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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.

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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

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 (12.000g, 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.

For HPLC analysis, samples were dissolved in 40 μL of 0.1% TFA (trifluoroacetic acid) in 30% acetonitrile and divided into two replicates. Pre-column derivatization of AVT and IT was performed according to the procedure previously reported (Gozdowska et al., 2013). For derivatization reaction, 20 μL of sample and 20 μL of 0.2 M phosphate buffer (pH = 9) were mixed, and 3 μL of NBD-F (4-fluoro-7-nitro-2,1,3-benzoxadiazole: 30 mg in 1 mL of acetonitrile) was added. The solution was heated at 60 °C for 3 min, cooled on ice, acidified with 4 μL of 1 M HCl and eluted in a HPLC column. Derivatized samples were measured with Agilent 1200 Series Quaternary HPLC System (Agilent Technologies, USA). Chromatographic separation was done on an Agilent ZORBAX Eclipse XDB-C18 column (150 mm × 4.6 mm I.D., 5 μm particle size). The gradient elution system was applied for separation of derivatized peptides. The mobile phase consisted of solvent A (0.1% TFA in H_2O) and solvent B (0.1% TFA in acetonitrile: H_2O (3:1). A linear gradient was 40–65% of eluent B in 20 min. Flow rate was set at 1 mL/min and the column temperature set at 20 °C. Injection volume was 47 μL and fluorescence detection was carried out at 530 nm with excitation at 470 nm. The two peptides were analyzed simultaneously in every sample and data expressed in pmol of peptide per tissue weight (mg).

Behavioral analysis

For the behavioral data, video recordings were analyzed using a computerized multi-event recorder (Observer XT, Noldus, Wageningen, The Netherlands) and following the ethogram of zebrafish agonistic behavior (Oliveira et al., 2011). The following behavior measures were taken: latency to the first interaction; fight resolution time; and frequency of aggressive (bite, chase and strike) and submissive (freeze and flee) behaviors in the last 5 min of the interaction (post-resolution phase). The last two measures were taken at the end of the interaction because at this stage of the fights behavioral phenotypes are well differentiated: Winners only express aggressive behaviors, whereas Losers only express submissive behaviors.

Statistical analysis

To assess differences between real opponent and mirror-elicited fights, the latency for the first attack, fight resolution time, and aggression frequencies were compared with a *t*-test. The link between social phenotypes (Winners, Losers, Mirror-fighters and Controls) and neuropeptide (AVT and IT) levels in different brain areas (i.e. olfactory bulbs, telencephalon, diencephalon, optic tectum, cerebellum and brainstem) were assessed using a linear mixed model (LMM) with a random effect for the subjects. Data were log transformed in order to meet the parametric assumptions of normal distribution and homoscedasticity. Tukey post-hoc tests were used to identify which groups within each factor were responsible for significant main effects. Planned multiple comparisons analyses (contrast effects z-tests) followed to compare nonapeptide levels within each brain area across different social phenotypes (Winners vs. Losers vs. Mirror-fighters vs. Controls). Effect sizes for these comparisons were also reported and reference effect size values (small: d > 0.2, medium: d > 0.5, and large: d > 0.8) used to interpret the mean difference of the effect (Cohen, 1988). Pearson correlations followed by Benjamini and Hochberg's method for p-value adjustment were used to assess the association between AVT and IT levels and behavioral data. Linear discriminant function analyses (LDA) were used on AVT and IT concentrations across all brain regions, to identify the variables that contribute the most to differentiate the 4 social treatments.

Sample sizes varied due to technical difficulties during nonapeptide quantification, or to outlier values (see Table S1 in electronic supplementary material for final sample size). Outliers were identified for each condition using the generalized extreme studentized deviate procedure with a p = 0.05 and a maximum number of outliers of 20% of the sample size (Rosner, 1983). Statistical analysis was performed on R (R CoreTeam, 2015), using the following packages: nlme (linear mixed

models), multcomp (multiple comparisons), and on STATISTICA V10 and SPSSV21. For all tests the significance level used was p < 0.05.

Results

Behavior

In real opponent fights, the latency to the first attack was longer (*t* = 2.31, df = 20, p < 0.05; Fig. 1A) and the time to solve the interactions was shorter (*t* = 13.84, df = 19, p < 0.0001) than in mirror-fights. There were no differences between the two types of interactions in the frequency of aggressive behaviors (*t* = 1.53, df = 14, p > 0.05; Fig. 1B).

