Involvement of neuropeptidergic neurons in the establishment of dominance in a teleost model of non-breeding aggression: neuropeptide-specific and status-dependent actions

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Article

Keywords: Dominance, AVT, IT, electric fish

Posted Date: January 4th, 2023

DOI: https://doi.org/10.21203/rs.3.rs-2419476/v1

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Abstract

The establishment of the dominant-subordinate status implies a clear behavioral asymmetry between contenders that arises immediately after the resolution of the agonistic encounter and persists during the maintenance of stable dominance hierarchies. Changes in the activity of the brain social behavior network (SBN) are postulated to be responsible for the establishment and maintenance of the dominant-subordinate status. The hypothalamic nonapeptides of the vasopressin/vasotocin (AVP/AVT) and oxytocin/mesotocin/isotocin (OXT/MST/IT) family are known to modulate the activity of the SBN in a context-dependent manner across vertebrates, including status-dependent modulations. We searched for status-dependent asymmetries in AVT/IT cell number and activation immediately after the establishment of dominance in the weakly electric fish, Gymnnotus omarorum, which displays the best understood example of non-breeding territorial aggression among teleosts. We used immunolabeling (FOS, AVT, and IT) of preoptic area (POA) neurons after careful designed intermale dyadic agonistic encounters. We show for the first time in teleosts, that AVT, but not IT, is involved in the establishment of the dominant-subordinate status. We also found status-dependent POA subregion-specific changes of AVT cell number and activation that confirm the involvement of AVT in the establishment of dominance likely achieved by the release of AVT from dominants’ AVT neurons.

Introduction

Social dominance emerges as a consequence of agonistic behavior, (i.e., any combative behavior involving struggle among individuals of the same species over limited resources)\(^1\). The establishment of the dominant-subordinate status implies the recognition of the fighting ability and motivation among opponents and usually requires aggression\(^2,3\). Once dominance is established, a stable hierarchy can suppress further aggression and unwanted fights among group members\(^4\). As a result, a clear status-dependent asymmetry in the behavior of contenders is observed immediately after the resolution of the agonistic contest, which can also consolidate in enduring social hierarchies. These behavioral asymmetries between dominants and subordinates are commanded by distinctive neuroendocrine mechanisms occurring in the highly conserved vertebrate social behavior network (SBN)\(^5-8\). Multiple neuromodulators, acting both via fast wired circuits and slow diffusive ways, shape the spatio-temporal pattern of activity of the SBN, thus providing the emergence of status-dependent behaviors and the maintenance of stable hierarchies\(^5,7,9\).

A widely used strategy for studying the short-term activation of the SBN is to identify the expression of early genes among its nodes using strictly controlled social behavioral experiments\(^10-15\). As a general rule, the expression of early genes in the SBN is higher in animals in social interaction with respect to isolated animals\(^16-18\). In particular, it has been found that the expression of early genes in the SBN is also higher in dominants than in non-interacting animals\(^16,18,19\) and can show status-dependent spatial patterns across the SBN\(^16\). Yet, early genes expression, as nonspecific markers of activity, may also be
enhanced in both dominants and subordinates and show no difference between them immediately after an agonistic encounter.

Hypothalamic nonapeptides of the vasoppressin-vasotocin (AVP/AVT) and oxytocin-isotocin (OXT/IT) family are well known to modulate the activity of the SBN in a context-dependent manner and are therefore involved in social status-dependent behaviors across vertebrates\(^{20-23}\). Within the enormous diversity of the neuropeptidergic control of the dominant-subordinate status, there are at least three general aspects that account for its complexity. First, AVP/AVT and the OXT/IT systems usually have complementary/antagonic roles. AVP/AVT are generally related to aggression and dominance\(^{24-27}\), while OXT/IT are associated to submissive behaviors and to induce a decrease in aggression\(^{28-30}\). Second, the same neuropeptide systems have distinctive actions depending on social status. For example, a differential pattern of activation of AVP/AVT neurons between dominants and subordinates has been reported in different vertebrates\(^{31-33}\), and the same pharmacological manipulations of the AVP/AVT system induce different actions in dominants and subordinates\(^{24,34-36}\). Third, while these pharmacological actions and status-dependent neuronal activation patterns occur during the emergence of dominance, long-term status-dependent changes are frequently observed in AVP/AVT cellular traits\(^{37,38}\) and less often in OXT/IT ones\(^{39}\).

