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SYMPOSIUM

Neuromolecular Regulation of Aggression Differs by Social Role during Joint Territory Defense

Chelsea A. Weitekamp,* Jessica Nguyen† and Hans A. Hofmann[1,*,†,‡]

*Department of Integrative Biology, University of Texas at Austin, Austin, TX 78705, USA; †Institute for Cell and Molecular Biology, University of Texas at Austin, 2415 Speedway, Austin, TX 78712, USA; ‡Institute for Neuroscience, University of Texas at Austin, 2415 Speedway, Austin, TX 78712, USA

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[1]E-mail: hans@utexas.edu

Synopsis In response to a territory intrusion, neighboring males of the African cichlid fish *Astatotilapia burtoni* engage in aggressive joint territory defense in a manner that depends on their social role. Here, we examine the possible function of several neuroendocrine and neuromodulator pathways previously implicated in the regulation of complex social behavior. We find that the neuromolecular regulation of aggression during joint territory defense is very much dependent on an individual's role in this context. In neighbors but not in residents, aggression is correlated to gene expression in the medial part of the dorsal telencephalon (area Dm), the putative homolog to the mammalian basolateral amygdala. This correlation is strikingly high for expression of the serotonin receptor 5-HT_{2c}, suggesting the serotonin system is important in regulating context-dependent behavior. Furthermore, by examining candidate gene expression co-variance patterns in area Dm and in the lateral part of the dorsal telencephalon (area Dl), the putative homolog to the mammalian hippocampus, we identify two main patterns: gene expression is co-regulated within, but not across, brain regions, and co-regulation is synergistic rather than antagonistic. Our results highlight the critical effect of social context on both behavior and its neuromolecular basis.

Introduction

In many species, particularly those with lek-mating systems, males maintain display territories in close proximity to other males (Bradbury 1981). Neighboring males often have established relationships, whereby they exhibit reduced aggression to one another, referred to as the "Dear Enemy" effect (Fischer 1954). If an intruder tries to usurp a familiar male's neighboring territory, it can be beneficial to cooperate in defense depending on the level of relative threat posed by the intruder. Under certain conditions, the cost of renegotiating territory boundaries with a new neighbor outweighs the temporary cost of engaging in joint defense (Getty 1987; Detto, Jennions and Backwell 2010). Cooperative defense requires that a territory holder is capable of assessing when an intruder is threatening his own territory versus the territory of his neighbor. This cognitive inference is likely adaptive, as it would be costly to respond to every nearby novel male as an intruder in one's own territory. Studying males of the African cichlid fish, *Astatotilapia burtoni*, we previously found that a territory intrusion elicits different behavioral responses from the resident and from his neighbor in a manner that depends on the size relationships between males (Weitekamp and Hofmann 2017). Importantly, we showed that neural activity patterns depend on an individual's role during joint territory defense, and provided evidence that the dopaminergic system regulates cooperative behavior. However, the possible role of other gene pathways in joint territory defense remains unknown. Here, we investigate the activity of several neuromodulatory and neuroendocrine pathways within two brain regions that are likely critical for context-dependent decision-making during joint territory defense.

Social behavior is, in part, controlled by the Social Decision-Making Network (SDMN), a system of interconnected fore- and midbrain regions that are highly conserved across species (O'Connell and Hofmann 2011, 2012a). Previous studies in *A. burtoni* have implicated two SDMN nodes, the lateral part of the dorsal telencephalon (Dl) and the medial part of the dorsal telencephalon (area Dm)—putative homologs of the mammalian hippocampus and basolateral amygdala (blAMY), respectively (O'Connell and Hofmann 2011), in male social habituation to a territorial neighbor and in cooperative territory defense (Weitekamp and Hofmann 2017; Weitekamp et al. 2017). These regions typically do not control aggressive behavior directly, but rather have a modulatory role in social behavior and mediate behavior based on context and stimulus salience, thus making them important candidates in which to examine the role of context in joint territory defense. The hippocampus is critical for spatial learning and memory, and provides access to stored information about social experiences (Eichenbaum 2000). The blAMY assesses the salience and value of social stimuli (Adolphs 2010). *In vivo* electrophysiology recordings show that neuronal activity in the amygdala responds to social interactions (Katayama et al. 2009). In addition, both regions have been implicated in social recognition (Maaswinkel et al. 1996; Kogan, Frankland and Silva 2000; Ferguson et al. 2001). Furthermore, they are reciprocally connected; optogenetic manipulation demonstrated that amygdala inputs to the hippocampus bi-directionally modulate social behavior (Felix-Ortiz and Tye 2014).