Nonapeptides levels in the brain

There were significant main effects of social status (LMM; $F_{3, 25}$ = 4.49, p < 0.05), and of brain area (LMM; $F_{5, 109}$ = 3.56, p < 0.01) on AVT levels, whereas no effect of the interaction between these two main factors was detected (i.e. social status x brain area, LMM; $F_{15,}$
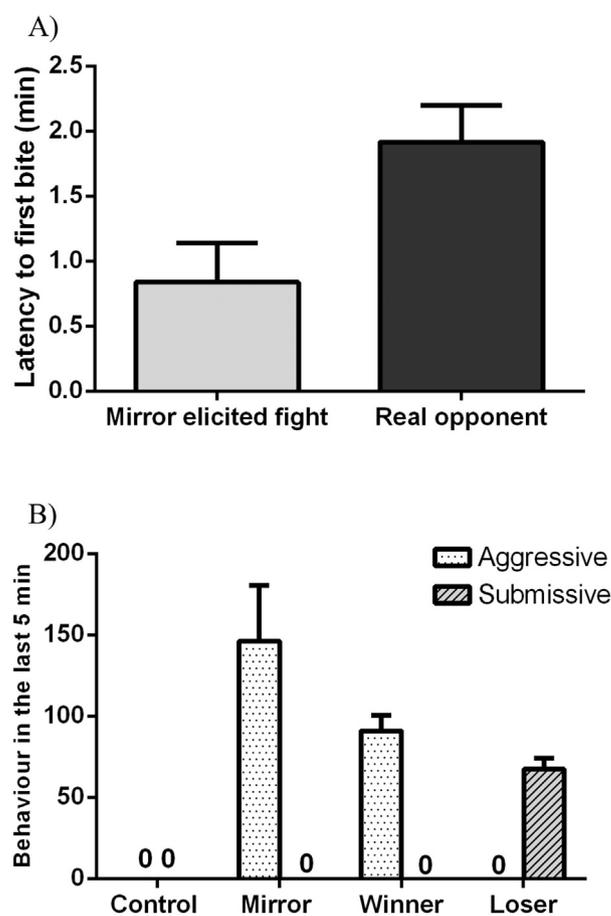

Fig. 1. Behavioral results. A) Latency to the first bite and B) mean number of aggressive acts performed in the last 5 min of the 30 min agonistic interaction; error bars represent the standard error of the mean and asterisks significant differences between treatments (p < 0.05).

$_{109}$ = 0.75, p > 0.05). Post-hoc analyses on the effect of social status revealed that AVT levels were higher both in Mirror-fighters and in Losers than in Controls (z = 3.17, p = 0.008, and z = 3.31, p = 0.005, respectively), whereas post-hoc analyses of the main effect of the brain area revealed that the diencephalon has higher AVT levels than either the optic tectum (z = −2.46, p = 0.007) or the brainstem (z = −3.66, p = 0.003). Planned comparisons of the effect of social treatment at each brain area revealed that all social phenotypes (i.e. Mirror-fighters, Winners and Losers) increased AVT levels in the telencephalon when compared to the Controls (z = 2.15, p = 0.03, d_s = 0.932; z = 2.78, p = 0.005, d_s = 1.741; z = 2.54, p = 0.01, d_s = 1.346; respectively, Fig. 2A). In the diencephalon, only Winners (z = 2.23, p = 0.02, d_s = 0.564) and Losers (z = 2.25, p = 0.02, d_s = 0.595) heightened AVT levels, and in the optic tectum and brainstem changes were only observed in the Losers (z = 2.17, p = 0.02, d_s = 1.331; z = 2.02, p = 0.04, d_s = 1.033), always using the Controls as a reference.

