. Photographs were taken with a digital camera (AxioCam MRc, Zeiss) using the AxioVision (Zeiss) image acquisition and processing software. Coded slides were processed by an observer blinded to treatment. The total number of IST-positive neurons was visually counted in the parvocellular and magnocellular cells of the POA. The number of neurons exhibiting co-localization of both IST and c-Fos was quantified by superimposing images generated by FITC and TRITC fluorescence filters. The total number of neurons positive for IST, and the number of IST and c-Fos co-localized neurons for each brain region was determined by averaging two to three sections per individual. The resulting data are presented as the average ratio of neurons co-expressing IST and c-Fos to the total number of IST neurons.

Data analysis

All analyses were conducted in PASW and significance was considered as $p<0.05$.

Behavior and hormones

Behavior and hormone measurements were not normally distributed and were log-transformed, resulting in normalized residuals. Between and within group changes in aggressive and paternal behavior were calculated using a repeated measures ANOVA with behavior as the dependent variable, treatment as a between-group factor and day as a within group factor. With aggression data, within-group sphericity was not assumed and therefore Greenhouse–Geisser statistics are reported for aggression and affiliative behavior. To investigate the group differences in each day post-spawn, a univariate ANOVA was performed with behavior as the dependent variable and treatment group as the independent variable. To determine differences in hormone levels between groups, a univariate ANOVA was carried out, with hormone as the dependent variable and treatment group as the independent variable. A Fisher's Least Significant Difference (LSD) test was applied post-hoc to determine between group differences.

c-Fos immunoreactivity and co-localization with IST

Counts of c-Fos immunoreactivity for each brain region and co-localization with IST neurons in the POA were not normally distributed and thus log-transformed, resulting in normalized residuals. For quantification of c-Fos induction, we performed an ANOVA to determine differences across treatment groups using cell counts as the dependent variable, treatment group as the independent variable, and the focal male mass as a covariate (to account for the relative size of the target region). An ANOVA was conducted post-hoc between each of the two groups, as a covariate was included in the model. For induction of IST neurons, an ANOVA was carried out with the ratio of c-Fos and IST co-localized neurons to total IST neurons as the dependent variable and treatment group as the independent variable. LSD post-hoc tests were used to determine between group differences.

Pharmacology

Behavior measurements were not normally distributed and therefore were log-transformed, resulting in normalized residuals. An ANOVA was carried out to determine differences between treatment groups (saline vs. IST receptor antagonist treatment groups) on each day of treatment with behavior as the dependent variable and drug as the independent variable.

Results

Male behavioral responses to changes in family structure

To better understand the behavioral responses associated with care for offspring, we quantified aggressive, paternal, and affiliative

behavior in males with a partner (biparental), without a partner (single father), or without the partner or brood present (lone) over the 4 days post spawning (Fig. 1). We found that each family structure differentially elicited social behavior in new fathers. As expected, affiliative behavior was only displayed by biparental males, whose mate was present (Fig. S5).

Aggression increased immediately after spawning (ANOVA, $F_{22,3} = 3.775$, $p = 0.018$), with biparental and single fathers displaying more aggression compared with pre-spawn levels (data not shown; LSD $p < 0.03$), while lone males did not increase aggressive displays ($p = 0.847$) (pre-spawn aggression mean ± standard deviation: 1.4 ± 0.86). After spawning, aggressive displays (Fig. 1A), which were mostly directed at the juvenile stimulus, increased over time within groups (ANOVA, $F_{34,2.106} = 3.382$, $p = 0.038$) and also differed across social treatment groups (ANOVA, $F_{34,2} = 7.662$, $p = 0.002$), with biparental males and single fathers displaying more aggression than lone males (LSD $p < 0.004$), although overall aggression did not differ between single fathers and biparental males (LSD $p = 0.698$). When analyzing differences on particular days, treatments differed only marginally in aggressive displays on Day 1 (ANOVA, $F_{34,2} = 2.967$, $p = 0.066$) and on Day 2 (ANOVA, $F_{34,2} = 3.242$, $p = 0.053$). However, this difference in aggression between treatment groups became significant on Day 3 (ANOVA, $F_{34,2} = 10.251$, $p = 3.82 \times 10^{-4}$) and Day 4 (ANOVA, $F_{34,2} = 9.990$, $p = 4.48 \times 10^{-4}$). On these days, males with a brood (biparental and single fathers) displayed more aggression compared to lone males (LSD: Day 3, $p < 0.001$; Day 4, $p < 0.004$), although the amount of aggression displayed did not differ (LSD: Day 3 $p = 0.699$, Day 4 $p = 0.274$) between biparental and single fathers.