There are a number of important signaling pathways within the amygdala and hippocampus that act to modulate social behavior. We chose to examine expression levels of the serotonin (5-HT) receptor 5-HTR_{2C}, the two canonical dopamine (DA) receptors, D1R and D2R, the sex steroid hormone receptors, androgen receptor α (ARα), and estrogen receptor α (ERα), as well as the isotocin (oxytocin ortholog) receptor ITR2. In addition to these candidate pathways, we also measured expression of two immediate-early genes (IEGs), egr-1 and c-fos. IEGs serve as markers of neural activity, and both IEG mRNA expression and protein levels change rapidly in response to social stimuli (Kovács 2008; Robinson et al. 2008; Taborsky and Oliveira 2012). For example, in male zebra finches, IEG expression is affected by song playback, changes with social context, and affects long-term memory formation (Mello et al. 1995; Vignal et al. 2005). Similarly, in *A. burtoni*, IEG activity in the preoptic area is increased when subordinate males ascend to dominance (Burmeister et al. 2005), and also correlates with aggressive behavior in a context-dependent manner (Weitekamp and Hofmann 2017).

The monoamines 5-HT and DA have wide ranging effects on physiology and behavior across species, particularly in mediating aggressive behavior and encoding stimulus salience. 5-HT neurotransmission typically has an inhibitory effect on aggression and varies with social status (Edwards and Kravitz 1997). 5-HT influences neural activity via a diverse family of 5-HT receptors. Of particular interest, the 5-HT_{2C} receptor is a G-protein coupled receptor involved in aspects of sensory processing, learning and memory, and anxiety (Popova et al. 2010). Interestingly, while most 5-HTR subtypes act to facilitate dopamine release, 5-HTR_{2C} mediates an inhibitory effect of 5-HT on DA release (Alex and Pehek 2007). DA mediates reward and encodes the salience of social stimuli, often via action on D1R and D2R (Berridge 2007; Riters 2012).

Expression levels of ARα, ERα, and ITR2 also appear to encode aspects of the salience of the social environment. For example, in the preoptic area, a neuroendocrine relay center, these genes are upregulated during territory defense in *A. burtoni* when a neighbor male is present compared to control conditions when the neighbor male is absent (Weitekamp, Nguyen and Hofmann, in revision). In the amygdala, ERα and OTR have been implicated in social recognition (Choleris et al. 2003, 2007). In the hippocampus, the role of these genes in the context of social behavior is not well understood (Ophir et al. 2012; Ervin et al. 2015). Finally, circulating hormone levels can bias sensorimotor integration and have been shown to interact with the above-mentioned pathways in mammals (Hull et al. 1999). Specifically, in *A. burtoni*, circulating levels of testosterone (T) and cortisol (Cort) respond to challenge in a context-dependent manner (Weitekamp, Nguyen and Hofmann, in revision; Weitekamp and Hofmann 2017). Importantly, the way in which these candidate gene and hormone pathways co-vary in a region specific manner is unknown, particularly for teleosts.

In the present study, we aimed to gain a more integrative understanding of the mechanisms mediating context-dependent aggression during joint territory defense between *A. burtoni* males. Territorial males of this species show habituation of aggression to familiar neighbors (Weitekamp et al. 2017). They engage in stereotyped border conflict displays over their shared territory boundary line (Fernald and Hirata 1977). Furthermore, a male will aggress an intruder in his neighbor's territory based on the

relative size difference between males (Weitekamp and Hofmann 2017). Males are also acutely aware of the social relationships between other individuals and can infer dominance rank through observation (Grosenick, Clement and Fernald 2007). We staged simulated territorial intrusions to facilitate joint territory defense between two males, the neighbor and the resident, and measured the gene expression response in the putative homologs of blAMY and hippocampus (areas Dm and Dl, respectively). During joint territory defense, we previously found associations with neural activity in the Dm in neighbors and neural activity in the Dl in residents. As such, we predicted that candidate gene expression would correspond to behavior, respectively, in these regions. Our results suggest that social role is highly salient, and further, that aggression is regulated by different neural subsystems between neighbors and residents.