There were no significant main effects either of social status (LMM; $F_{3, 25}$ = 0.049, p > 0.05) or of brain nuclei (LMM; $F_{5, 93}$ = 1.99, p = 0.08) on IT brain levels. However, the interaction between these two factors was significant (LMM; $F_{15, 93}$ = 2.43, p < 0.01). Planned comparisons within each brain region revealed a decrease of IT levels in the olfactory bulbs of Winners in comparison with the other behavioral phenotypes (Controls: z = −2.95, p = 0.003, d_s = 0.957; Mirror-fighters: z = −3.62, p = 0.0003, d_s = 1.054; Losers: z = 2.82, p = 0.004,

d_s = 0.845, Fig. 2B). In contrast, there was an increase of IT levels in the diencephalon of Losers in comparison to all other phenotypes (Controls: z = 2.74, p = 0.006, d_s = 1.277; Mirror-fighters: z = 2.44, p = 0.014, d_s = 1.223; Winners: z = 2.15, p = 0.03, d_s = 1.222), and a decrease in the cerebellum in comparison with either Controls (z = −2.07, p = 0.03, d_s = 1.051) or Winners (z = −2.60, p = 0.009, d_s = 1.153).

Linear discriminant analysis (LDA) for AVT in all regions revealed a single significant function (χ^2 = 38.89, p < 0.01) that explained 95.6% of the variance (Fig. 3A). This discriminant function was most heavily loaded by the cerebellum (−2.77), optic tectum (2.68) and telencephalon (2.17) followed by the olfactory bulbs (1.10), suggesting that AVT levels in these three areas are the best predictors for distinguishing between the examined behavioral phenotypes: Winners (group centroid = 1.109), Losers (group centroid = 4.749), Mirror-fighters (group centroid = −4.95), and Controls (group centroid = −0.45). This LDA correctly classified 100% of the animals for all experimental groups. Regarding IT levels, LDA also revealed one significant function (χ^2 = 36.51, p < 0.01) that explained 99.8% of the variance found (Fig. 3B). This function was most heavily loaded by the telencephalon (10.52) and diencephalon (− 10.46), followed by olfactory bulbs (2.45), cerebellum (2.08) and optic tectum (1.12), indicating that the areas that are the best predictors of different social phenotypes differed between AVT and IT. The discrimination between groups was not so evident for
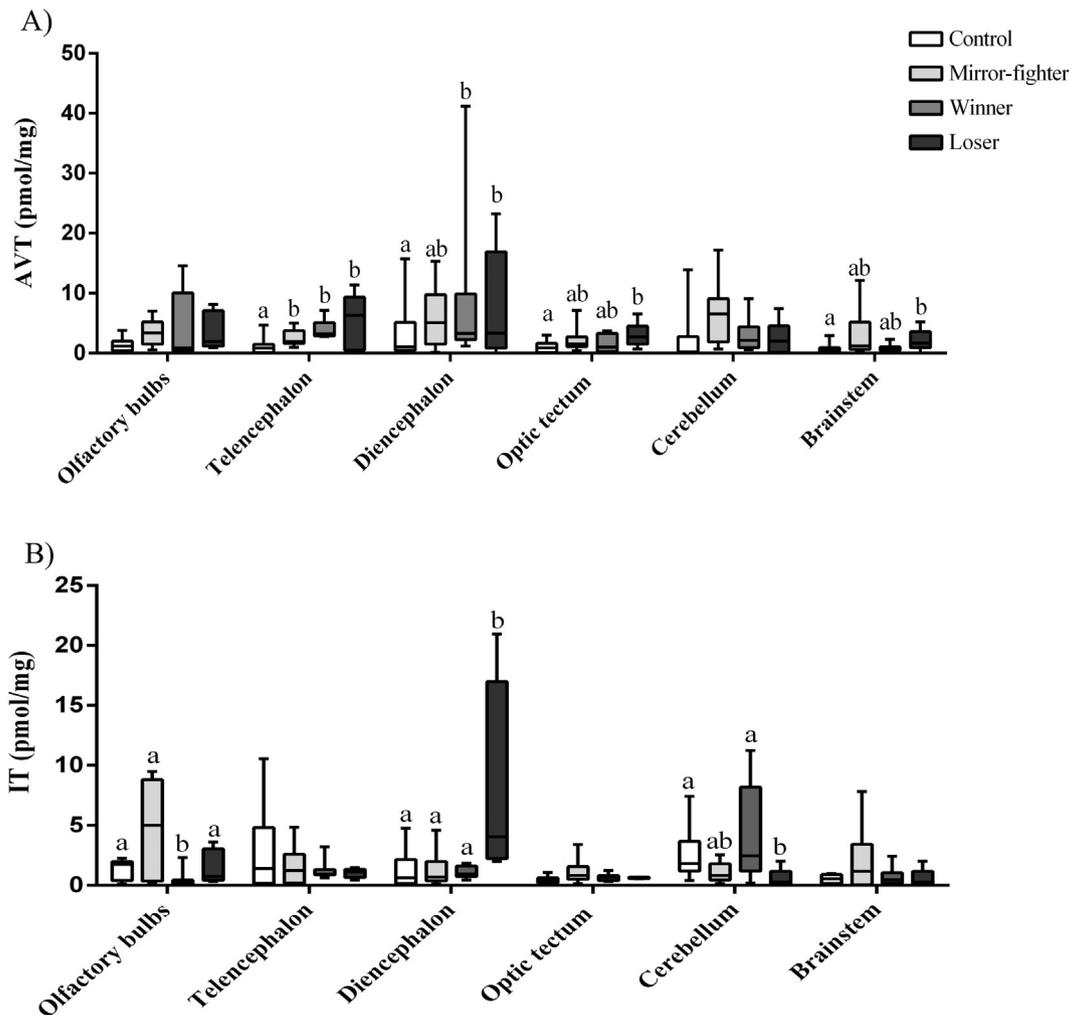