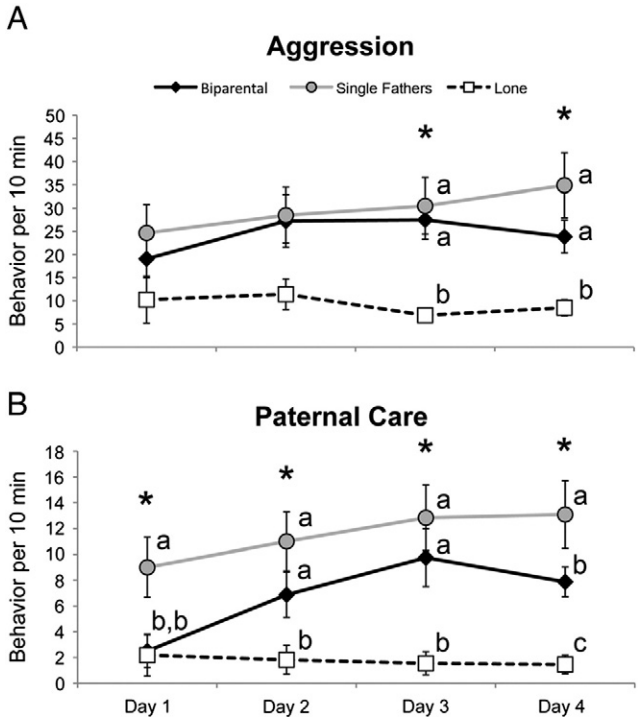

Fig. 1. Male behavioral responses to changes in family structure. Aggressive (A) and paternal care (B) behavior is shown for biparental males (black diamond, n = 11), single fathers (gray circles, n = 12), and lone males (whites squares, n = 11) for four consecutive days post-spawn. Data are represented as the mean ± SEM; stars (*) represent a significant difference by ANOVA between groups on a particular day and groups not labeled with the same letter are significantly different with a Fisher's LSD post-hoc test.

Paternal care (Fig. 1B) increased significantly over time within groups (ANOVA, $F_{34,3} = 8.965$, $p = 2.82 \times 10^{-5}$) and levels of paternal behavior differed between treatment groups (ANOVA, $F_{34,2} = 17.208$, $p = 9.40 \times 10^{-6}$). Overall, single fathers displayed more paternal care than biparental males (LSD $p = 0.024$) and lone males (LSD $p = 1.90 \times 10^{-6}$). Additionally, biparental males display more paternal care than lone males (LSD $p = 0.006$). Interestingly, lone males sometimes displayed paternal care immediately after removal of the brood, as they would fan over the site where the brood was located prior to removal. There was a robust behavioral change on Day 1 (ANOVA, $F_{34,2} = 6.155$, $p = 0.006$), with single fathers displaying more paternal care than biparental males (LSD $p = 0.013$) and lone males (LSD $p = 0.003$), while biparental males and lone males did not differ (LSD $p = 0.532$). On Day 2, there was still significant variation in paternal care across groups (ANOVA, $F_{34,2} = 8.337$, $p = 0.001$), with both single fathers and biparental males displaying more care behavior than lone males (LSD $p < 0.016$), although single fathers and biparental males did not differ (LSD $p = 0.156$). A similar pattern was observed on Day 3 (ANOVA, $F_{34,2} = 21.732$, $p = 1.26 \times 10^{-6}$) and Day 4 (ANOVA, $F_{34,2} = 25.536$, $p = 2.79 \times 10^{-7}$), where single fathers and biparental males again displayed more paternal care than lone males (Day 3: LSD $p < 8.81 \times 10^{-5}$; Day 4: LSD $p < 3.80 \times 10^{-5}$). Biparental and single fathers did not differ from each other on Day 3 (LSD $p = 0.228$) but did show a significant difference on Day 4 (LSD $p = 0.044$).

