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Opposite effects of nonapeptide antagonists on paternal behavior in the teleost fish *Amphiprion ocellaris*

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ABSTRACT

The nonapeptides isotocin (IT) and arginine vasotocin (AVT), along with their mammalian homologs oxytocin and arginine vasopressin, are well known regulators of social behaviors across vertebrate taxa. However, little is known about their involvement in paternal care. Here, we measured the effect of an IT and an AVT V1a receptor antagonist on paternal behaviors in the primarily paternal teleost *Amphiprion ocellaris*. We also measured the effect of the IT receptor antagonist on aggression in dyadic contests between two non-reproductive fish to assess specificity of the effect on paternal behaviors. Individual differences in levels of paternal behaviors (nips, fanning the eggs, and proportion of the time in the nest) were consistent across spawning cycles when no treatments were administered. The IT receptor antagonist severely reduced paternal behaviors but had no effect on aggression, whereas the AVT V1a receptor antagonist increased paternal behaviors. These results support the idea that IT signaling is crucial for the expression of paternal behavior in *A. ocellaris*. Based on a previous study showing that the AVT V1a antagonist decreases aggression in dyadic contests, we hypothesize that the antagonist enhances paternal behavior indirectly by reducing vigilance and aggression, thereby alleviating effort directed towards other competing behaviors and allowing for the increased expression of paternal behaviors.

1. Introduction

Parental care is a costly investment on the part of the caregiver as it presents a trade-off between current parental investment and opportunities for future reproductive events (Clutton-Brock, 1991; Trivers, 1974). Often, this trade-off is different for each sex. Male care is less common, as males produce a large number of metabolically inexpensive gametes, and generally have higher reproductive success siring as many offspring as possible (Kokko and Jennions, 2012; Trivers, 1972). Thus, paternal care is predicted to occur only when the cost of desertion is high, or future reproductive opportunities are low (Gross and Sargent, 1985). Conversely, females produce fewer gametes that are larger in size, and generally have higher reproductive success when effort is directed towards egg survival (Gross, 2005; Trivers, 1972). High rates of maternal care has consequently led to a bevy of studies on the evolution and underlying neural regulation of female parental care. However, unlike female parental care, there have been relatively few studies examining mechanisms underlying male parental care, despite the fact that in many species exhibiting male parental care, paternal effort is of equal importance to maternal care, and in some species even more important than female care for offspring survival (Kleszczyńska et al., 2012; Ripley and Foran, 2010; Rodgers et al., 2006; Trainor and Marler, 2002).

While paternal care is relatively uncommon, teleost fishes are a unique group among vertebrates in which male parental care is the predominant parental care strategy (Baylis, 1981; Magee et al., 2006; O'Connor et al., 2009; Pradhan et al., 2014). Recent work has presented evidence of a highly evolutionarily conserved social decision-making network. More specifically, the brain regions, neuropeptides, and hormones involved in the regulation of social behaviors share similar pathways across vertebrate taxa (O'Connell and Hofmann, 2011). Thus, teleosts present interesting opportunities to gain insight into the regulation of vertebrate parental care (Amundsen, 2003; Gross and Sargent, 1985; Ridley, 1978). To date, the few species of teleosts where paternal care has been explored present confounding results, which may in be in part due to the often simultaneously occurring social displays such as courtship and territory defense (Kleszczyńska et al., 2012; O'Connell et al., 2012). Hence, the high homology of the underlying circuitry involved in the regulation of social decisions allows insights to be gained broadly about fathering by studying species where high levels of paternal care are exhibited and in which care can be isolated form other confounding social behaviors (Amundsen, 2003; Goodson, 2005; O'Connell et al., 2012).

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The anemonefish, *Amphiprion ocellaris* is a predominantly paternal care species in which male care is critical for offspring survival (DeAngelis and Rhodes, 2016). Their obligate symbiosis with protective host sea anemones and highly monogamous lifestyle has removed opportunities of finding additional mates. (Godwin, 2009; Mitchell and Dill, 2005). Hence, the substantial effort of parental care does not come at the expense of seeking additional reproductive partners, as in other species where additional mating opportunities are more pervasive. This leads to an increased fitness by directing effort towards current brood survival and enables research to be directed specifically at paternal care in isolation of other confounding social displays. In addition, *A. ocellaris* is particularly well suited for laboratory studies on paternal care due to their small home ranges and adaptability to aquarium conditions (Iwata et al., 2008). They also have a short generation time (1 year) and spawn readily in captivity. Parental behaviors are easily observed and quantified, and sex differences in parental care have already been established. Males are the predominant caretakers, and spend the majority of their lives caring for eggs (DeAngelis and Rhodes, 2016).

As an initial foray into the regulation of the high levels of paternal care displayed by *A. ocellaris,* the vasopressin and oxytocin systems were logical candidates for exploration. The vasopressin and oxytocin systems are well situated for functioning in parental care as cell bodies containing these neuropeptides reside in the preoptic area of the hypothalamus, an area of the brain well known for regulating reproductive behaviors (Insel and Young, 2000; Kline et al., 2011). The cell bodies and dendrites heavily express sex steroid receptors, and project axons all over the brain, the terminals of which release the neuropeptides onto multiple neuron types (Foran and Bass, 1999). Receptors for these neurochemicals are known to be expressed in crucial brain areas that comprise the social decision-making network such as ventral midbrain, basal ganglia, and hippocampus (O'Connell and Hofmann, 2011). Nonapeptide cells in the brain receive information from the gonads via the blood and relay that information to the rest of the brain for making social decisions related to reproduction (Maruska and Fernald, 2011). While a relationship between these highly conserved neuropeptides and maternal care and other social behaviors has been identified, the extent to which these nonapeptides play a role in paternal care remains unclear.