Fig. 2. Nonapeptide levels in different brain areas following different types of social experiences [social isolation (Control); fighting own image on a mirror (Mirror-fighter); winning a real opponent fight (Winners); losing a real opponent fight (Losers)]: (A) Arginine vasotocin (AVT); (B) isotocin (IT). A standard boxplot is presented with a dark line representing the median, the box representing the lower quartile (Q1) and the upper quartile (Q3), and the whiskers minimum to maximum values. Different letters indicate differences between treatments (p < 0.05).
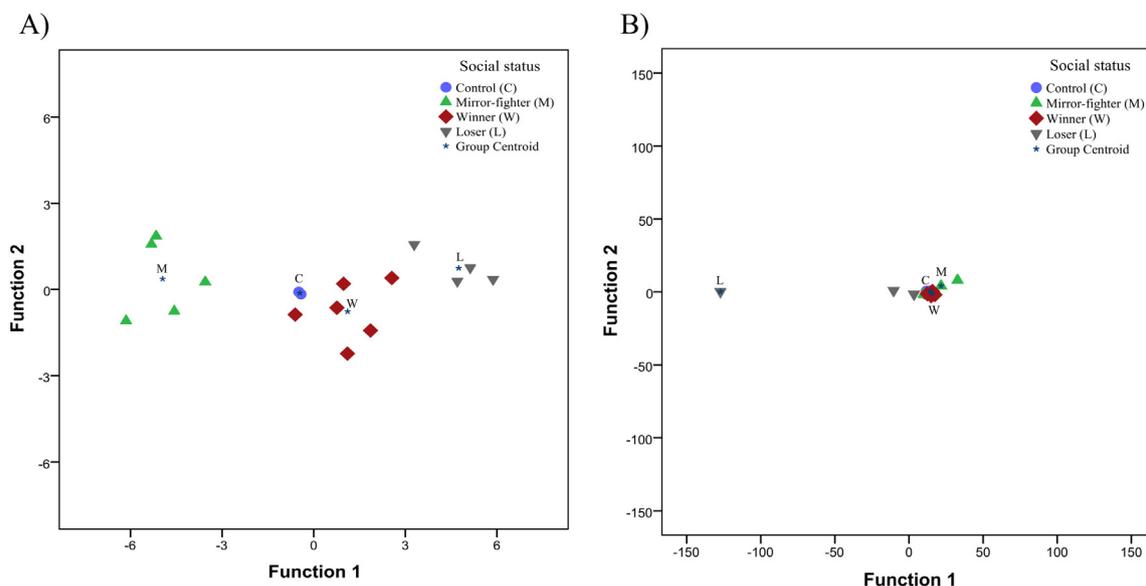
A)

B)

Fig. 3. Linear discriminant function analysis of nonapeptide levels. A) AVT discriminant functions, B) IT discriminant functions. Discriminant scores are plotted and stars represent the centroid of each social group.

the IT LDA function: Controls (group centroid = 14.26), Mirror-fighters (group centroid = 21.62), Winners (group centroid = 15.70) and Losers (group centroid = −127.2); and it correctly classified 100% of the animals in the Controls, 66.7% of Mirror-fighters, 80% of Winners and 33% of Losers, with an overall classification success of 75%.