Androgen responses to changes in family structure

In order to understand the hormonal response to a dramatic change in family structure, free circulating testosterone and 11-KT were measured (Fig. 2). We found no significant variation in testosterone levels between treatment groups (ANOVA, $F_{29,2} = 2.347$, $p = 0.120$). However, there was a significant difference in 11-KT levels between groups (ANOVA, $F_{24,2} = 21.129$, $p = 9.4 \times 10^{-6}$). Specifically, 11-KT was higher in lone males compared to biparental males (LSD $p = 2.70 \times 10^{-6}$) and single fathers (LSD $p = 0.0002$). Across all focal males, testosterone and 11-KT levels were positively correlated (Spearman's rho = 0.691, $p = 0.0001$).

c-Fos induction in candidate brain regions

To investigate which brain regions may be involved in the regulation of paternal care, we measured c-Fos induction in three candidate brain regions (Fig. 3). We focused on homologous regions implicated in paternal care in mammals, including the lateral septum (Liu et al., 2001; Wang et al., 1994) and preoptic area (de Jong et al., 2009; Kirkpatrick et al., 1994; Lee and Brown, 2002). Although the BNST is involved in paternal care in rodents (Kirkpatrick et al., 1994), the homologous region in teleosts is ambiguous. While an extended amygdala has been identified in teleosts (O'Connell and Hofmann, 2011), this region has a neurochemical profile more similar to the medial amygdala than the BNST. Furthermore, we quantified c-Fos in the central part of the dorsal telencephalon (Dc), as this region has been shown to regulate paternal care in the bluegill sunfish (Demski and Knigge, 1971). We found significant differences in c-Fos induction across treatments in Vv (lateral septum homologue; ANOVA, $F_{24,2} = 4.821$, $p = 0.020$), but not in the parvocellular POA (ANOVA, $F_{27,2} = 0.617$, $p = 0.548$) nor in area Dc (ANOVA, $F_{28,2} = 0.859$, $p = 0.436$). Within Vv, single fathers showed higher c-Fos induction compared to lone males (ANOVA, $F_{17,1} = 5.884$, $p = 0.029$), although c-Fos induction in the Vv of biparental males did not differ from lone males (ANOVA, $F_{15,1} = 3.01 \times 10^{-6}$, $p = 0.999$) or single fathers (ANOVA, $F_{16,1} = 4.571$, $p = 0.052$). However, there was a non-significant trend for higher induction in single fathers compared to biparental males. Furthermore, c-Fos induction in Vv was positively correlated across all animals with

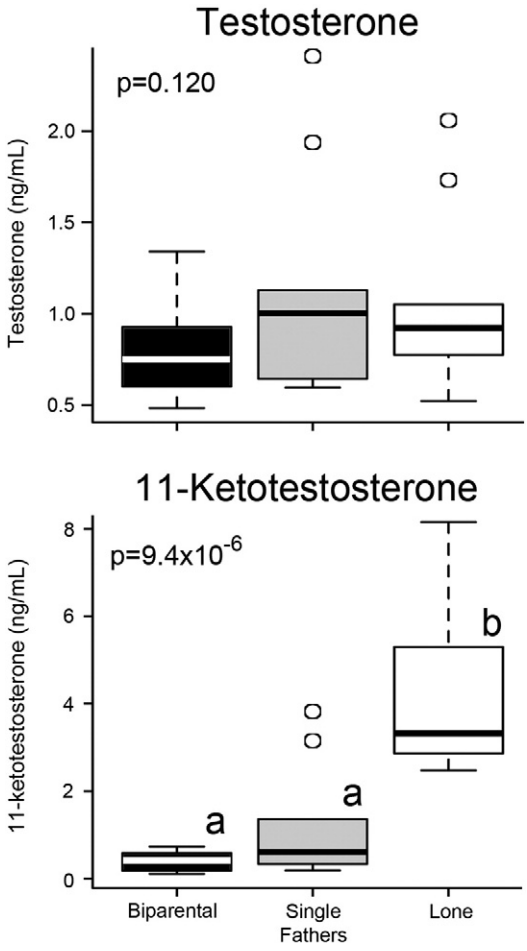

Fig. 2. Circulating levels of 11-ketotestosterone, but not testosterone, are lower in males displaying paternal care. Testosterone (top panel) and 11-ketotestosterone (11-KT) levels (bottom panel) in biparental males (black, n = 8), single fathers (gray, n = 9) and lone males (white, n = 7) represented by box and whisker plots, where the box delineates the first and third quartiles, the horizontal line shows the median, the whiskers indicate the maximum and minimum values, and circles represent outliers. ANOVA p-value is indicated in the corner while groups not sharing the same letter are significantly different with a Fisher's LSD post-hoc test.

paternal behavior displayed 1 h prior to sacrifice (rho = 0.438, p = 0.032).