In teleost fish, the localization of AVT and IT somata and fibers follow a general common pattern with two main cell groups (magnocellular and parvocellular) located in the preoptic area (POA), which are homologous to the amniote supraoptic and paraventricular nuclei of the hypothalamus\(^{40-43}\). Several teleost model systems (e.g., *Astatotilapia burtoni*, *Danio rerio*) and approaches (e.g., immunohistochemistry, *in situ* hybridization) have been used to identify neuropeptidergic cellular correlates of the dominant-subordinate status\(^{38,39,41,44,45,46}\). However, most previous studies have only focused a) on the status-dependent asymmetries in the number and size of AVTergic neurons\(^{38,39,41,44}\) but not of IT neurons; and b) on the cellular traits of long-term stable social hierarchies\(^{38,39,41,44}\) but not of the emergence of the dominant-subordinate status.

*Gymnotus omarorum* (Gymnotiformes, Gymnotidae\(^{47}\)), is a neotropical weakly electric fish that displays a clear-cut example of pure territorial aggression during the non-breeding season\(^{36,48-52}\). While dominants are highly aggressive even after the conflict is solved, subordinates signal submission in a precise sequence of locomotor and electric traits\(^{36,48,50}\). The presence and neuroanatomical distribution of AVT and IT neurons have been previously described in this species as well as their wide projections to brain areas including those involved in the control of aggression and electromotor behaviors\(^{42,43}\). The administration of IT does not modify either locomotor or electromotor activity in isolated individuals\(^{43}\), while the effect of IT on the agonistic encounter of *G. omarorum* has not been previously evaluated. In contrast, the behavioral asymmetry between dominants and subordinates in *G. omarorum* is somehow paralleled by status-dependent AVT modulations\(^{36}\). While dominants’ aggression requires an endogenous liberation of AVT, the administration of AVT to subordinates induce them to increase their electric
signaling of submission\textsuperscript{36}. Although these status-dependent actions of AVT suggest distinctive activation patterns of the AVT system between dominants and subordinates during the emergence of dominance, no short-term plastic changes of neuropeptidergic neurons have been evaluated in the agonistic behavior of \textit{G. omarorum} so far.

An elegant way of evaluating the involvement of neuropeptidergic neurons during the establishment of the dominant-subordinate status is to search for short-term plastic changes of AVP/AVT and OXT/IT cells associated to the expression of early genes. This immunohistochemical approach has been carried out in several amniote species\textsuperscript{24,31−33}, but not in teleosts. In this study, we took advantage of the non-breeding aggressive behavior of \textit{Gymnotus omarorum} to focus on the cellular changes of POA neuropeptidergic neurons of both dominants and subordinates during the agonistic encounter. To do this, we conducted careful behavioral experiments including two conditions of social interaction (fighting and non-fighting male dyads) that implied a similar global activation of the POA measured by the expression of the early gene fos. In these similarly socially activated brains, we were thus able to search for short-term plastic status-dependent changes of AVT + and IT + cells by immunohistochemistry.

\section*{Results}

\subsection*{Status-dependent asymmetry in behavior}

In this study, we conducted non-breeding male-male dyadic agonistic encounters in the weakly electric fish, \textit{Gymnotus omarorum}\textsuperscript{47}, that displays the best understood example of gonadal independent territorial aggression among teleosts\textsuperscript{48−52,53}. Male-male dyads with body size asymmetry of 5–20\% were assigned into two experimental groups: the non-fighting group (n = 6), in which each fish was placed in a separate compartment for all the recording time allowing them to interact electrically but not physically; and the fighting group (n = 10) in which fish were initially placed in separate compartments before the gate was lifted allowing them to physically interact (Fig. 1a, Movie S1, see Methods). After lifting the gate, all fighting dyads displayed a robust agonistic behavior as previously described elsewhere\textsuperscript{48,51} in which a clear dominant-subordinate status was achieved in less than 25 min with the expected contest outcome: the larger fish always won the fight (Fig. 1a).

The agonistic encounter followed the phases of evaluation, conflict and post-resolution as previously described (Fig. 1b; Batista et al 2012). The asymmetry in the behavior of dominant and subordinate \textit{G. omarorum} is outstanding immediately after the conflict is solved (post-resolution, Fig. 1b; Movie S2). Although there is no significant difference between contenders in the time they are in motion (Fig. 1c, \(p = 0.08\)), there is a clear asymmetry in the type of behaviors they display (Fig. 1d (\(p < 0.001\)). Dominants are highly aggressive and persist attacking, while subordinates retreat and signal submission in a precise sequence of electric traits, which is in line with previous reports\textsuperscript{36,48,50}.