Methods

Astatotilapia burtoni descended from a wild caught stock population were maintained in stable naturalistic communities, as described previously (O'Connell and Hofmann 2012b), until transfer to the experimental paradigm. All work was done in compliance with the Institutional Animal Care and Use Committee at The University of Texas at Austin.

Behavioral paradigm

A total of 15 experimental units were set up, consisting of 2 adjacent 10 gallon tanks separated by an opaque divider. Each tank contained two nonreproductive females and one territorial male, as well as terra cotta flowerpot shards which served as the male bower and refuge for females. Paired males originated from different community tanks. To ensure a nonreproductive state of females, brooding females were taken from community tanks, stripped of their brood, and immediately placed in experimental tanks. Prior to the start of the experiment, the opaque dividers were left in place for 1 week to allow the fish to acclimate. At the start of the experiment, the opaque dividers were removed for 1 h twice daily at 1000 h and 1500 h for 4 days. This protocol is sufficient time for the paired males to become familiar with each other (Weitekamp and Hofmann 2017). Following the 4-day social habituation period, on Day 5 at 1000 h we added a territorial intruder contained within a transparent cylinder to one of the paired tanks, randomly assigned. The male in the tank containing the cylinder thus became the resident male, while the adjacent tank contained the neighbor male (Fig. 1A). The intruder originated from a different community tank from the resident or neighbor so that the males were unfamiliar. To increase variation in behavior, we varied the size differences between all three males. The sizes and size differences between males are given in Supplemental Table 1. The females in the tanks do not interact with the males outside of active avoidance.

We video recorded the behavior of resident and neighbor males for 1 h beginning at 1000 h on Day 5. At the conclusion of the hour, resident and neighbor were netted, weighed, and measured for standard length. Blood was drawn from the dorsal aorta (always within 4 min after capture) using heparinized 26G butterfly infusion sets (Becton Dickson, Mountain View, CA, USA). Plasma was stored at −80 °C for hormone analysis. Males were killed by rapid cervical transection. The brain was removed, embedded in OCT compound (Tissue Tek; Fisher Scientific Co., Pittsburgh, PA, USA), and frozen on dry ice. The process from catching the individuals to embedding their brain was consistently under 5 min. Brains were stored at −80 °C.

Behavior

The behavior of both resident and neighbor was scored using JWatcher. The behaviors quantified included lateral displays to the intruder, forward displays to the intruder, lateral displays to the partner, forward displays to the partner, and chases to females. We scored behavior for 10 min, beginning 20 min after the start of the experiment. This time point captures variation in behavior and has been used in several previous studies of *A. burtoni* male behavior. Chases to females occurred at a low frequency and did not differ between males, thus they were excluded from further analysis. Forward and lateral displays were tightly correlated and thus summed. Therefore, the variables in the final analyses were "Aggressive displays to intruder," "Aggressive displays to partner," "Aggressive displays" (sum of former variables), and "Partner aggressive displays."

RNA extraction and quantitative real-time Polymerase Chain Reaction (RT-PCR)

Brains were sliced on a cryostat in the coronal plane at 300 um. A tissue corer tool (Fine Science Tools, Foster City, CA, USA) with a diameter of 300 um was used to separately isolate Dm-1 and Dlv (Fig. 1B). Two punches per brain region (one from each hemisphere) were taken from a single slice and were pooled in ice-cold homogenization working solution. Given the large size of these regions, the tissue punch was likely specific. RNA was extracted using the Maxwell 16 LEV simplyRNA Tissue Kit (Promega