Induction of c-Fos in IST neurons in the POA

As IST may play an important role in paternal care, we quantified c-Fos induction in preoptic IST neurons by co-localizing c-Fos and IST (Fig. 4). The total number of IST neurons did not differ between experimental groups in the parvocellular (ANOVA, $F_{21,2} = 0.192$, $p = 0.827$; mean ± standard deviation: biparental males: 21.2 ± 13; single fathers: 28.1 ± 20; lone males: 25.5 ± 15) or magnocellular POA (ANOVA, $F_{25,2} = 0.574$, $p = 0.572$; mean ± standard deviation: biparental males: 28.1 ± 20; single fathers: 35.9 ± 21; lone males: 25.1 ± 8). However, c-Fos induction in IST neurons (ratio of c-Fos containing IST neurons to total IST neurons) significantly differed between treatment groups in the parvocellular POA (ANOVA, $F_{21,2} = 3.999$, $p = 0.037$), but not in the magnocellular POA (ANOVA, $F_{25,2} = 2.576$, $p = 0.099$). Specifically, single fathers had a greater proportion of c-Fos co-expressing parvocellular IST neurons compared to biparental (LSD $p = 0.025$) and lone (LSD $p = 0.035$) males, with a similar, albeit non-significant, pattern in magnocellular IST neurons. Lone and biparental males did not differ in induction of parvocellular IST neurons (LSD $p = 0.884$).

Functional role of the IST receptor in regulating paternal care

These patterns of c-Fos induction in preoptic IST neurons led us to functionally test the role of the IST receptor in mediating paternal care in *A. nigrofasciata* males using pharmacological manipulations. We administered an IST receptor antagonist to biparental (control) males, and measured changes in behavior (Fig. 5). Although the IST receptor antagonist did not alter paternal care on Day 1 ($F_{16,1} = 0.103$, $p = 0.753$), the IST receptor antagonist did block the rise in male parental care typically seen on Day 2 after spawning ($F_{16,1} = 7.141$, $p = 0.018$). Importantly, blocking the IST receptor did not alter affiliative behavior toward the mate (Day 1, $F_{16,1} = 0.033$, $p = 0.859$; Day 2, $F_{16,1} = 2.797$, $p = 0.117$) nor did it affect aggressive behavior directed towards the juveniles (Day 1, $F_{16,1} = 1.263$, $p = 0.280$; Day 2, $F_{16,1} = 1.087$, $p = 0.315$).

Discussion

We have shown that IST facilitates paternal care, likely via peptide release from the parvocellular POA neurons. The involvement of IST in paternal care in a monogamous cichlid suggests that the involvement of oxytocin in promoting paternal care is the result of convergent evolution of endocrine systems and provides new insights into the neuroendocrine basis and evolution of fatherhood.

Paternal care and androgens

Aggressive defense of a territory is often linked to androgen levels (Gleason et al., 2009). However, a universal pattern that would link aggression, paternal care, and androgen levels across vertebrate species has yet to emerge (Lynn, 2008; Wynne-Edwards and Timonin, 2007). Paternal aggression is dependent on high androgen levels in the blue-banded goby, *Lythrypnus dalli* (Rodgers et al., 2006) and the California mouse, *Peromyscus californicus* (via aromatization to estradiol; Trainor and Marler, 2002). Here we found that convict cichlid fathers increase aggression despite having lower androgen levels compared to lone males. Similarly, many teleost males displaying paternal care maintain high levels of aggression when circulating androgen levels are low, including three-spined stickleback (*Gasterosteus aculeatus*; Páll et al., 2002a,b), the Azorean rock-pool blenny (*Parablennius parvicornis*; Ros et al., 2004), bluegill sunfish (Neff and Knapp, 2009), and the cooperatively breeding cichlid *Neolamprologus pulcher* (Desjardins et al., 2008). In other species, including humans and frogs, males also have decreased androgen levels when caring for offspring (Gettler et al., 2011; Townsend and Moger, 1987). It may be that aggression as a form of indirect paternal care is mediated by other neurochemicals in species where aggressive nest defense is necessary for offspring survival yet androgen levels remain low.