Non-status dependent difference was found in POA activation (FOS+).
The organization of the POA in nucleus preopticus ventricularis anterior (PPa) and posterior (PPp), as was previously described\(^{42,43}\), is shown in Fig. 2. The presence of FOS labeling was studied in POA transversal sections (Fig. 2) in fighting and non-fighting animals by immunohistochemistry. FOS labeling was widespread and uniformly distributed from the nearness of the ventricle to hypothalamic and telencephalic areas. In sections at the level of the PPa (Fig. 2a-b) and PPp (Fig. 2c-d), no differences in FOS labeling amount were evident. The mean number of FOS + cells was also not different in PPa or in PPp sections of the POA during social interaction between fighting and non-fighting males (Fig. 2e).

Potential and fighting subordinates did not show significant differences in the mean number of FOS + cells per area in PPa or PPp sections of the POA (Fig. 2e, \(p > 0.5\)). Potential and fighting dominants did not exhibit significant differences in the number of FOS + cells in PPa or PPp sections of the POA (Fig. 2e, \(p > 0.3\)). We found no correlation between the mean number of FOS + immunolabeled cells and the percentage of time in which fighting dominant and fighting subordinate isolated males display locomotor activity (Fig. 2f, \(p = 0.28\)).

**Status-dependent changes in AVT+ cells but not in IT+ cells**

We searched for short-term immunohistochemical changes in AVT + and IT + cells during social interaction by comparing dominants and subordinates of the fighting group with potential dominants and subordinates of the non-fighting group (Fig. 1a, see Methods). The immunolabeling for AVT and IT was compared by separate in PPa and PPp sections of the POA in non-fighting and fighting dominants and subordinates (Fig. 3, see Methods). The general distribution of AVT + and IT + cells in PPa and PPp sections of the POA resembled the pattern previously described for this species\(^{42,43}\) and was similar across experimental groups as shown in examples of non-fighting and fighting dominants and subordinates (Fig. 3b-j). Quantification of the number of immunoreactive cells showed no difference in the number of IT + cells between fighting and non-fighting males, or between fighting and non-fighting subordinates and dominants in PPa or PPp sections of the POA (Fig. 3k, all \(p > 0.35\)). In contrast, a clear status-dependent pattern as well as differences between fighting and potential dominants emerged for AVT + cells (Fig. 3l). While potential and fighting subordinates did not show significant differences in the number of AVT + cells (all \(p > 0.6\)), fighting dominants showed fewer AVT + cells than potential dominants in PPp sections of the POA (as illustrated by the selected slices shown in Fig. 3i-j, \(p < 0.001\)).

Figure 4 (a-d) shows the double immunolabeling for AVT and FOS in a PPa section of the POA (Fig. 4a-d) of a fighting dominant male. The nuclei were stained with DAPI (Fig. 4a), which allows the clear identification of double immunostained cells (Fig. 4d, inset). While FOS labeling showed its characteristic nuclear signature, (Fig. 4b), AVT labeling was present in somata and cell fibers (Fig. 4c), resulting in a distinctive double labeling for FOS and AVT (Fig. 4d).

Potential and fighting subordinates did not show significant differences in the activation of AVT + cells (proportion of AVT/FOS + cells) in PPa or PPp sections of the POA (Fig. 4e, all \(p > 0.3\)). However, fighting dominants showed a significantly lower activation of AVT + cells in PPa sections of the POA than potential dominants (Fig. 4e, \(p = 0.004\)), while no significant differences were found in PPp sections (\(p =\)
There was no correlation between the mean number of double immunolabeled AVT+/FOS+ cells and the percentage of time in which fighting dominants and subordinates males displayed locomotor activity (Fig. 4f, p = 0.09).

**Discussion**

Previous studies across vertebrates have explored the role of nonapeptides in dominance in breeding males competing for mates and reproductive resources, hampering the distinction of the mechanisms strictly responsible for dominance. To avoid this bias, this study takes advantage of the well-documented non-breeding territorial aggression of *Gymnotus omarorum* to delve into previously unexplored cellular mechanisms. Combining double immunolabeling of POA neurons with careful designed behavioral experiments, we show for the first time in teleosts, that AVT, but not IT, is involved in the establishment of the dominant-subordinate status in *G. omarorum* and that dominants, but not subordinates, release AVT during the contest. Unlike previous findings showing long-term status-dependent asymmetries in AVT/IT cells traits in teleosts, we searched for status-dependent asymmetries in nonapeptidergic cells immediately after the establishment of dominance.