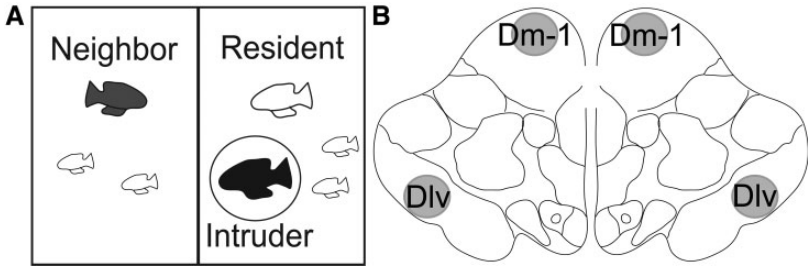

Fig. 1 Experimental design and tissue collection. (**A**) Following 4 days of social habituation between neighbor and resident, a territorial intruder was added to the tank of the resident for 1 h. (**B**) Approximate locations where tissue punches were micro-dissected from pallial areas Dm-1 (subregion 1 of the medial part of the dorsal telencephalon) and Dlv (ventral portion of the lateral part of the dorsal telencephalon), the putative homologs of the mammalian basolateral amygdala and hippocampus, respectively.

Corporation, Madison, WI, USA) following manufacturer instructions, which included DNase treatment. RNA samples were eluted into 40 uL of nuclease-free water. RNA was reverse transcribed to cDNA using the GoScript Reverse Transcription System (Promega Corporation, Madison, WI, USA).

Quantitative RT-PCR was used to measure the mRNA levels of target genes. In both brain regions, Dm-1 and Dlv, we measured expression levels of *d1r, d2r, 5-htr2c, arα, erα, itr, egr-1*, and *c-fos*. Primer sequences are provided in Weitekamp, Nguyen and Hofmann, in revision. For each sample, target gene expression was measured in triplicate in the ViiA^{TM} 7 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) using GoTaq qPCR Master Mix (Promega). Amplification efficiency for each primer pair was determined using standard curves made from serial dilutions of cDNA. To determine relative gene expression of each sample, we used the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001). Values were normalized by the amount of *18s* present in each sample.

Hormone analysis

Free circulating testosterone (T) and cortisol (Cort) were measured using ELISA (Enzo Life Sciences, Farmingdale, NY, USA). For the T assay, cross-reactivity is 100%, 14.6%, 7.2%, 0.72%, and 0.4% for T, 19-hydroxytestosterone, androstendione, dehydroepiandrosterone, and estradiol, respectively, and less than 0.001% for dihydrotestosterone and cortisol. For the Cort assay, cross-reactivity is 100%, 27.7%, 4%, 3.74%, and 0.12% for Cort, corticosterone, progesterone, and T, respectively. Plasma samples were diluted 1:30 and processed as previously described (Kidd et al. 2010). T levels were measured for all males ($n = 15$ residents, $n = 15$ neighbors). Cort levels were measured for a subset of males for which there was sufficient remaining plasma ($n = 9$ neighbors, $n = 8$ residents).

Statistics

All statistical tests were performed using R v. 3.3.1. Data were checked for normality using the Shapiro–Wilk test. To examine differences in gene expression, hormone levels, and behavior between neighbors and residents, we used Welch two-sample *t*-tests. The resulting *P*-values were adjusted using the Benjamini–Hochberg correction. To examine which genes best predict behavior, we used the R package glmulti which ranks models based on Akaike Information Criterion scores, corrected for small sample sizes (AICc) (Calcagno and Mazancourt 2010). We used glmulti for neighbors and residents separately, with behavior as the dependent variable and candidate gene expression levels as the independent variables. To gain a measure of model fit, we then performed linear regression analysis on the best supported model for both neighbor and resident. To examine how hormone levels vary with behavior, we used linear regression analysis. Then, to examine how gene expression, T levels, and aggressive behavior co-vary between neighbors and residents, we created clustered correlation matrices using R package gplots. Cort was excluded from this analysis because of insufficient sample size. We performed hierarchical bootstrap resampling using the package pvclust to gain significance measures for each cluster. Clusters highly supported by the data ($P < 0.05$) are surrounded by bold black squares on the heatmaps and are highlighted by bold lines on the dendrograms.