Neural substrates of paternal care

The widespread use of IEGs as neural activity markers has led to an increased understanding of the neural circuits underlying complex social behavior (Burmeister et al., 2005; Hoke et al., 2004). To our knowledge no other study has characterized IEG induction in fathers with and without their own offspring present. We have employed this approach to identify brain regions regulating paternal care in convict cichlids. Our results indicate that the putative lateral septum homolog of teleosts (Vv) may be involved in paternal care, as induction of c-Fos in Vv is correlated with paternal behavior. Interestingly, male prairie voles exposed to pups also showed c-Fos induction in the lateral septum (Kirkpatrick et al., 1994), although this study used naïve males. Given our results, it is particularly intriguing that nonapeptide action in the lateral septum is required for paternal care in prairie vole (Wang et al., 1994), although this

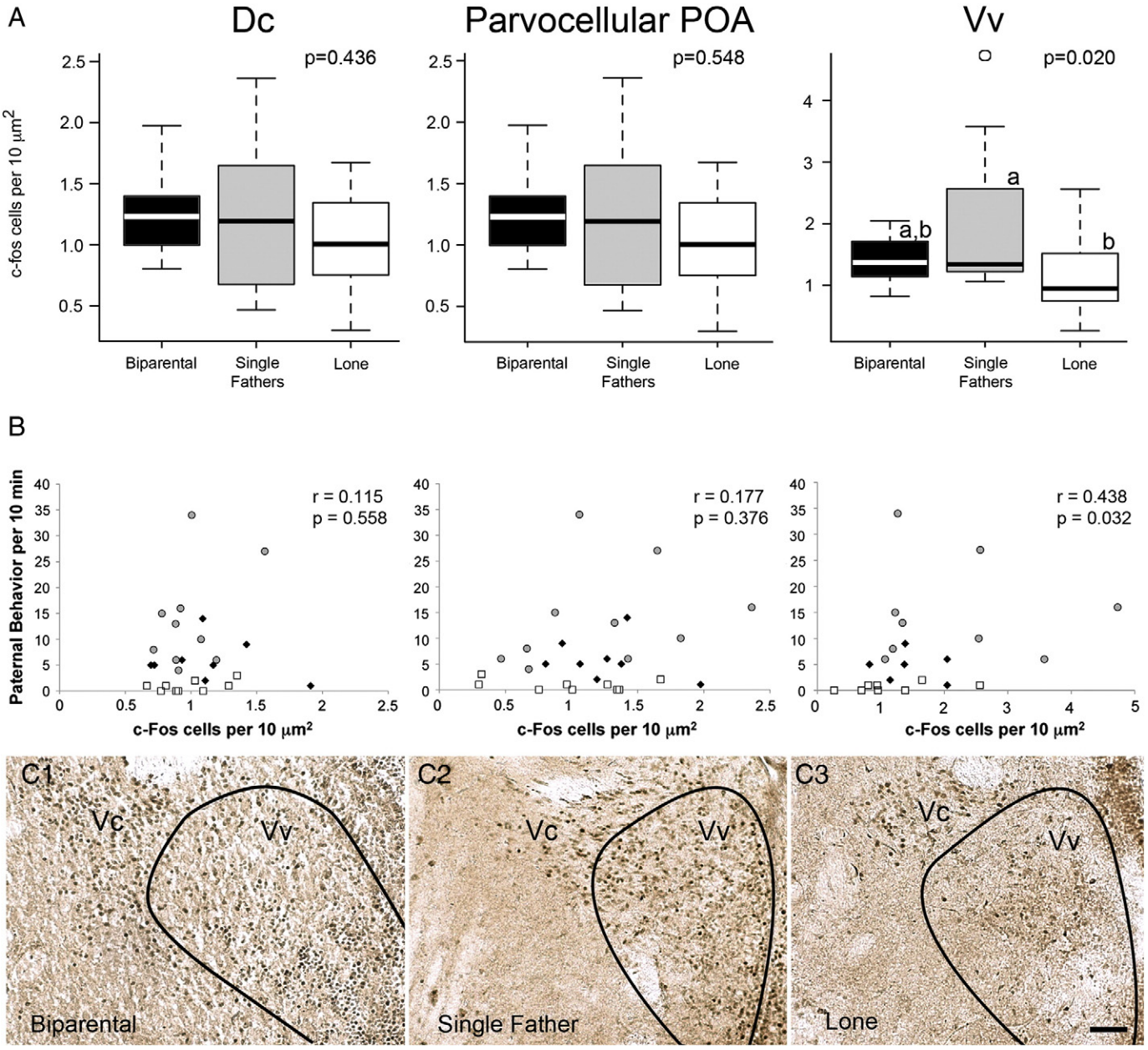

Fig. 3. Neuronal induction of c-Fos in convict cichlid fathers after social manipulation. Induction of c-Fos in candidate brain regions (A), including the central part of the dorsal telencephalon (Dc), the parvocellular preoptic area (POA), and the ventral part of the ventral telencephalon (Vv), are shown in biparental males (black, n = 7), single fathers (gray, n = 9), and lone males (white, n = 8) represented by box and whisker plots. ANOVA p-value is indicated in the corner while groups not sharing the same letter are significantly different with a Fisher's LSD post-hoc test. Correlations between c-Fos induction and paternal care are shown (B) for biparental males (black diamond), single fathers (gray circles), and lone males (whites squares). Representative micrographs of c-Fos induction in the Vv are shown for each experimental group (C). Scale bar is 50 μm.