The neuropeptide oxytocin (OT) has been well studied for its role in female parental care; its release at parturition is a catalyst for an array of behavioral and physiological changes critical for offspring survival (Bartz et al., 2010; Francis et al., 2000). While less understood in males, it has been suggested that OT plays a similar role in paternal behavior. In humans, OT rises in males following contact with an infant (Feldman et al., 2010); in the monogamous California mouse *Peromyscus californicus*, OT levels are higher in expectant fathers (Gubernick et al., 1995); and in teleost *Amatitlania nigrofasciata* blockade of the IT receptor reduced paternal effort (O'Connell et al., 2012). These results support a conserved function of OT in the promotion of parental care.

Like OT, the neuropeptide AVP/AVT has a well-documented role in the regulation of social behaviors across a wide array of vertebrates, and has been suggested to be more important in regulating male social behaviors (Insel and Young, 2000). In teleosts, AVT is broadly implicated in behaviors leading to reproduction, but its specific function varies depending on species and social status (Foran and Bass, 1999; Insel and Young, 2000; Kleszczyńska et al., 2012). More specifically, AVT has been implicated in the regulation of dominance, aggression, and courtship (Huffman et al., 2015; Semsar et al., 2001; Yaeger et al., 2014). AVP/AVT is clearly important in a variety of male social behaviors, but surprisingly few studies have addressed its role in paternal care, and therefore how AVT signaling functions to promote or inhibit male parental care remains unclear.

The goal of this study was to determine the extent to which AVT and IT signaling play a role in the modulation of paternal care in a species where high paternal effort can be isolated in the absence of other confounding co-occurring social behaviors often exhibited in other species. In this study we hypothesize that IT signaling is critical for high levels of paternal care, and thus, blockade of IT signaling will reduce total parental effort. Given that blockade of AVT V1a receptors reduced aggression in *A. ocellaris*, and the diversity of roles reported for AVT in the regulation of multiple different competing social behaviors, we were not certain how blockade of V1a receptors would affect paternal care.

2. Materials and methods

2.1. Animals and husbandry

A. ocellaris, bred in the laboratory from a female obtained from ORA (Oceans Reefs and Aquariums, Fort Pierce, FL), and a wild caught male (location unknown; obtained through the pet trade) were used. Tank conditions for all individuals were set to mimic the natural environment, with a temperature of 79 °F, photoperiod of 12:12 (lights on at 7:00 am and off at 7:00 pm), pH of 8.2 and specific gravity of 1.026. Individuals were housed in groups of 2 or 3 in 20-gallon aquariums, and allowed over a year for consistent spawning (spawn period of <20 days) prior to the onset of behavioral observations or experimental manipulations. Each tank contained one clay pot (4-inch diameter) to serve as the nest site where the fish deposit their eggs. Lengths and weights were taken one week prior to the start of the experiment and then again at the conclusion of the study. In experiment 1, mean body weight and standard length were 2.97 g (range 1.60–5.70 g) and 53 mm (45–70 mm) for males and 7.38 g (4.60–9.60 g) and 70 mm (46–96 mm) for females. In experiment 2, mean body mass and standard length of the males were 2.60 g (range 1.88–3.44 g) and 45 mm (38–53 mm). Fish used in experiment 1 were approximately 24 months of age, while fish used in experiment 2 were approximately 18 months of age. All fish in experiment 1 had previously been observed caring for a batch of fertilized eggs and were established as reproductively mature. The fish in experiment 2 were non-reproductive males taken from a holding tank, which contained >20 fish (i.e., no reproductive pairs could be established). Adequate measures were taken to ensure minimal pain and discomfort for all animals used in experimental procedures. All procedures were approved by the University of Illinois Institutional Animal Care and Use Committee.

2.2. Experiment 1: Effects of AVT and IT antagonists on paternal behavior

2.2.1. Baseline measures of parental behavior, sex differences and consistency across breeding cycles

Aquariums that contained reproductive pairs ($N = 8$) were recorded daily for a 10-minute behavioral observation period (between 2:00–3:00 pm daily) over the entire spawn cycle. Behavioral analysis for spawn period 1 (SP1) began on the day the eggs were laid (day 0) and ended when eggs hatched into the larval phases (7–9 days). Parental behaviors were scored using JWatcher behavioral event recording software (Blumstein and Daniel, 2007). The amount of time spent in the nest, as well as the total number of nips and fans were quantified for the reproductive male and female in each aquarium. Nips are defined as mouthing the eggs to keep them clean of debris and fungus, while fanning is the process of using the pectoral and caudal fins to aerate the eggs. Total numbers of parental behaviors (the sum of nips and fans) were analyzed. This procedure was repeated again during spawn period 2 (SP2) to test the extent to which individual variation in behavior was consistent across spawn periods, before starting the pharmacological manipulations.

2.3. Pharmacological manipulations

Only males received the pharmacological manipulations. Following the analysis above, during the subsequent spawn period 3 (SP3),