In this study, we used the immunolabelling of the early gene FOS as a non-specific marker of the activation of the SBN node POA in dominants and subordinates of fighting and non-fighting dyads of *G. omarorum* (Fig. 2). Previous reports extensively documented that social stimuli cause the activation of SBN nodes and therefore an increase in the expression of early genes. In particular, the expression of early genes in fighting animals is higher with respect to control ones but there are no reported differences between dominants and subordinates, probably because both contenders have their SBN similarly activated during the contest. Our experimental design assumed, and our results confirmed, that the social interaction between fighting and non-fighting dyads implied a similar expression of the early gene fos (Fig. 2). We also excluded the possibility that FOS expression was related to fish movement (Fig. 2). It is also interesting to note that the number of FOS+ cells is not significantly different between the PPa and PPp despite the PPa has more nonapeptidergic neurons than the PPp (Fig. 3). Therefore, the symmetric activation of the POA between dominants and subordinates of both fighting and non-fighting dyads can be considered the best scenario to evaluate distinctive nonapeptidergic mechanisms involved in the emergence of the dominant-subordinate status. Indeed, we consider the even FOS expression among status and experimental groups as the preliminary requirement to validate the interpretation of the observed status-dependent differential profile of AVT+ neurons.

The combination of immunodetection of AVT/IT and early gene expression during the establishment of the dominant-subordinate status (minutes-hours after, short-term) has been carried out in several amniote species but not in teleosts and has focused on AVP/AVT neurons but not on OXT/IT neurons. It is thus remarkable that we were able to test both nonapeptidergic cells in the same experiment and to find a very clear differential effect of AVT and IT in the establishment of the dominant-subordinate status of *G. omarorum*. While no IT cell changes were observed neither between dominants and subordinates, nor...
between fighting and non-fighting dyads, AVT cellular traits were status-dependent and different between fighting and non-fighting dyads (Fig. 3). This cellular plasticity of AVT neurons is in line with previous reports of the role of AVP/AVT as modulator of aggression in mammals\textsuperscript{27}, birds\textsuperscript{57}, amphibia\textsuperscript{25}; teleosts\textsuperscript{23,26,38,58}, and in the study species \textit{G. omarorum}\textsuperscript{36}. On the other hand, we found no IT cellular correlates of the agonistic behavior in \textit{G. omarorum} although it is recognized that OXT/IT has clear anti-aggressive effects in species of several classes of vertebrates\textsuperscript{59–62}.

The main results of this study show a smaller number of AVT + cells and less percentage of activated AVT + cells in fighting than in non-fighting dominants (Figs. 3; 4). Although it is not possible to find a general common pattern, it is interesting to note that similar approaches in non-related species have also shown short-term negative correlations of AVP activation with dominance and levels of aggression\textsuperscript{11,31}. In teleosts, in turn, nonapeptidergic cellular traits have been only explored in models of long-term stable hierarchies. These studies have shown phenotype-dependent asymmetries in the number\textsuperscript{38} and size of AVTergic neurons\textsuperscript{39,41} and in the amount of AVT measured by immunohistochemistry\textsuperscript{41} or by \textit{in situ} hybridization\textsuperscript{44}. On the other hand, only one previous study in teleosts has reported a greater number of IT neurons in dominants with respect to subordinates\textsuperscript{39}.