study only investigated vasopressin. IST fibers are present in the lateral septum of teleosts (Dewan et al., 2008, 2011; Goodson et al., 2003) and IST receptors are expressed in this region (Huffman et al., in press), further supporting an evolutionarily conserved role of this brain region in the regulation of paternal care. Given this information, more research in other vertebrates is needed to determine the extent to which isotocin (and its tetrapod homologues oxytocin and mesotocin) mediates paternal care in the lateral septum.

The POA has also been implicated in mammalian parental care, as medial POA lesions disrupt parental behavior in male and female California mice (Lee and Brown, 2002), and fathers exposed to unfamiliar pups show increased c-Fos induction in the medial POA compared to naïve males (de Jong et al., 2009). Similarly, electrical stimulation of the POA in male bluegill sunfish elicits paternal care (Demski and Knigge, 1971), suggesting the POA's role in paternal care is functionally similar in mammals and teleosts. Although overall c-Fos induction in the parvocellular POA did not differ between fathers and lone males, we did find that single fathers had higher c-Fos induction specifically in parvocellular IST neurons. This result emphasizes the importance of incorporating functional identification of neuronal cell types into IEG studies. de Jong et al. (2009) also found preoptic c-Fos induction in paternal California mice, though not specific to nonapeptidergic neurons. This discrepancy may be due to species differences or variation in experimental design, as our paternal males cared for their own offspring while the mice were presented with unfamiliar pups.

Lastly, we did not find any significant variation in c-Fos induction in Dc, although this region has been implicated in paternal care in bluegill sunfish (Demski and Knigge, 1971). There are several possible explanations for this result. First, it should be noted that in sunfish