Results

Behavior and hormones

We found that intruder directed aggression was higher in residents compared to neighbors ($t = -2.68$, $P = 0.014$; Fig. 2A). However, partner directed aggression and total male aggression were not significantly different ($t = 0.84$, $P = 0.41$; $t = -1.63$, $P = 0.116$; Fig. 2B and C. There were no significant

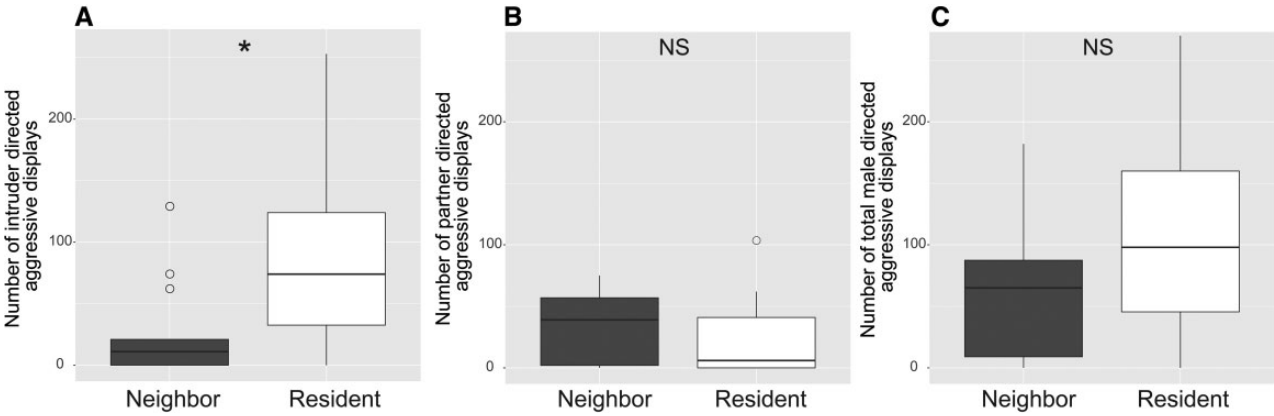

Fig. 2 The number of intruder-directed aggressive displays were higher in the resident (**A**), while partner directed displays and total male directed aggressive displays did not differ between neighbor and resident (**B** and **C**). Box plots show the median, upper and lower quartiles, and range. Circles are outliers. Asterisk indicates significance ($\alpha = 0.05$).

differences in hormone levels between neighbors and residents (Supplemental Table 2).

Gene expression

There were no significant differences in levels of gene expression between neighbors and residents after applying the Benjamini–Hochberg correction (Supplemental Table 2).

Correlations between behavior, hormones, and gene expression

In the neighbor, *5-htr2c* in Dm-1 best predicted aggressive displays to the intruder (Akaike weight = 0.47; $R^2 = 0.46$, $P = 0.011$) and to the partner (Akaike weight = 0.48; $R^2 = 0.5$, $P = 0.007$). As such, we report total male directed aggression (Akaike weight = 0.84; $R^2 = 0.72$, $P < 0.001$; Fig. 3A) for the remainder of the analyses. Interestingly, *5-htr2c* in Dm-1 did not correlate with aggressive displays in the resident ($R^2 = 0.02$, $P = 0.62$; Fig. 3A). In the resident, no measures of gene expression ranked above the null model. However, neighbor aggressive displays were best predicted by gene expression of *egr-1* in Dl in the resident (Akaike weight = 0.36; $R^2 = 0.44$, $P = 0.01$; Fig. 3B). There was no relationship between these variables in the neighbor ($R^2 = 0.01$, $P = 0.68$; Fig. 3B).

Circulating levels of testosterone correlated with total male directed aggression in neighbors ($R^2 = 0.54$, $P = 0.003$; Fig. 3C) but not in residents ($R^2 = 0.16$, $P = 0.14$; Fig. 3C). Cort levels did not correlate with aggression in neighbors ($R^2 = 0.2$, $P = 0.22$) or residents ($R^2 = 0.29$, $P = 0.17$). T and Cort themselves were positively correlated in neighbors ($R^2 = 0.61$, $P = 0.012$), but not in residents ($R^2 = 0.17$, $P = 0.31$).