In line with previous evidence in \textit{G. omarorum} showing status-dependent effects of AVT administration\textsuperscript{36}, we found that both AVT cell number and activation change in dominants but not in subordinates. Fighting dominants showed fewer AVT + cells and a lower percentage of activated AVT + cells than non-fighting dominants (Figs. 3; 4). Both results support the interpretation that AVT is less detectable in the POA of actual dominants and allow us to hypothesize that AVT is released from the POA of dominants during the establishment of the dominant-subordinate status. Several arguments support this prediction: a) the number of AVT + neurons is expectable for a non-breeding adult of this species\textsuperscript{43}; b) the possibility that differences in cell traits are due to random variations between individuals is ruled out by the use of linear mixed models with random intercepts for each animal\textsuperscript{64}; c) given the time course of the experiment (2 h) it is not likely that the decrease in the number of AVT + cells observed in dominants is due to the destruction of neurons\textsuperscript{65}; d) instead, as the immunolabelling for AVT is designed to detect this nonapeptide inside the cell, its release will ultimately result in the disappearance of the AVT staining and therefore in less AVT + cells; and e) an endogenous release of AVT related to dominants’ aggression levels has been previously shown by pharmacological experiments in this species\textsuperscript{42}. Short-term cellular changes in the activation of AVP-AVT neurons associated to agonistic behavior have been previously reported in several species\textsuperscript{11,31–33} with no clear general pattern of action, which is not surprising given the well-known context-dependency and species-specific actions of hypothalamic nonapeptides\textsuperscript{66}. However, no previous reports showed short-term changes in the number of AVT neurons associated to the establishment of dominance, which we understand is a remarkable finding of this study given its clear functional meaning.

We found differences in the number and activation of AVT POA cells occurring in different regions of the POA. In fighting dominants, changes in AVT + cell number were only observed in the PPp POA (Fig. 3)
while changes in the activation of AVT + cells were only observed in the PPa POA (Fig. 4). In a conserved profile across teleosts, these regions of the POA contain different nonapeptidergic cell types; namely parvocells and magnocells are more abundant in the rostral PPa POA, while gigantocells only occur in the caudal PPp POA [41–43]. If the decrease in the number of AVT + cells is a clear indication of AVT release and considering that it is only evident in the PPp POA, we can speculate that our results suggest that AVT gigantocells are probably involved in dominants’ AVT release. This is in line with the functional regionalization of the POA in teleosts proposed by Greenwood [44], by which its caudal portion was found to be associated with dominance and aggressive behaviors while its rostral portion was found to be related to submissive displays. Interestingly, larger or more numerous giganto-AVT cells are also often observed in dominants after long-term stable hierarchies in several teleost species [44,38,67–69]. On the other hand, only one previous study to our knowledge found a similar decrease in the activation of AVT cells in the mammalian PVN, homologous to the PPa POA [31].

This is the first study to identify short-term nonapeptidergic cellular correlates of the establishment of the dominant-subordinate status in teleosts. Immunolabelling of FOS expression (as indicator of activation) and of AVT and IT (to identify the type of nonapeptide neuron) following a set of agonistic encounters allowed us to show that: a) AVT + cells but not IT + cells are associated to the dominant-subordinate status; b) dominants but not subordinates display changes in the number and activation of AVT + cells that suggest the release of AVT by dominants during the agonistic contest; c) the most clear evidence of AVT release occurs only in the PPp POA where AVT gigantocells occur. Finally, we found short-term changes in AVT cell number associated to the establishment of dominance for the first time in vertebrates.

**Methods**

**Fish maintenance**

In this study we used 32 wild-caught non-breeding adult males of Gymnotus omarorum [47] (body-length 15 to 31 cm; body-weight 9.2 to 72 g). Sex in G. omarorum is not externally apparent and was determined before the perfusion by gonadal inspection as described elsewhere [49]. In all cases, the gonadal inspection was performed between 30 to 50 days before behavioral experiments. All males were non-sexually mature with gonadosomatic index always below 1% as expected for non-breeding teleost fish (0.07 to 0.5% for G. omarorum). All animal procedures were reviewed and approved by institutional and national guidelines and regulations for animal welfare (Comisión Honoraria de Experimentación Animal, Universidad de la República, Protocol Number 008/002), and all experiments were performed in compliance with relevant guidelines and recommendations. All animal studies reported also followed the recommendations in the ARRIVE guidelines. To achieve reliable and repeatable behaviors, our collection, transportation, housing, and recording conditions were adjusted to minimize stress.
Fish were collected during the non-breeding season (May–July) in a freshwater lagoon in Laguna del Sauce (34°51’S, 55°07’W, Department of Maldonado, Uruguay) using a “fish detector”, an electronic audio amplifier connected to a pair of electrodes, as described elsewhere. Fish were housed in individual compartments within 500-l outdoor tanks for 10 days before the behavioral experiments. Water temperature in the tanks ranged from 8 to 16°C; light-dark cycle was around 10:14; and water conductivity was maintained below 150 µS/cm, resembling the characteristics of the non-breeding natural habitat. Aquatic plants (Eichhornia crassipes, Pistia stratiotes) provided shelter for the fish. Fish were fed Tubifex tubifex.