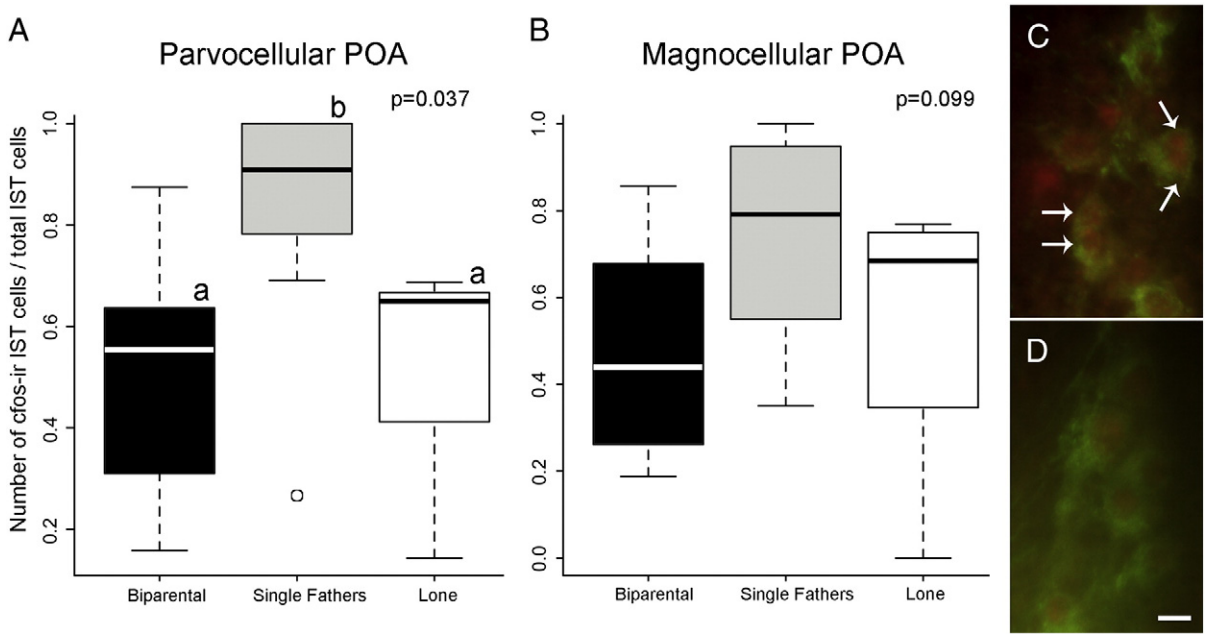

Fig. 4. IST neurons show more c-Fos induction in males displaying paternal care. Induction of IST neurons in the parvocellular POA (A) and magnocellular POA (B) is represented by box and whisker plots for biparental males (black, n = 6), single fathers (gray, n = 9) and lone males (white, n = 6). ANOVA test p-values are shown in the corners while groups not sharing the same letter are significantly different with a Fisher's LSD post-hoc analysis at $p<0.05$. Representative micrographs of c-Fos (red nucleus) and IST (green cytoplasm) co-localization are shown from a single father (C) and biparental male (D) in the POA. Arrows indicate cells coexpressing c-Fos and IST. Scale bar is 10 μm.

only males display offspring care. This fundamental difference between the two species may be associated with corresponding variation in the neural substrates underlying paternal behavior. Secondly, it is possible that there is not an overall change in Dc c-Fos induction, but that induction shows a different time course or is limited to a particular cell type. Finally, social stimulation of Dc may induce the expression of IEGs other than c-Fos. Further functional studies in convict males and other teleosts are required to understand the role Dc might play in regulating paternal care.

An important aspect of examining IEG induction in our study is that we examined c-Fos expression 4 days after spawning (and the subsequent social manipulation) and 1 h after the final observation period. Given this experimental design, it is possible that brain region-specific responses could be observed using a different IEG, such as *egr1*. Furthermore, different patterns might have been observed if c-Fos induction was measured on Day 1 rather than Day 4. However, given the strong positive correlation we observed between paternal care and Vv c-Fos induction, we suggest that the observed IEG pattern is at least in part reflective of the animal's behavior immediately prior to tissue sampling, although the overall level of paternal care obviously depended on the experimental manipulation any given male experienced.

IST modulation of male behavior in a family group

Our pharmacological results suggest that the IST receptor modulates paternal behavior in male convict cichlids, as blocking the IST receptor inhibited the normal increase in paternal care on Day 2 in biparental males. Very few other studies have examined the function of oxytocin in paternal care and any evidence for a functional role of oxytocin in mammalian paternal care is inconclusive. Treatment of male prairie voles with oxytocin receptor agonists or