Integrative analysis of co-variance patterns

To gain a systems-level understanding of the covariance patterns between behavior, circulating hormones, and brain region-specific gene expression patterns, we conducted a hierarchical clustering analysis with bootstrapped *P*-values. This approach revealed several significant clusters of gene expression in both the neighbor and the resident. In neighbors, we identified four significant clusters, listed from top to bottom (Fig. 4): (1) aggression, T levels, and *5-htr2c*, *d1r*, *c-fos*, and *egr-1* expression in Dm; (2) *erα*, *d2r*, *itr*, and *arα* expression in Dm; (3) *egr-1*, *c-fos*, and *5-htr2c* expression in Dl, and (4) *erα*, *arα*, *itr*, and *d2r* expression in Dl. In residents, five significant clusters were identified: (1) *egr-1* and *c-fos* expression in Dl; (2) *d2r*, *d1r*, *itr*, and *erα* expression in Dm; (3) T levels and aggression; (4) *itr*, *5-htr2c*, *erα*, and *arα* expression in Dl; and (5) *d2r* expression in Dl and *5-htr2c*, *egr-1*, and *c-fos* expression in Dm. Note that none of the significant co-variance clusters contain negative correlations.

Discussion

In the present study, we examined in the putative homologs of both hippocampus and blAMY the role of several neuromodulatory and neuroendocrine pathways in the context of joint territory defense by males of the highly social cichlid fish, *A. burtoni*. Remarkably, we find that aggressive behavior in response to a simulated territorial intrusion appears to be controlled by different neuromolecular pathways dependent on an individual's role during joint territory defense. In the neighbor, aggressive behavior and circulating T levels correlated strongly with Dm expression of *5-htr2c*, which formed a co-expression cluster with the two IEGs, *c-fos* and *egr-1*, and *d1r* (neighbor cluster 1). In

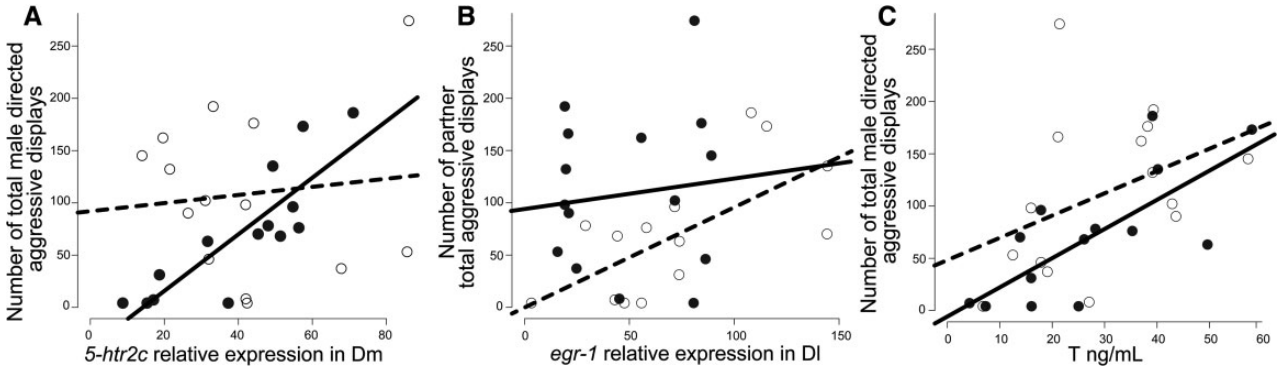

Fig. 3 (**A**) Aggressive displays were positively correlated with *5-htr2c* expression in Dm (medial part of the dorsal telencephalon) in neighbors but not residents (**B**) Partner aggressive displays were positively correlated with *egr-1* expression in Dl (lateral part of the dorsal telencephalon) in residents but not neighbors (**C**) Aggressive displays were positively correlated with circulating testosterone in neighbors but not residents. Filled circles and bold lines = neighbor; open circles and dashed lines = resident.