Relationship between nonapeptides and behavior

Correlation analysis of the relationship between nonapeptide and nonapeptide levels and behavior revealed non-significant after p-value adjustments.

Discussion

The results presented here show that an acute social interaction is associated with rapid changes in nonapeptide levels in the brain. Indeed, Winners, Losers and Mirror-fighters had different patterns of nonapeptides levels in the brain. When compared to Control fish, Losers presented higher AVT levels in the forebrain (telencephalon and diencephalon), optic tectum, and brainstem, higher IT levels in the diencephalon and lower IT levels in the cerebellum. On the other hand, Winners exhibited increased AVT levels in the forebrain (telencephalon and diencephalon) and reduced IT levels in the olfactory bulbs. Finally, Mirror-fighters showed increased levels of AVT in the telencephalon. Therefore, AVT seems to be more involved in the response to an acute agonistic interaction than IT, which is in line with previously reported results that have associated AVT with aggressive behavior and IT with affiliative behaviors (Ross and Young, 2009). This view is also supported by the fact that the discriminant analysis using AVT levels to classify individuals into social groups was much more successful than that using IT levels, indicating that AVT levels better discriminate individuals of the different social phenotypes than IT.

The three social phenotypes (Winners, Losers and Mirror-fighters) generated by the behavioral paradigm used in this study can be contrasted among themselves and with a non-interacting Control in order to infer a conclusion on specific aspects of the social interaction that are linked with the observed changes in brain nonapeptides. Thus, differences in nonapeptide levels between either Winners or Losers and Controls, which are not present in Mirror-fighters, can be interpreted as being associated with changes in social status (i.e. increase in Winners; decrease in Losers). Conversely, differences in

nonapeptide levels between either Winners or Losers and Controls, which are also present in Mirror-fighters, should reflect those aspects of fighting behavior, which are common in these three groups, and should not be associated with any changes in social status since Mirror-fighters do not experience any change in status. Finally, differences in nonapeptide levels between Mirror-fighters and Controls that are not present in either Winners or Losers, should reflect specific aspects of their fighting behavior and again should not be related to a shift in social status.

Following the rationale presented above, the increase in AVT levels observed in the telencephalon of Winners, Losers and Mirror-fighters when compared to Controls is expected to reflect a common aspect of both interaction types (i.e. real-opponent and mirror fights) that is also shared by both Winners and Losers of the real opponent interaction. Given that Winners and Losers have distinct behavioral experiences in the post-resolution phase of the fights, and given that Mirror-fighters never solve the fight, the common factor coupled with the common AVT response across these three groups must reside in the pre-resolution phase of the fights. This can be the expression and/or perception of display behaviors, which are specific to the pre-resolution phase (Oliveira et al., 2011). Interestingly, an evolutionary conserved social decision-making network that is mostly located in the forebrain has been recently described in vertebrates (O'Connell and Hofmann, 2012, 2011). Most nodes of this network are known to express AVT receptors (Goodson, 2005; O'Connell and Hofmann, 2011), which allow this peptide to regulate social decision-making at multiple target areas in the telencephalon. Thus, increased AVT levels in the telencephalon related to the assessment phase of the interaction may reflect AVT modulation of social decision making in a competitive context. In line with this argument, a comparative study in butterfly fishes has shown several associations between the density of AVT-ir varicosities in different nuclei of the telencephalon and types of social behavior (Dewan et al., 2011). In particular, the density of varicosities in the ventral nucleus of the ventral telencephalic area (Vv), a putative fish homolog of the lateral septum, has been identified as the best discriminator between aggressive and non-aggressive species (Dewan et al., 2011). Interestingly, the role of the lateral septum in aggression has also been noted in birds, in which intraseptal administration of AVT increases aggression in non-territorial species, and decreases it in territorial species (Goodson, 1998). By analogy, given that zebrafish

is a shoaling species, one could expect that higher brain AVT levels also are associated with increased aggressive response. However, in this study we found no evidence for a positive association between aggressiveness and AVT levels in the telencephalon (which includes the Vv). In support of this result, pharmacological administration of AVT to dominant zebrafish males inhibits aggressive behavior (Filby et al., 2010). However, one should keep in mind that here only major brain areas were analyzed and thus we cannot discriminate the contribution of each specific brain nuclei to the total AVT measured in the telencephalon.