**Recordings**

Fish were placed in an experimental setup that allowed simultaneous video and electric recordings as described elsewhere. The experimental tanks, four 50-l glass aquaria (55 × 40 × 25 cm) were fitted with two pairs of orthogonal electrodes attached to each tank wall. The day–night cycle and the physicochemical parameters (water temperature, conductivity, and pH) of indoor tanks matched those of the outdoor housing tanks. All the experiments were performed in total darkness illuminated by an array of infrared LEDs (L-53F3BT, Fablet & Bertoni Electronics) located above the tank. An infrared-sensitive video camera (SONY CCD-Iris and RoHS CCD Digital Video Camera) was focused on the bottom of the tank. Electric signals of freely moving fish were detected by two pairs of fixed electrodes, connected to two high-input impedance amplifiers (FLA-01, Cygnus Technologies Inc.). Images and electric signals were captured by a video card (Pinnacle Systems, PCTV HD pro stick) and stored in the computer for further analysis. The fish remained in the recording tank at constant temperature (10–16°C) for 4–5 h before the experiments in separate compartments in which each contender could perceive a distorted and low-amplitude signal from the other fish.

**Behavioral experiments**

We analyzed the agonistic behavior of *G. omarorum* in nocturnal dyadic male-male encounters. Animals were randomly assigned to a dyad in which body weight difference ranged from 5 to 20% to predict the contest outcome: the dominant male (larger one) and the subordinate (smaller one).

During agonistic encounters, we used a gate protocol in which territory is the only resource that individuals fight for, providing symmetric resources and resource values for both contestants: equally-sized plain territory, same residence time, and the same previous experience.

**Behavioral paradigm**

Male-male dyads were assigned to two experimental groups, non-fighting group (n = 6) and fighting group (n = 10) in which the difference is the agonistic encounter experience (Fig. 1A). To minimize stress in animals, before behavioral test, males of each dyad were individually housed in tanks inside the experimental station for at least six hours. One hour before the agonistic encounter, each male of the dyad was housed in a compartment of the same tank divided by a removable plexiglass gate. This gate allowed the perception and electric interaction of fish but avoided physical interaction (Fig. 1a).
In the fighting group, 15 min after the lights were turned off the gate was lifted, and if the conflict was solved less than 25 min after each male (dominant = larger one; and subordinate = smaller one) was individually housed in a tank (Fig. 1a). The resolution of the conflict was determined when the subordinate retreated three times without attacking back (Fig. 1a). Two hours after the gate was lifted both males were simultaneously anesthetized, intracardiacally perfused, and brains were dissected for immunohistochemistry (Fig. 1a). In the non-fighting group, the protocol was similar, but the gate was not lifted (Fig. 1a).

**Behavioral Data Processing**

In all experiments we measured the time of movement of animals (measured in percentage), number of attacks and post-resolution number of retreats in subordinates.