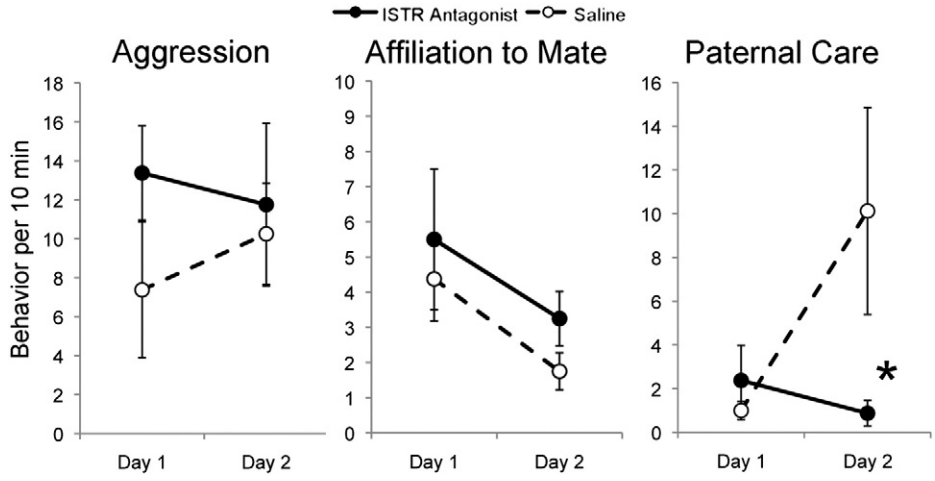

Fig. 5. IST receptor antagonist treatment blocks rise in paternal care. Biparental males were given either an IST receptor antagonist (n = 8) or saline (n = 8) for 2 days after spawning. Data are represented as the mean ± SEM for 2 days post spawn; *ANOVA $p \leq 0.05$.

antagonists failed to change their behavior towards pups (Bales et al., 2004), although blocking both vasopressin and oxytocin receptors simultaneously impaired some aspects of paternal behavior. It should be noted, however, that this study manipulated naïve males, not fathers, which might have altered expression of vasopressin and oxytocin receptors (Wang et al., 2000). Interestingly, intranasal oxytocin increases responsiveness of human fathers to their children (Naber et al., 2010), and similarly, in monogamous marmoset monkeys, oxytocin increases paternal care (Saito and Nakamura, 2011). Given the evidence presented here, it may be fruitful to further investigate the role of oxytocin in paternal care across vertebrates.

IST does not appear to play a significant role in affiliative behavior towards the mate in an established pair bond. Importantly, affiliative behavior towards the female is maintained at high levels even after spawning, suggesting that these behaviors may be important for pair bond maintenance. A previous study in convict cichlids used a broad nonapeptide receptor antagonist to show that AVT and/or IST mediate the formation of a pair bond but not its maintenance (Oldfield and Hofmann, 2011). Such a separation of the molecular mechanisms underlying the establishment of a pair bond from those regulating maintenance of the pair bond has also been described in prairie voles (Lim and Young, 2006), and the present study provides further evidence that IST likely does not play a significant role in pair maintenance in this species. Finally, it is important to note that the stress resulting from the removal of the mate cannot be excluded as a possible confounding variable in our results. In the yellow-toothed cavy (*Galea monasteriensis*), a monogamous rodent, disruption of a pair bond by short-term female mate removal resulted in increased cortisol levels in males (Adrian et al., 2008). Similar investigations in male-only parental care species would be required to fully eliminate this potential confound.

Evolutionary convergence in neuroendocrine mechanisms of paternal care

Although paternal care has evolved independently several times in both the mammalian, avian and teleost lineages (Reynolds et al., 2002), the neuroendocrine similarities in paternal care across teleosts and mammals are striking (studies testing the role of neuropeptides or brain regions in paternal birds and amphibians are sorely lacking). We have described here that the lateral septum and preoptic area likely are both important for paternal care in the convict cichlid, mirroring findings in mammals (Lee and Brown, 2002; Wang et al., 1994). Additionally, we have discovered an important role for IST in cichlid fathers, as observed in mammalian fathers (Naber et al., 2010). This role of the oxytocin system facilitating paternal care also parallels the role of oxytocin in maternal care (Francis et al., 2000). We therefore tentatively suggest that as paternal care evolved numerous times independently in different vertebrate lineages (likely as a consequence of very different selection pressures and ecological constraints; see Werren et al., 1980), ancient neuroendocrine and neuroanatomical substrates were recruited repeatedly to converge on similar social phenotypes. Complementary studies in birds and amphibians are urgently needed in order to determine to what extent other independent evolutionary transitions to paternal care relied on such deep molecular and neural homologies (Shubin et al., 2009).

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Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.yhbeh.2012.03.009.

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