Following the same rationale the increase of AVT in the diencephalon shared by Winners and Losers, but not observed in Mirror-fighters is expected to reflect a component of the agonistic interaction that is missing in a mirror elicited fight. In this case, both phenotypes share the experience of the post-resolution phase of the fight, characterized by extended chases and attacks directed by the Winner towards the Loser. Overt-aggression is known to be more energy consuming than displaying (Castro et al., 2006; Ros et al., 2006), and therefore the post-resolution phase can arguably be considered more stressful that the assessment phase. If this is the case, the observed increase in AVT may be related to social stress experienced by both social phenotypes at the post-resolution stage of the fight. This interpretation is supported by data from another study where both Winners and Losers, but not Mirror-fighters have elevated cortisol levels (Teles et al., 2016), and by the fact that AVT in the POA plays a role in stress regulation (Gilchriest et al., 2001; Olivereau and Olivereau, 1990). Alternatively, the shared increase in AVT levels within the diencephalon between Winners and Losers may have different origins. In fish, two AVT-producing cell populations have been described in the diencephalon: the parvocellular nuclei, that appears to be more involved in the stress response, namely in the regulation of cortisol release by the action of AVT on the hypothalamic-pituitary-interrenal axis (Gilchriest et al., 2001; Olivereau and Olivereau, 1990), and the magnocellular/gigantocellular cluster, which likely regulates aggression (Dewan et al., 2008; Greenwood et al., 2008; Larson et al., 2006b). Thus, in Winners and Losers, the different neuronal populations may be activated in the post-resolution phase: in Winners, there are the magnocellular population related to the expression of overt-aggression; and in Losers the parvocellular population engaged in the stress response (Larson et al., 2006b). Finally, in this respect it is also worth mentioning that the results presented here for peptide levels contrast with previously reported results for the gene expression, where dominant males had higher expression of the *avt* gene in the hypothalamus than subordinates (Filby et al., 2010). However, changed gene expression that reflects changed activity of peptide-producing machinery it is not a good measure of the presence of mature peptide active at the site of action. As mentioned above, only the nonapeptides that emerge from the complex prepro-peptides in process of maturation when they are carried all along the axon to the target areas, are biologically active at the target site. Moreover, the different time frames of aggression used in the two studies may explain these divergences, since in the present study a short-term (30 min) interaction was used, whereas the gene expression profiles were performed on social stable hierarchies after 24 h of social interaction.

The pattern of brain nonapeptides levels observed exclusively in Losers, i.e. higher AVT in the optic tectum and brain stem, higher IT in the diencephalon and lower IT in the cerebellum can be interpreted as being associated with the loss of social status experienced by these animals. In teleost fish, the optic tectum and rhombencephalon receives AVT fibers (Saito et al., 2004), and both areas have been implicated in the regulation of visual and motor responses to sensory stimulation (Iwasaki et al., 2013), as well as in escape behaviors (Herrero et al., 1998). Thus enhancement of AVT signaling in these areas in Losers may reflect sensory-motor integration related to defensive behaviors exhibited exclusively by Losers. The high IT levels found in Losers'

diencephalon might be associated with a downregulation of aggressive behavior. This process has already been described in Syrian hamsters (*Mesocricetus auratus*), in which OT administration to the POA and the anterior hypothalamus decreases aggression, whereas administration of OT receptor antagonist facilitates it (Harmon et al., 2002).

Finally, the lower IT levels in the olfactory bulbs exclusively observed in Winners, can also be related to status-acquisition. In fish, IT fibers reach the granular layer of the olfactory bulbs (Saito et al., 2004) and olfaction plays a major role in intra-specific communication (Barata et al., 2007; Simões et al., 2015) and social recognition (Gerlach et al., 2008). Thus the lower levels of IT in the olfactory bulbs of Winners may reflect the social regulation of olfactory memory formation.

In summary, this study showed different patterns of nonapeptides across different brain areas in zebrafish that experienced different social interactions (i.e. Winners, Losers and Mirror-fighters). These results strongly suggest the occurrence of differential social modulation of AVT and IT across different brain regions putatively involved in social behavior, hence suggesting a role for these nonapeptides in the regulation of social behavior in a context dependent manner.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.yhbeh.2016.05.020.

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