**Immunohistochemistry**

Fish were anesthetized by immersion in 0.05% 2-phenoxy-ethanol (Sigma, P-1126) and then perfused with saline followed by 4% paraformaldehyde in phosphate buffered saline (PBS, 25–35 ml; pH = 7.4). Coded brains were processed by an observer blinded to treatment. Brains were dissected, post-fixed overnight in the same fixative at 4°C, rinsed in 0.1M PBS and cryoprotected in 30% sucrose 24 hours at 4°C before embedding in Cryomatrix and storage at −80°C for no more of 4 weeks. Brains were then sectioned on a cryostat at 50 µm and transverse sections were processed for double immunolabeling for FOS and AVT as was previously described. Free floating sections were rinsed in PBS, and non-specific binding sites were blocked with normal 10% donkey serum ((DS) + 0.3% Triton in PBS 0.1M; PH 7.4) for 1 h. Sections were incubated for 36 h in primary antibodies (anti-FOS, 1:500, goat, polyclonal, donated by Dr James Goodson, Indiana University, United States; anti-OXT 0.5:500 Millipore mouse, monoclonal, MAB5296, Millipore and anti-AVP, 2:500, rabbit, polyclonal, Immunostar, #20069), dissolved in 0.1M PBS + 0.3% Triton X-100 + 5% DS with Sodium Azide 0.01%). Sections were rinsed (3×10 min in PBS) and incubated for 2 h at room temperature with secondary antibodies (Alexa Fluor 594 (red) donkey anti-goat IgG (H + L), 1:200, Invitrogen, Cat#A11058; Alexa Fluor 488 (green) donkey anti-mouse IgG (H + L),1:200, Invitrogen, Cat#A21202, and Alexa Fluor 680 (false color magenta) donkey anti-rabbit IgG (H + L), 1:200, Invitrogen, Cat#A21109) dissolved in 0.1M PBS + 0.3% Triton X-100 + 5% DS with Sodium Azide 0.01%). All sections were then rinsed (3×10 min in PBS), mounted with a anti fade nuclear staining. The POA cytoarchitecture was identified following. Double immunolabeling for FOS and AVT was performed and quantified in PPa and PPp sections of the POA in both experimental groups of animals and an example is showed in PPa section of a dominant male (Fig. 2a). Control sections were incubated with the primary antiserum (anti-AVP) pre-absorbed with an excess of AVT (1 µg/ml; Cat. 66-0-09, American Peptide Company) and no labeling was present (Fig.S1 b). The primary antiserum (anti-AVP) was preincubated 24 hours in 10 µM IT (1:500; Bachem), and the slices showed no difference with plain AVP antibody staining (Fig.S1 c). Control sections were incubated with the primary antiserum (anti-FOS) pre-absorbed with an excess of FOS (1 mg/ml) for 3 h at room temperature and no labeling was observed (Fig.S1 e). The use of OXT antiserum has been
previously validated for *G. omarorum*⁴³. Control experiments omitting the primary and secondary antibody were routinely performed.

The number of FOS+ cells, the proportion of AVT+/FOS+ expression and the percentage of movement of animals were quantified to check that no correlation between those measures was present (Fig. 3e, f). Even more the percentage of movement between experimental groups was not significant different (Fig. 1c).

**Quantification and Statistical Analysis**

Brain sections were viewed with a laser confocal microscopy (Zeiss LSM 880) using the following lasers sequentially: Diode 405 nm, HeNe 594nm and HeNe 633nm. Confocal images were imported into Image J 1.52i⁷⁰ software. The z-stacks were generated and then exported to GIMP 2.8.16 software to do quantification and to Inkscape v0.48 for figure adjustment. Images were adjusted in contrast and brightness. We used standardized methods to quantify FOS+ cells¹¹,⁵⁶ To measure the density of FOS+ cells, the counts of FOS+ nuclei were conducted within standardized polygons (100 µm²) on the digital photomicrographs. Dots were placed over each labeled cell (in a separate layer) and the dots were then counted. The raw cell counts were ultimately converted into the number of FOS+ nuclei per 100µm² of tissue.

We also quantified the total number of AVT+ and IT+ cells per slice following ⁴²,⁵⁶. Only somata with a distinct perimeter and at least one neurite was measured. AVT and IT immunostaining was done on non-adjacent slices that were 50 µm thick, to avoid counting AVT+ or IT+ cells twice. We used monochrome photomicrographs to measure the number of double-labeled cells for FOS and AVT. We measure the percentage of these neurons that are also FOS-labeled following ⁵⁶,⁵⁷.

General and generalized linear mixed models⁶⁴ were used to analyze immunohistochemical quantification data, and were implemented in R. Experimental condition (fighting, non-fighting), status (dominant, subordinate), anatomical descriptors and behavioral predictors were included as fixed effects; individual was included as a random intercept effect. Pairwise comparisons were calculated using the emmeans package. Poisson linear mixed models were used for cell counts (AVT+ or FOS+ cells). Logistic mixed regression models were used for proportions (FOS+/AVT+). In Fig. 1 behavioral displays were analyzed using unpaired t-tests.

**Declarations**

**Acknowledgments**

We thank to Dr James Goodson and Marcy Kingsbury who kindly donated the antibodies used in this research. We also thank to Dr Eva Fischer and Gustavo Somoza for the critical comments on this manuscript. This work was partially supported by PEDECIBA.
Author Contributions

Conceptualization, A.S. and P.P.; Methodology, P.P., A.C., V.F.; A.S.; Investigation, P.P., A.S.; Resources; A.S.; PP Writing – Original Draft, P.P, A.S.; Writing – Review & Editing, all authors; Funding Acquisition, A.S.; P.P; Supervision, A.S.

Additional Information

Competing Interests Statement

All authors declare no competing interests

Data availability statement

The datasets generated for this study are available on request to the corresponding author.