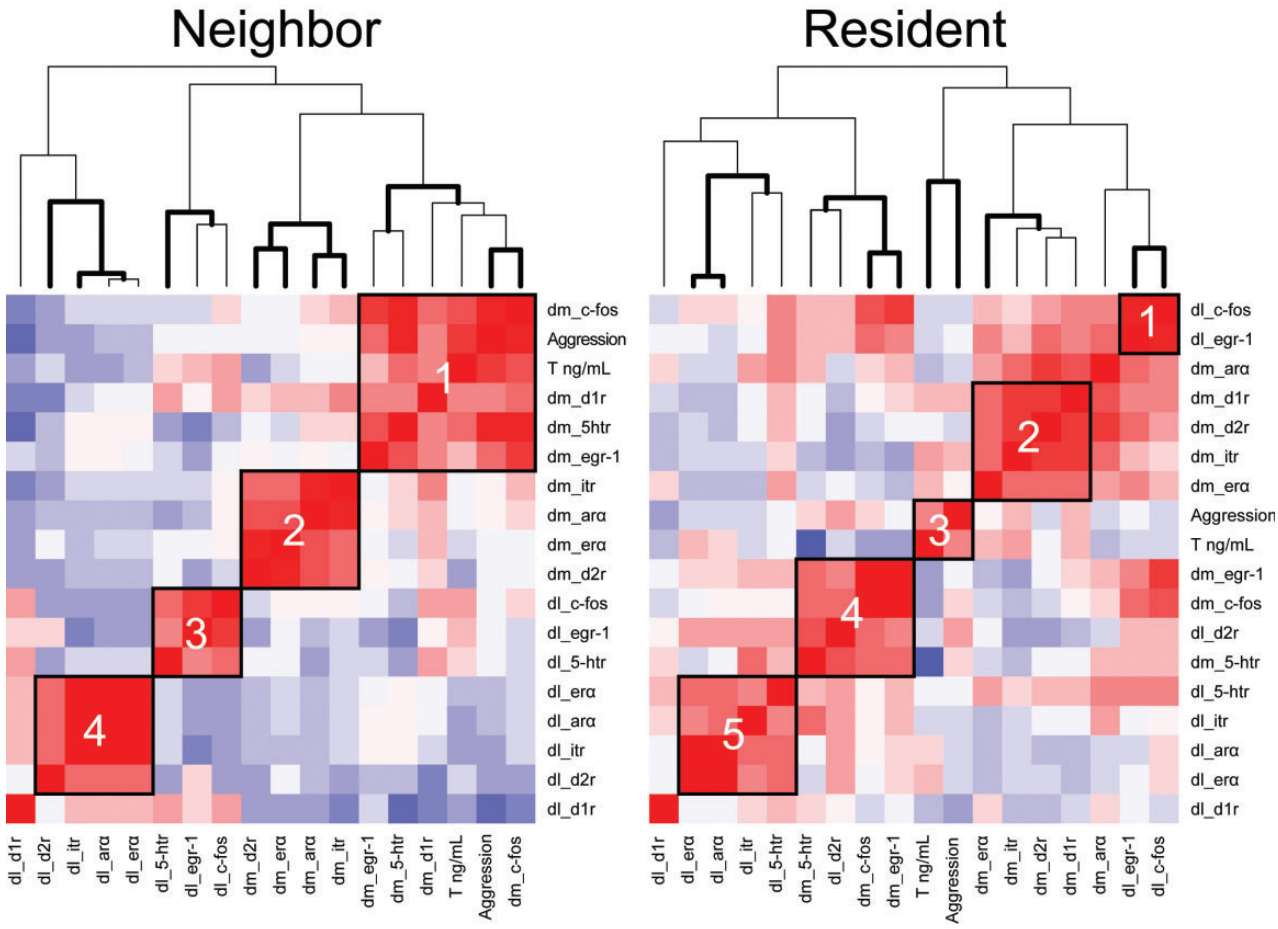

Fig. 4 Heatmaps and dendrograms representing the correlation matrices of gene expression in areas Dm (medial part of the dorsal telencephalon) and Dl (lateral part of the dorsal telencephalon), circulating T, and aggression for neighbors and residents following 1 h of a simulated territorial intrusion. Significant clusters are shown by bold black squares on the heatmaps and significant subclusters are shown by bold lines on the dendrograms. Red indicates positive correlations, blue indicated negative correlations.

contrast, aggressive behavior in the resident clustered only with circulating T levels (resident cluster 3), and did not correspond to any measure of gene expression. Our results reveal that the way in which an intruder stimulus is perceived, based on social context, affects the molecular regulation of aggression in the Dm.

