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Agonistic interactions elicit rapid changes in brain nonapeptide levels in zebrafish

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ABSTRACT

The teleost fish nonapeptides, arginine vasotocin (AVT) and isotocin (IT), have been implicated in the regulation of social behavior. These peptides are expected to be involved in acute and transient changes in social context, in order to be efficient in modulating the expression of social behavior according to changes in the social environment. Here we tested the hypothesis that short-term social interactions are related to changes in the level of both nonapeptides across different brain regions. For this purpose we exposed male zebrafish to two types of social interactions: (1) real opponent interactions, from which a Winner and a Loser emerged; and (2) mirror-elicited interactions, that produced individuals that did not experience a change in social status despite expressing similar levels of aggressive behavior to those of participants in real-opponent fights. Non-interacting individuals were used as a reference group. Each social phenotype (i.e. Winners, Losers, Mirror-fighters) presented a specific brain profile of nonapeptides when compared to the reference group. Moreover, the comparison between the different social phenotypes allowed to address the specific aspects of the interaction (e.g. assessment of opponent aggressive behavior vs. self-assessment of expressed aggressive behavior) that are linked with neuropeptide responses. Overall, agonistic interactions seem to be more associated with the changes in brain AVT than IT, which highlights the preferential role of AVT in the regulation of aggressive behavior already described for other species.

Introduction

In a wide range of vertebrate species, nonapeptides of the vasotocin family [e.g. arginine vasopressin (AVP) and oxytocin (OT) in mammals; arginine vasotocin (AVT) and isotocin (IT) in teleosts] have emerged as key regulators of social behavior (Goodson and Bass, 2001). Among teleost fish these effects include the regulation of aggressiveness (Godwin and Thompson, 2012; Goodson, 1998; Yaeger et al., 2014) and social status acquisition (Almeida and Oliveira, 2015; Almeida et al., 2012; Greenwood et al., 2008; Huffman et al., 2015; Larson et al., 2006a; Lema et al., 2015). However, there is considerable variation in the function of nonapeptide circuits related to AVT/AVP and IT/OT, which appears to be species- and context-dependent (Goodson, 2008). Among fish, AVT and IT administration could either increase or decrease aggression and courtship depending on the species (Godwin and Thompson, 2012). In general, the AVT/IT neurosecretory system in fish consists of three main cell groups distributed along the ventral portion of the preoptic area (POA) [gigantocellular (gPOA), magnocellular (mPOA), and parvocellular (pPOA), reviewed in (Urano and Ando, 2011)], which project fibers to multiple target areas, such as ventral telencephalon, diencephalon, and various mesencephalic structures, in addition to projections to the neurohypophysis (Saito et al., 2004), suggesting a diffuse neuromodulatory role for these peptides. Therefore, the nonapeptide regulation of social behavior may occur at multiple target areas and at different levels. First, it can be related to the number of nonapeptide producing cells and their activity (e.g. as indicated by cell body size) in the relevant cell group(s) in the POA. In some teleost species, the expression of social dominance has been associated with the number or size of AVT-ir cells in mPOA or gPOA, whereas social submission has been associated with the number or size of AVT-ir cells in pPOA (e.g. zebrafish, *Danio rerio* (Larson et al., 2006a); African cichlid, *Astatotilapia burtoni* (Greenwood et al., 2008) butterfly fishes (Dewan et al., 2011)). In contrast, in other

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species, social submission has been associated with changes in cell populations in the mPOA and gPOA (e.g. African cichlid *Oreochromis mossambicus* (Almeida and Oliveira, 2015)), and aggressive behavior with variations in size of AVT-ir cells in the pPOA instead (Pupfish, *Cyprinodon nevadensis amargosae* (Lema, 2006)). Secondly, the sensitivity of the target tissue to nonapeptides, as indicated by the local expression of mRNA for their receptors, may also be linked to their regulation of social behavior. For example, in zebrafish, the V1b and oxytocin-like receptors are overexpressed in the hypothalamus of dominant males (Filby et al., 2010), and in pupfish (*C. nevadensis amargosae*) transcripts encoding V1a1 are expressed at higher levels in the telencephalon and hypothalamus of subordinate males, whereas the V1a2 variant is more abundant in the telencephalon of dominants (Lema et al., 2015). Thirdly, since the prepro-vasotocin and prepro-isotocin are produced in the cell body and are then transported to the target areas via axonal transport where the final, bioactive nonapeptides are released at the synapses, the local availability of these peptides may itself be involved in regulation of behavior. So far few studies have measured local peptide concentrations at regions of interest in the brain in order to link it with the expression of different social behaviors. Cichlids subordinate males present higher AVT levels in whole brain and pituitary than dominants, and no difference between social status was detected for IT (Almeida et al., 2012; Reddon et al., 2015). In the three-spined stickleback, both AVT and IT levels are higher in whole brain of dominant males, whereas nonapeptides' levels in females' brain are related to breeding and egg deposition, rather than to aggression (Kleszczyńska et al., 2012; Kulczykowska and Kleszczyńska, 2014). Finally, among different wrasse species brain AVT/IT levels have been shown to vary with the degree of cleaning (mutualistic) behavior (Kulczykowska et al., 2015). The examples provided above suggest that an association between AVT/IT system and social status in fish is not conserved. Since only the nonapeptides, which emerge from the prepro-peptide complex, are biologically active at the target site, a good approach to study such diversity is the direct measurement of these peptides in the brain areas where they are hypothesized to act as neuromodulators.