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Figures

a

Fig 1

Status dependent behavioral assimetry in dyadic agonistic encounters in *G. omarorum.*
(a) Behavioral Paradigm: short-term establishment of dominant-subordinate status in non-breeding males of Gymnotus omarorum. Each fish is placed in a separate compartment of the tank. 15 min after the light is turned off, the gate is removed and the agonistic encounter begins in the fighting group. After 25 min each animal is moved to separate tanks. Animals are sacrificed after 120 min. t: time.

(b) Characteristic agonistic posture during conflict phase in a dyad. Image shows an attack from the dominant and a retreat response from the subordinate. Time structure of agonistic encounter in experiment. Agonistic behavior has three different stages: evaluation phase, from time 0 (gate removal) to the occurrence of the first attack; contest phase from the occurrence of the first attack to conflict resolution; and post-resolution. Post-resolution phase starts after conflict resolution and has an arbitrary duration of 95 min.

(c) Percentage of movement in fighting dominants and subordinates in post-resolution phase. No significant difference is observed between fighting dominants versus subordinates (p=0.08).

(d) Locomotor displays in the post resolution phase. Fighting dominants show significant more attacks than subordinates (p<0.001). Fighting subordinates show more retreats than dominants (p<0.001)
Figure 2

Non difference in POA activation (FOS+) in fighting and non-fighting dominants and subordinates.

(a,c) Schematic brain diagram of transversal section of the POA showing the PPa (a) and the PPp (c). Inset in a and (c) are magnified in (b) and (d)

(b, d) Immunolabeling for FOS in PPa (b) and PPp (d) sections of the POA

(e) No significant difference in the number of FOS+ cells in PPa and PPp sections of the POA in fighting nor non-fighting dominants and subordinates (all p>0.3).

(f) No correlation was found between the mean number of FOS+ cells and the percentage of time in which fighting dominants and subordinates displayed locomotor activity (p=0.28).

* Ventricle. DLd, dorsolateral telencephalon, dorsal subdivision; DC, central division of dorsal forebrain; DD, dorsal division of the dorsal forebrain; DL, dorsolateral telencephalon; Vp, ventral telencephalon, posterior subdivision Dr, dorsal; OC, optic chiasm; FB: forebrain bundle; PPa, nucleus preopticus ventricularis anterior; PPp, nucleus preopticus ventricularis posterior.
Figure 3

Status-dependent AVT cellular asymmetry in dyadic agonistic encounters in *G. omarorum*.

Schematic brain diagram of transversal section of the POA in a rostral (a) and caudal (f) section showing AVT and IT+ cells distribution. Diagram shows IT-ir cells (blank dots) and AVT-ir cells (black dots).

Distribution of IT-ir cells in a PPa section of potential dominant (b) and fighting dominant (c). Distribution of IT-ir cells in a PPp section of potential dominant (g) and fighting dominant (h). Distribution of AVT-ir cells in a PPa section of potential dominant (d) and fighting dominant (e). Distribution of AVT-ir cells in a PPp section of potential dominant (i) and fighting dominant (j). Scalebars: A, F: 500 µm; B-E; G-J: 50 µm * ventricle

(k) Number of IT+ cells per slice in the POA. PPa showed higher number of IT+ cells than PPp sections (p<0.001). No significant differences between potential and fighting dominants were found (all p>0.35).
(l) Number of AVT+ cells per slice in the POA. Fighting dominants show fewer AVT+ cells than potential dominants in PPp sections (p<0.001). n = 10 per fighting group; n=6 per potential group.

Figure 4

Status-dependent changes are present in activated FOS+/AVT+ cells

(a, d) Double immunolabeling for FOS and AVT in the POA during establishment of dominance in G. omarorum. Inset in second panel: FOS+ nuclei. Upper inset in fourth panel: double immunolabeled cell for AVT and FOS. Lower inset in fourth panel: cell immunolabeled for AVT. Asterisk indicates ventricle. Scalebar 50 µm; insets: 10 µm.

(e) Percentage of double immunolabeled FOS+/AVT+ cells of the POA. Fighting dominants show less FOS+/AVT+ cells than potential dominants in PPa sections (p=0.004).

(f) No correlation was found between the proportion of FOS+/AVT+cells and the percentage of time in which fighting dominants and subordinates displayed locomotor activity (p=0.09).

Supplementary Files

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