We find a striking positive correlation between *5-htr2c* receptor gene expression in the putative amygdala homolog and aggressive behavior in neighbor males, a relationship absent in residents. 5-HT is typically considered to have an inhibitory effect on aggressive behavior (Bell et al. 2007). However, the role of 5-HT in mediating aggression varies temporally, spatially, and with social context (Summers et al. 2005). For example, following aggressive territory defense, males of the lizard *Sceloporus jarrovi* have higher 5-HT and DA levels in the forebrain (Matter et al. 1998). In addition, after 1 h of aggressive interaction, dominant *Anolis carolinensis* males have elevated 5-HT in the amygdala, while subordinates do not, suggesting context-dependent regulation (Summers et al. 2003). Similarly, in *A. carolinensis*, motor output for territorial displays appears to be regulated by 5-HTR_{2c} activity, in a direction consistent with our results (Baxter et al. 2001). It is possible that the association we identified was the result of an acute (within 1 h) change in receptor expression in response to behavior, though it may be more likely that pre-existing variation in expression levels between males affected their behavioral responses. Examination of *5-htr2c* expression before aggressive interactions will offer further insight into the extent to which these receptors are dynamically regulated.

In a previous study, we found that neural activity in the putative hippocampus homolog Dlv correlated with the neighbor's behavior to the intruder in the brain of the resident (Weitekamp and Hofmann 2017). This was identified by detection of c-Fos protein via immunohistochemical staining followed by stereological analysis. Intriguingly, we found a similar relationship between neighbor aggression and mRNA gene expression of the immediate early gene *egr-1* in the brain of the resident using quantitative real-time PCR on tissue punches of Dlv. In *A. burtoni*, resident males modulate their aggressive response to the intruder in a manner that depends on both the size and behavior of their partner, the neighbor. Neural activity in this region appears to serve a role in encoding this response. The neural circuits through which IEG activity in the hippocampus are acting to mediate this behavior remain to be uncovered.

We found that gene expression is highly co-regulated within brain regions but not across (with one exception, see below). Furthermore, we identified one group of genes which was significantly clustered in males of both behavioral roles (within cluster 2 in each), which consisted of Dm gene expression of *d2r*, *erα*, and *itr*, suggesting these pathways may interact in the blAMY homolog to mediate aggression independent of social context. Interestingly, in mammals, transcription of OTR in the medial amygdala is regulated by ERα (Young et al. 1998). During social interaction in male mice, gene expression of *otr* and *erα* was highly positively correlated in the medial amygdala (Murakami et al. 2011). Furthermore, in female prairie voles, partner preference formation requires both OTR and D2R acting in concert (Liu and Wang 2003). The specific mechanisms through which D2R, ERα, and ITR may interact to regulate social behavior remain to be elucidated.

In residents, we found that *d2r* expression in the hippocampus homolog correlates with *egr-1, c-fos*, and *5-htr2c* expression in Dm. It is well known that DA signaling in the hippocampus is involved in learning and memory processes, and D2R specifically may regulate hippocampal neuronal excitability (Yoon et al. 2015). Interestingly, in mice, optogenetic inhibition of the projections between the ventral hippocampus and blAMY increased social interaction, while activation reduced social interaction in the context of intruder exploration (Felix-Ortiz and Tye 2014). D2R may be acting to coordinate the response between these two regions. This putative relationship deserves further investigation.

Finally, none of the significant clusters identified contained negative correlations, suggesting the prevalence of synergistic co-regulation, as opposed to antagonistic regulation. Notably, this is also true for the two IEGs measured, which were positively correlated both between themselves and with other measures of gene expression. Aggressive territory defense may be a highly salient stimulus that results in coordinated neural activity. Indeed, the patterns of neural activity and modulation by neurochemicals in brain regions of the social decision-making network have been shown to change in a manner that reflects the valence of social stimuli (Goodson and Thompson 2010), including for aggressive behavior (Goodson et al. 2009). It would be interesting to examine the co-variance patterns between these candidate genes and brain regions before exposure to the conspecific intruder in order to determine the extent to which these pathways are being dynamically regulated.

Conclusion

In this study, we presented a conspecific intruder to two familiar territorial male cichlids, one serving as the resident and the other as the neighbor. The males did not differ overall in displays of total male directed aggression, and yet we found that the

neuromolecular regulation of this behavior was strongly dependent on their respective social role. Our results provide further evidence that social context is highly salient and results in a complex interaction between aggression and the systems mediating reward, motivation, and learning and memory.

Supplementary data

Supplementary Data available at *ICB* online.

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