In the present work, we used zebrafish (*D. rerio*) males to study the link between changes in social status and brain nonapeptides' levels. Zebrafish males establish dominance relationships through agonistic interactions, and the behavior expressed in these interactions is well characterized. (Oliveira et al., 2011; Teles et al., 2013). At the start of the interaction both opponents exhibit the same behavioral repertoire (displays, circle, and bites). After the fight is solved and a Winner and a Loser emerge, an asymmetry of expressed behaviors is observed, where all aggressive acts are initiated by the dominant (Winner) and the subordinate (Loser) only displays submissive behavior (Oliveira et al., 2011). In zebrafish, the outcome of a fight can have a significant impact in subsequent interactions, since the Winner of an encounter is more likely to win its next interaction, whereas the Loser decreases its probability of success, indicating the relevance of past experience in agonistic interactions (Oliveira et al., 2011). In the behavioral paradigm used here, acute (30 min) agonistic encounters between conspecifics produced three behavioral phenotypes: (1) Winners of real opponent interactions, that hence increased their social status; (2) Losers of real opponent interactions, that concomitantly decreased their social status; and (3) Mirror-fighters, that fought their own image on a mirror, but that despite expressing aggressive behavior and observing it in its opponent (i.e. the mirror image) did not experience a change in social status, since they did not either won or lost the mirror fight. Thus, herein we assessed to what extend the changes in nonapeptide levels are coupled with the changes in social status (increased in Winners; decreased in Losers) and with the expression/perception of aggressive behavior independently of changes in social status, as experienced by Mirror-fighters.

Methods

Animals

Thirty-two adult wild-type zebrafish (*D. rerio*) males of the AB strain were used in this experiment. Animals were bred and held at the Fish Facility of Instituto Gulbenkian de Ciência (IGC, Oeiras, Portugal) in mixed sex groups under a 14L:10D photoperiod and with a water temperature of 28 °C. Water quality was monitored daily for pH and conductivity (7 and 700 μSm respectively), and weekly for ammonia (0.01–0.1 ppm), nitrite (<0.2 ppm) and nitrate (<50 ppm)concentrations. Animals were fed twice a day.

Behavioral paradigm

Fish were tested in an agonistic behavior paradigm previously described for zebrafish (Oliveira et al., 2011; Teles et al., 2013). In brief, animals were grouped in size matched pairs and each pair randomly assigned to one of the following conditions: real opponent fights (mean length ± SEM: 2.78 ± 0.03 cm; mean body mass ± SEM: 0.28 ± 0.01 g); mirror elicited fights (mean length ± SEM: 2.67 ± 0.04 cm; mean body mass ± SEM: 0.27 ± 0.01 g); no social interaction (mean length ± SEM: 2.82 ± 0.05 cm; mean body mass ± SEM: 0.31 ± 0.01 g). Dyads were left overnight in the experimental tank (12.5 × 20 × 15 cm) that was beforehand divided with an opaque PVC partition into two visually isolated areas. After this period, the partition was removed and fish were allowed to interact with a conspecific in the real opponent interaction, or with their own mirror image on a mirror, in the mirror-elicited fight, for a period of 30 min. In the reference group (Control) no social interaction occurred; on each side a partition was also removed, but the opaque PVC divider between the two animals remained in place preventing any visual contact between the two fish. Thus, four behavioral phenotypes emerged: Winners (n = 8) and Losers (n = 8) of real opponent interactions, Mirror-fighters (n = 8) that experience unsolved fights, and Controls (n = 8) non-interacting fish. Behavioral interactions were recorded with a digital camera for subsequent analysis.

Brain collection

Immediately after the encounter animals were sacrificed with an overdose of tricaine solution (MS222, Pharmaq; 500–1000 mg/L) and sectioning of the spinal cord. The brain was macrodissected under a stereoscope (Zeiss; Stemi 2000) into six areas: olfactory bulbs (OB), telencephalon (TL), optic tectum (OT), diencephalon (DE), cerebellum (CB), and brainstem (BS). Immediately after collection brain tissue was placed on dry ice and stored at −80 °C until further processing. In order to standardize the time between sacrifice and brain tissue collection between individuals, only one fish from each dyad was used for nonapeptide quantification.

Quantification of nonapeptides by high performance liquid chromatography with fluorescence detection (HPLC-FL)

Brain areas were weighed and sonicated in 1 mL of Milli-Q water (Microson™ XL, Misonix, USA) acidified with glacial acetic acid (3 μL), and placed in a boiling water bath for 3.5 min. The homogenates were then centrifuged (12.000*g*, 20 min, 4 °C), and the supernatants loaded into solid phase extraction (SPE) columns (100 mg/1 mL, C18 Bakerbond, J.T. Baker) previously conditioned with 3 mL methanol and 3 mL Milli-Q water. To purify the samples, columns were washed sequentially with 1 mL of 5% acetic acid, 1 mL Milli-Q water and 1 mL of 5% methanol, and the peptides eluted with 2 mL mixture of ethanol: 6 M HCl (2000:1, v/v). The eluate was evaporated to dryness in a Turbo Vap LV Evaporator (Caliper Life Sciences, USA) and samples frozen, and stored at −80 °C until further processing.